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Life Without Thyroid Hormone Receptors

Sten Göthe

Stockholm 2003
On the front cover: An adult male TRα-/β-/ mouse (five months old) devoid of all known thyroid hormone receptors.

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Everything you can imagine is real
Pablo Picasso
Thyroid hormone (TH) has many important functions during development and in the adult individual. It reaches its target cells via the blood system and binds its receptor (TR) located within the cell nucleus where the receptor regulates target genes. This hormone-mediated gene regulation is a finely tuned interplay of numerous parameters. These include i) usage of several isoforms of receptors (TRα1, TRβ1, TRβ2, TRβ3) encoded for by two distinct genes, ii) receptor-mediated repression of target gene expression in the absence and activation in the presence of T3, and iii) enzymatic control of the relative concentrations of the active thyroid hormone (T3) versus prohormone (T4) through deiodinase activity. There are also target genes that respond in the opposite way to T3. This thesis describes mice that live without TRs. What have we learned from them?

The mice show a milder phenotype than mice with a congenital incapacity of producing T3. In contrast to not having T3, being without TRs is less severe. This is due to the ability of TRs to act as potent transcription factors in the absence of hormone. The mice devoid of all known TRs were generated from mice lacking either the TRα or the TRβ gene. Since deficiency for either single TR gene resulted in a much weaker phenotype as compared to fully TR deficient animals, our data indicate that the TR isoforms can substitute for each other in a variety of physiological and developmental functions. Despite the importance of T3, life is possible without TRs. However, the absence of TRs leads to an extreme dysregulation of T3 and retarded growth.

T3 is important for skeletal development. It acts via growth hormone (GH) and insulin-like growth factor 1 (IGF-1). However, whether it has a direct effect on bone has been the subject of controversy. The mice lacking all TRs exhibit retarded bone development combined with low levels of GH and IGF-1. Supplementing the mice with GH normalises growth, suggesting that the retarded growth is caused by the low production of GH and IGF-1. However, the defective ossification in the epiphyses was not rescued by GH, which supports the hypothesis that T3 exhibits a direct effect on bone.

Effects of T3 on brain development are known clinically from neurologic cretinism derived from low foetal T3 hormone levels and which leads to an almost vegetative life. The effects of T3 has been extensively studied particularly on cerebellar development. We therefore aimed to determine if T3 has any effect on the adult brain. Assaying solely for rapid responses in the cerebrum, we found almost 150 genes responding to T3, and notably, a third of these were also observed in mice having no TRs.
Publications

This thesis is based on the following articles, which will be referred to by their roman numerals:


Reference is also made to the following articles:


Contents

INTRODUCTION .................................................................................................................9
 Thyroid hormone, its receptors and their function .................................................9
 Thyroid hormone ........................................................................................................10
 Thyroid hormone receptors ..................................................................................12
 Nongenomic and extra-receptor mechanisms ......................................................15
 Diseases .....................................................................................................................16
 Target tissues .............................................................................................................17
 Bone ...........................................................................................................................18
 Brain ..........................................................................................................................21
 AIMS OF THE THESIS ...............................................................................................23
 MATERIALS and METHODS ......................................................................................24
 The creation of a knockout mouse ..........................................................................24
 Species differences .................................................................................................26
 RESULTS and COMMENTS .......................................................................................27
 TRs are nonessential for life (Paper I) .....................................................................27
 Any remaining TRs? .................................................................................................27
 A delicate existence .................................................................................................28
 Out of control ............................................................................................................28
 TR effects on bone (Paper I & Paper II) ...............................................................29
 Reduced GH restrains growth ...............................................................................29
 T3 seems to act directly on bone ............................................................................30
 Anomalous adipose tissue ......................................................................................30
 Testes maximus ........................................................................................................31
 TR effects in brain (Paper III) .................................................................................31
 Many were called but few were chosen ...............................................................31
 Other collaborations ...............................................................................................32
 TRs in heart ...............................................................................................................32
 TRs in skeletal muscle .............................................................................................33
 TRs in thermogenesis ..............................................................................................34
 GENERAL DISCUSSION .............................................................................................36
 TRα1-/-β-/- mice vs other TR deficient mice .........................................................36
 Common and diverse functions of TRα1 and TRβ .............................................36
 Deficiency of T3 vs deficiency of TR .....................................................................38
 Deficiency of T3 vs deficiency of GH ....................................................................40
 Various responses to T3 in adult brain .................................................................41
 SUMMARY ................................................................................................................43
 ACKNOWLEDGEMENTS ............................................................................................44
 REFERENCES .............................................................................................................46
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AF-1, 2</td>
<td>Activation function 1, 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>D1, 2, 3</td>
<td>Iodothyronine deiodinase type 1, 2, 3</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual x-ray absorptiometry</td>
</tr>
<tr>
<td>ES cell</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HPT</td>
<td>Hypothalamus-pituitary-thyroid</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5 trisphosphate</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>NCoR</td>
<td>Nuclear receptor corepressor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation signal</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear receptors</td>
</tr>
<tr>
<td>nTRE</td>
<td>Negative thyroid hormone response element</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pQCT</td>
<td>Peripheral quantitative computerised tomography</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>RTH</td>
<td>Syndrome of resistance to thyroid hormone</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator of retinoic acid and TRs</td>
</tr>
<tr>
<td>T3</td>
<td>Triiodothyronine</td>
</tr>
<tr>
<td>T4</td>
<td>Thyroxine</td>
</tr>
<tr>
<td>TH</td>
<td>Thyroid hormone</td>
</tr>
<tr>
<td>TR</td>
<td>Thyroid hormone receptor</td>
</tr>
<tr>
<td>TRAP</td>
<td>TR-associated protein</td>
</tr>
<tr>
<td>TRE</td>
<td>Thyroid hormone response element</td>
</tr>
<tr>
<td>TRH</td>
<td>Thyrotropin releasing hormone</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
</tbody>
</table>
INTRODUCTION

No other hormone affects such a wide range of cells and tissues as thyroid hormone. Evolutionary, it can be traced back to the first creatures evolving a vertebra about 500 million years ago. During evolution, thyroid hormone took on novel applications. Amphibians, emerging some 370 million years ago, have both isoforms of the receptor. Thyroid hormone in amphibians have a spectacular permissive function on metamorphosis, not completely dissimilar from e.g. their role in human brain development. With the evolution of the homeotherms some 200 million years ago, thyroid hormones took up regulation of oxygen consumption, thermogenesis, and lipogenesis. While new applications were co-opted, some of the developmental function persisted, as documented in the irreversible arrest in bone and brain ontogeny in thyroid hormone deficient mammals. Leaving water for a terrestrial life entailed new problems. Iodine, an essential component of thyroid hormones, is available from the ocean. Thus, salt-water vertebrates, the first forms to develop a thyroid gland, are not at risk of iodine deficiency. Iodine availability can on the other hand be rate limiting for terrestrial vertebrates, including humans, depending on the proximity to the ocean and the iodine content of water and the soil. About 1 billion people in the world have some form of thyroid disease. In Sweden thyroid hormone diseases is the second most common endocrine disease after diabetes. Non-institutional care include some 30 000 patients, and 2300 patients are treated in hospitals every year. Unfortunately, the diverse functions of thyroid hormones can cause patients with thyroid disorders to encounter general practitioners unfamiliar to the hallmarks of thyroid disease, sometimes resulting in delayed diagnosis and therapy.

Thyroid hormone, its receptors and their function

Throughout the body thyroid hormone and its receptors interplay to control expression of specific target genes. Several factors contribute to the tissue specificity. Importantly, whereas increased levels of thyroid
hormone may have a particular effect on a specific gene, reduced levels generally have the opposite effect on that gene.

**Thyroid hormone**

Thyroid hormone (TH) is produced in the thyroid gland situated in the front of the neck. Here thyroglobulin iodinates the amino acid tyrosine, resulting mainly in monoiodothyronine (T1) and diiodothyronine (T2), but also in the active hormone triiodothyronine (T3) and the prohormone thyroxine (T4). The first two most probably make up a storage for emerging deficiencies.

Release of TH from the thyroid gland is under control of the anterior pituitary hormone thyroid stimulating hormone (TSH). This is composed of an α- and β-subunit, where the α-subunit is shared with the gonadotropins (luteinizing hormone and follicle-stimulating hormone) also released from the pituitary. The β-subunit is distinct and confers on the intact TSH its specific biological activity. TSH acts in the thyroid gland via G protein coupled receptors on thyroid follicular cells. The control of TH is taken one step further in that TSH in turn is under control of the thyrotropin releasing hormone (TRH) in the paraventricular nucleus of the hypothalamus. This is a tripeptide derived from a pro-TRH peptide, and it reaches the anterior pituitary via the hypothalamic-pituitary portal circulation acting via the IP3/DAG pathway on pituitary thyrotrophs to increase release of TSH. This circle of activation is closed in that both TSH and TRH are negatively regulated by TH, repressing both the TSHα and the TSHβ subunit genes in the anterior pituitary and the TRH gene in the hypothalamic paraventricular nucleus. This mechanism therefore controls the release of THs in a strict negatively regulated feedback loop (Figure 1).

The predominant form of TH released from the thyroid gland into the blood is T4. Compared to T3, it is in a ratio of 14:1 in humans (or 5:1 in rat). Almost all of the circulating T3 and T4 is bound to proteins, predominantly thyroxine-binding protein but also transthyretin and albumin, although a small percentage of both T3 and T4 also circulate in the free form (Werner et al., 1996).
The relative amounts of T₃ and T₄ (and derivates) are under control of the three iodothyronine selenodeiodinase enzymes D₁, D₂, and D₃. Whilst T₄ is produced entirely by the thyroid gland, the majority of circulating T₃ (80% in humans) is derived from the deiodination of T₄. This occurs in various organs such as the liver and the kidney. The selenodeiodinases are differently abundant: D₁ is ubiquitously expressed and is considered the central component for maintaining circulating T₃ levels. D₂ is present in a limited number of tissues maintaining the local T₃ levels where the intracellular T₃ level is critical such as in brain and brown adipose tissue. D₃ is responsible for the inactivation of T₄ and T₃ in tissues where efficient control of hormone levels is desirable, e.g. in the brain (Bianco et al., 2002).

Little is known about how thyroid hormones enter the cell. This was earlier believed to occur via passive diffusion, whereas recent studies have documented the existence of saturable transport systems related to amino acid transporters that mediate the movement across the plasma membrane. These may not be TH specific but nonetheless play a role in thyroid hormone bioavailability (Hyde et al., 2003).
Thyroid hormone receptors

TRs are encoded by two different genes, the TRα gene, in humans located on chromosome 17, and the TRβ gene, on chromosome 3 (or 11 and 14, respectively, for mice) (Sap et al., 1986; Weinberger et al., 1986). The TRs belong to a family of nuclear receptors (NRs) that act as sequence specific transcription factors modulated by ligand binding. In a recent attempt to unify the nomenclature for all receptors in this family, the TRα gene (also called c-erbA-1, THRA in human or Thra in mouse) was renamed NR1A1, and the TRβ gene (c-erbA-2, THRBA, and Thrb) NR1A2 (Nuclear Receptors Nomenclature Committee, 1999).

Gene regulation by TRs occurs both in the presence and in the absence of ligand. Positively regulated genes are activated by the TR-T3 complex and are generally repressed by the unliganded TR. For some genes in this group the unliganded receptor (aporeceptor) may however merely act as repressor, allowing T3 to relieve repression without activating the expression above basal levels. Other TR target genes may be unaffected by the unliganded receptor but are activated above basal level by the T3-TR complex. For other genes T3 can relieve repression that the unliganded TR exerts on a target gene to a fully activated state (Figure 2). In contrast, the negatively regulated genes are repressed by the T3-bound TR (holoreceptor). A classical example of such a gene is that for TSHβ. Many reports, relying on transfection studies, have also suggested that the aporeceptor can activate negatively regulated genes; however only few of these claims have been verified in animal experiments (Feng et al., 2000; Flores-Morales et al., 2002; Wong et al., 1997). These findings have paved the way for research within this area, and similar gene regulatory functions have been described for other NRs, e.g. those for vitamin A and D.

TRs share with its family a common structural organisation with three major domains. The first domain (A/B) includes a constitutively active transactivation region (AF-1). The second domain (C) contains a DNA binding domain (DBD) responsible for specificity in interaction with hormone responsive elements in the promoter region of the DNA, and is also involved in receptor dimerisation. A third short flexible domain (D) acts as a "hinge" and contains a nuclear localisation signal (NLS) (Zhu et al., 1998), separating the DBD from the last major domain (E),
the ligand binding domain (LBD). The LBD contains in addition to the T3 binding pocket a second nuclear localization signal and a ligand induced transactivation function (AF-2), important for interaction between TRs and coactivators (Tone et al., 1994).

A TR can bind DNA as a monomer (i.e. alone), or as a homodimer (i.e in pairs), but preferably binds as a heterodimer with the retinoid X receptor (RXR), another member in the family. Important for dimerisation is a heptad repeat in the LBD (Forman and Samuels, 1990). To positively regulate target genes, TR binds to thyroid hormone response elements in DNA (TRE), that consist of two half sites with the consensus sequence G/AGGTC/GA. They are arranged either as inverted palindromes, or as direct repeats separated by four nucleotides. To control negatively regulated genes, TRs bind to negative TREs (nTRE) on which target genes are induced when unliganded TRs bind. The nTREs are less well characterised than the TREs. Examples of negatively regulated genes are those for TRH, TSH\(\alpha\) and TSH\(\beta\) (Tagami et al., 1997).

Unliganded TRs recruit corepressors for suppression of positively regulated target genes. Important for interaction between TR and corepressors is the D domain of the TR (Chen and Evans, 1995; Horlein et al., 1995). Examples of corepressors are NCoR and SMRT that in a corepressor complex with HDAC deacetylate histones so that access of

\begin{figure}
\centering
\begin{tikzpicture}
\begin{axis}[
    name=plot1,
    ybar=15pt,
    bar width=20pt,
    xtick=data,
    enlarge x limits=0.25,
    symbolic x coords={Hyperthyroid,Basal level,Hypothyroid},
    xtick style={draw=none},
    ytick={0,1},
    yticklabels={0,1},
    ymin=0,ymax=1.5,
    nodes near coords,]
\addplot table [x=Label, y=Height] {data.csv};
\end{axis}
\end{tikzpicture}
\caption{The mode of regulation by TRs can vary in a gene dependent manner. Some genes respond to T3 with a relief of repression only (A). Others are only activated (B). Some genes are both derepressed and activated by T3 (C).}
\end{figure}
basal transcriptional machinery to the DNA is restricted. NCoR and SMRT interact rather promiscuously with other nuclear receptors. There are also corepressors that only interact with a subset of NRs, for instance Alien, a corepressor that interacts with TR but not RAR, RXR, or GR (Dressel et al., 1999).

Following T3 binding, a TR alters conformation and the corepressor complex dissociates, relieving repression and causing replacement of the corepressor complex by a coactivator complex. Many of the proteins in this process possess the capacity to acetylate histones (i.e. they have histone acetyltransferase activity). This remodels the chromatin structure of the DNA to facilitate access of general transcription factors to the promoter. The first coactivator complex is replaced by a second (TRAP containing), less involved in chromatin remodelling but interacting with the basal transcription machinery.

The several isoforms of the TRs provide diversity in T3 mediated gene regulation (Lazar, 1993). The TRα gene can be alternatively spliced to either a TRα1 or a TRα2 product. The TRα1 protein is a bona fide receptor. The TRα2 protein however cannot bind T3 nor any other known ligand, binds only weakly to a subset of TREs, interacts poorly with the dimerising partner RXR, and, because it lacks the AF-2, does not interact with coactivators (Lazar et al., 1988; Mitsuhashi et al., 1988; Wu et al., 1998). Two truncated forms of TRα have also been described, Δα1 and Δα2, which are products of an internal promoter. They lack the DBD, and have unknown functions (Chassande et al., 1997).

Also unable to bind T3 is the rev-erbα protein, encoded by a gene juxtaposed to the TRα gene (Lazar et al., 1989). The rev-erbα gene is on the other DNA strand, and the two genes have overlapping coding sequences in their 3'-extremes. It lacks the AF-2 domain and consequently does not interact with coactivators. It binds DNA as a mono- or homodimer, and is thus regarded as a constitutive repressor (Adelmant et al., 1996; Harding and Lazar, 1995).

The TRβ gene encodes several T3 binding proteins, TRβ1, TRβ2, TRβ3 and Δβ3, produced by use of separate promoters and alternative splicing. The first three function as classical nuclear hormone receptors, whereas Δβ3, even though it binds T3, cannot bind DNA due to its lack of the DBD. The function of Δβ3 is however unclear (Williams, 2000).
TRs are expressed in almost all tissues. However, to carry out the many different effects in various tissues the isoforms are differentially expressed (Hodin et al., 1990). Both TRα and TRβ are expressed in human oocytes (Zhang et al., 1997). In the fetus, TRα1 is the major isoform, expressed even before the onset of thyroid gland development, whereas TRβ1 is mostly restricted to cerebrum and inner ear. At adult ages both TRα1 and TRβ1 are widely expressed, with TRα1 being the major form in heart, muscle, brain and brown adipose tissue, whereas TRβ1 is the major form in e.g. liver and kidney. Expression of TRβ2 is restricted to the hypothalamus, anterior pituitary, retina, inner ear, and restricted parts of the central nervous system. The expression pattern of TRα2 in brain parallels that of TRα1, although it is expressed at 2- to 10-fold higher levels (Bradley et al., 1989). The Δα1 and Δα2 forms are highly expressed in lung and intestine, TRβ3 has its highest expression in lung, liver, and kidney, and Δβ3 in lung and spleen. The rev-erbα gene is very widely expressed.

**Nongenomic and extra-receptor mechanisms**

Reports of alternative pathways of hormone action, other than those exerted in the cell nucleus, appeared in the literature several years ago. The mechanisms for these remained elusive and were disputed by many. Recently however, several reports have elucidated novel mechanisms of cytoplasmic signaling by NRs. They are characterized by insensitivity to inhibitors of transcription and protein synthesis and by their rapid onset of action (within seconds to minutes).

One of the nongenomic effects recently described for TRs recently concern mitochondrial functions (Wrutniak-Cabello et al., 2001). It was proposed that a truncated form of TRα1 or possibly TRβ3 that both lack an extended A/B domain largely remain in the cytoplasm (Andersson and Vennstrom, 1997), and that such an isoform not only associates with mitochondria but also are imported into this organelle (Casas et al., 1999). The proposed functions of TRs in the mitochondria are unclear. In some cases nongenomic actions may have connections to the genomic effects of the same hormone. For example, by nongenomic mechanisms, TH can promote serine phosphorylation of nuclear TR.
Transcriptional activity of nuclear receptor proteins can be altered by such modifications (Davis et al., 2000).

Extra-receptor effects of TH that are not mediated by TRs have also been described. Some data support the existence of specific G protein coupled receptors at the cell surface that TH interacts with to activate signal transducing kinases such as those involved in the mitogen-activated protein kinase (MAPK) pathway. This was shown to modulate the cellular activities of certain cytokines and growth factors (Davis et al., 2000; Lin et al., 1999). Additionally, effects on ion fluxes at the plasma membrane (Huang et al., 1999; Incerpi et al., 1999; Sakaguchi et al., 1996), intracellular protein trafficking (Chen et al., 1999; Lin et al., 1996; Safran et al., 1992; Zhu et al., 1998), and actin polymerisation (Siegrist-Kaiser et al., 1990) have been described.

Diseases

A goiter is an enlarged thyroid gland, and may well be present in several of the following conditions.

An amount of T3 insufficient to support normal body function is called hypothyroidism. Iodine deficiency is globally the leading cause of this, whereas in iodine sufficient regions, the most common cause is thyroid dysgenesis. Congenital hypothyroidism, with lack of TH during brain development, can result in severe brain damage and cretinism. Endemic cretinism is caused by iodine deficiency in the pregnant mother in areas of iodine deficiency. This leads to irreparable brain damage and cretinism, even if the newborn infant is treated with TH. Sporadic cretinism is usually caused by defective thyroid gland function in the fetus and infant due to misdevelopment of the gland, failure to take up iodine, or defects in the enzyme machinery for making T3 and T4 and is characterised by retardation of physical and mental development. In developed countries congenital hypothyroidism is usually detected by perinatal screening and is curable by treatment with T4 starting in early infancy (Werner et al., 1996). Adult hypothyroidism is fairly common and can have different causes of which some are well understood, others not. Hashimotos disease is caused by autoantibodies directed against the TSH receptor in the thyroid gland. The antibodies block the stimulating
effects of TSH, leading to a low production of TH (Amino, 1988). Other
causes are related to either iodine deficiency in the food intake, or to the
inhibitory effects of certain compounds in the food. A classical example
of this is the widespread hypothyroidism seen in populations that use
cassava as their staple food. Thiocyanates in cassava inhibit iodine uptake
(Werner et al., 1996). The classical myxodema is most frequently found
in women, and some estimates suggest that >5% of women 50 years or
older benefit from replacement treatment.

Hyperthyroidism is increased levels of T3. One of the most common
disorders is Graves’ autoimmune disease, caused by stimulatory anti-
TSH receptor antibodies leading to excessive and uncontrolled
production of T3 (Sanders et al., 2003). Other common causes involve
thyroid cancer, that cause excessive growth of the thyroid gland beyond
the control of the TSH receptor. Thyrotoxic storm is a relatively rare
but life threatening syndrome of highly exaggerated levels of T3,
including high fever, high tachycardia, and CNS signs varying from
apathy, confusion, and even coma (Werner et al., 1996). It is sometimes
caused by Amiadarone, the only TR specific antagonist used in the
clinic.

The syndrome Resistance to Thyroid Hormone (RTH), is characterised
by mutations in either of three regions of the LBD of TRβ. The
resulting lower affinity to ligand leads to elevated T3 levels,
hypothyroidism at the tissue level, and inappropriately normal TSH
levels. The symptoms of the patients are quite variable. Although the
severity of the disease generally parallels that of the reduction in
affinity, the penetration of a particular mutation can be highly variable
in carrier members of a given family. Some individuals appear
hypothyroid and other hyperthyroid, maybe even in different tissues
(Yen, in press).

**Target tissues**

Thyroid hormone, often referred to as the major metabolic hormone,
affects virtually every cell in the body. This thesis focuses on two target
tissues: bone and brain.
Bone

Thyroid hormone is of major importance for appropriate bone development and maintainance in adult. The risk for osteoporosis in hyperthyroid patients has been known for a century (von Recklinghausen, 1891).

Skeletal development is perturbed by both deficiency or excess of T3: hypothyroidism at young age results in short stature, and in decreased bone remodelling in adult life. On the other hand, childhood hyperthyroidism causes increased bone turnover that may lead to premature growth cessation and consequently also short stature, whereas T3 excess in adult life can lead to osteoporosis.

The skeletal system consists of connective tissues, bone and cartilage. Three major cell types contribute to the skeleton: chondrocytes, which form cartilage; osteoblasts, which deposit bone matrix; and osteoclasts, which resorb bone. Chondrocytes and osteoblasts are of mesenchymal origin, whereas osteoclasts derive from the hematopoietic system. Once embedded in bone matrix, osteoblasts mature into terminally differentiated osteocytes. Formation of the skeleton, or ossification, occurs by either a direct (intramembraneous) or an indirect (endochondral) process. At embryonic development, intramembraneous ossification occurs by direct transformation of mesenchymal cells to osteoblasts, principally to reconstitute the skull. However most of the skeleton is made via endochondral ossification in which mesenchymal cells condense, and differentiate to chondrocytes, to form a foetal cartilage template that differentiates into chondrocytes that are subsequently transformed into bone. Ossification centres appear in the middle part of the bone, diaphysis and metaphysis, and in the distal part, epiphysis. In the ossification centres chondrocytes produce an extracellular matrix of collagen X and angiogenic factors. Finally this leaves a zone between the epiphysis and the metaphysis where endochondral bone formation continues in a coordinated way during postnatal life. This is the epiphyseal growth plate. Chondrocytes within this cartilage model initially undergo rapid proliferation producing a large amount of extracellular matrix, then cease proliferation to become hypertrophic. Once fated to hypertrophy, chondrocytes greatly increase their intracellular volume, they start expressing stage specific matrix
components such as collagen type X and the matrix subsequently calcifies. As mineralization proceeds, the tissue is invaded by blood vessels, and the cartilage is removed and replaced by bone. There are two types of bone trabecular bone (cancellous) and cortical (compact). The trabecular bone is found primarily in vertebrae and the ends of long bones. The cortical bone is found in the shafts of long bones (Bilezikian et al., 2002).

Postnatally, longitudinal bone growth takes place through a similar pattern of endochondral ossification in the growth plates located at the epiphyses (ends) of long bones. Bone growth takes place at the growth plate, at the border between the middle of the bone (metaphysis, diaphysis) and end of the bone (epiphysis). The growth plate can be divided into four layers depending on the stages of the chondrocytes. First, the germinal layer consists of slowly proliferating prechondrocytes. Secondly, the proliferative layer, containing actively proliferating chondrocytes neatly organised in columns in an extracellular matrix of type II and proteoglycans. Thirdly, the hypertrophic zone, consisting of chondrocytes that have ceased to proliferate and differentiate. The fourth layer is the calcifying zone, which contains chondrocytes that have undergone cell death and a mineralised extracellular matrix. Eventually the chondrocytes and the extracellular matrix are removed and replaced by bone cells with a mineralised matrix. As mineralization proceeds, the tissue is invaded by blood vessels.

A particular feature of the skeleton is that it is constantly subject to remodelling; the coordinated resorption and formation of new bone to maintain mechanical strength and quality. This is regulated by hormones and local factors, which direct osteoclasts and osteoblasts to renew the skeleton in cycles. Osteoclasts are recruited at a resorption site to resorb bone, and are subsequently replaced by osteoblasts to produce new bone matrix. The skeleton has several million small remodelling sites, that require 2-5 years to renew (Ott, 2002).

TH act as important regulator of bone development, in the growth plate, and in bone remodeling. It has a key role in endochondral ossification in the hypertrophic layer of the growth plate. It has been shown that T3 in vitro can stimulate maturation of hypertrophic chondrocytes (Wakita et al., 1998). Hypothyroidism in rats causes growth retardation due to growth plate dysgenesis in which
hypertrophic chondrocyte differentiation fails to progress (Harvey et al., 2002). Another observed in vitro effect is that T₃ has been found to stimulate osteoclastic bone resorption in organ cultures (Mundy et al., 1976), similarly to hyperthyroidism, which accelerates bone turnover and shortens the normal bone remodeling cycle. A conclusion from these studies is that hyperthyroid patients may have increased bone loss, increased osteoclastic activity, and hypercalcemia.

Other hormones that play major roles in regulating childhood growth include growth hormone (GH) and insulin-like growth factor 1 (IGF-1), whilst the major contributor during adolescence are the sex steroids. GH stimulates conversion of T₄ to T₃ (Sato et al., 1977), and the rat GH gene was the first for which a TRE was found (Brent et al., 1989; Flug et al., 1987). TH and GH interact in stimulating IGF-1. It is known from studies of knockout mice that an intact GH/IGF-1 signalling system is required for normal growth of the skeleton, and that targeted disruption of either the GH receptor (Zhou et al., 1997), IGF-1 or IGF-1 receptor (Baker et al., 1993) leads to severe growth retardation and delayed bone development (Robson et al., 2002; Williams et al., 1998).

As TRs are expressed in osteoblasts, osteoclasts, and chondrocytes (where the four splice forms TRα₁, TRα₂, TRβ₁, and TRβ₂ have been found to be expressed) (Abu et al., 2000), as well as the bone growth

**Figure 3.** T₃ stimulates bone growth via GH and IGF-1. Additionally, T₃ may also have direct effects on bone growth.
plate as studied in rat (Ballock et al., 1999; Milne et al., 1999), T3 clearly has a potential role in bone.

There has been a long controversy as to whether the T3 effects on bone are direct or via its interactions with GH and IGF-1 only (Figure 3), which is a question we discuss in paper II.

**Brain**

For decades it was thought that the adult brain is insensitive to T3. Lately however, indications and evidence that T3 affects gene regulation in the adult brain have emerged.

The development of the brain is critically dependent on adequate levels of T3 in the fetus and mother. Mild or perhaps transient maternal hypothyroidism may even in the absence of detectable abnormalities at birth result in altered intellectual development (Glinoer, 2001; Haddow et al., 1999; Pop et al., 1999).

Adults with altered T3 levels develop a number of significant neurological and psychological changes, that might even occur in reaction to small T3 deviations. The hypothyroid patient can develop symptoms such as psychotic behavior, delusions, intellectual deteriorations, ataxia, and sleepiness. Correspondingly, a hyperthyroid patient can develop emotional lability, anxiety, delirium, and seizures. Most importantly these adult neurological and psychological symptoms can be corrected by proper adjustment of the circulatory TH (Ahmed et al., 1993; Laurberg, 1990).

Brain development begins from a neural tube at the back side of the early embryo. The most frontal part of the neural tube subdivides into three primary brain vesicles. The first (proencephalon) will give rise to centers for mental activities and hormonal regulation, the second (mesencephalon) will become the midbrain, and the third (rhombencephalon) the cerebellum and the center for respiratory movements. The most well characterised target for T3 during development is the cerebellum where hypothyroidism result in disturbances in myelination, neuronal migration, synaptogenesis, and
arborization. A number of T3 responsive cerebellar target genes have been identified; most of these share very similar expression patterns (Oppenheimer and Schwartz, 1997). During postnatal cerebellar development in hypothyroid animals, expression of target genes for TRs is delayed. However, the retardation of cerebellar development is eventually overcome and results in normalisation of the gene expression pattern.

In the foetal brain, expression of TRα1 and TRα2 preceeds that of TRβ isoforms. Expression of all isoforms occurs in human foetal cerebral cortex from the 10-16th weeks, although at weeks 11-13 only TRα1 and TRα2 are expressed. Proteins for all receptors are detectable at 15-25 weeks. At birth, while the expression of TRα1 and TRα2 increases twofold there is a dramatic (40-fold) increase in TRβ1, coinciding with the postnatal surge in T3 (Strait et al., 1990). Except for this short postnatal period, TRα1 levels comprise about 80% of the T3 binding activity in the total brain, regardless of age (Schwartz et al., 1992).

Despite extremes of availability, brain T3 concentration and turnover rates are kept within narrow limits (Dratman et al., 1983; van Doorn et al., 1984) suggesting that brain T3 homeostasis is strongly defended. Nevertheless, the persistent belief of the adult brain as a nonresponsive tissue for T3 has been reconsidered in light of abundance of T3 and TRs in brain and the actual clinical observations of altered CNS function. Reports of T3 responsive genes in adult brain have still so far been few.

The aim of paper III is to identify genes in the adult brain that are regulated by T3, and to determine if they are regulated by TRs.
AIMS OF THE THESIS

The overall aim of this thesis was to investigate the role of thyroid hormone and its receptors on gene regulation, development and physiology, with particular attention to the skeleton and brain.

Specific questions were:

- What is worse: to lack TH or TRs?
- How does absence of all TRs compare to lack of a single TR?
- What is the role of TH in the bone?
- Do TRs regulate expression of genes in the adult brain?
- Can TH affect gene expression in the brain in the absence of TRs?
MATERIALS and METHODS

The creation of a knockout mouse

The single knockout strains TRα1-/ and TRβ-/ were previously made (Forrest et al., 1996b; Wikstrom et al., 1998). The compound knockout mouse, the TRα1-/β-/- mouse, which is the focus of this thesis, was made by intercrossing the single knockout mice.

For construction of the TRα1 single knockout mouse, the coding sequence specific for the TRα1 gene was replaced by its alternative splicing counterpart, that for the non ligand binding TRα2. The TRβ single knockout mouse was made by deletion of a 3 kb section of the DNA binding region that eliminates the function of TRβ1 and TRβ2 (and most likely TRβ3 if it exists in mouse).

The TRα1 and the TRβ single knockout mice were each made via the embryonic stem (ES) cell technique (Doetschman et al., 1987; Evans and Kaufman, 1981; Martin, 1981; Thomas and Capecchi, 1987). In this technique the altered gene is electroporated into totipotent ES cells derived from a blastocyst. In a few of the ES cells homologous recombination occurs so that the altered gene replaces the corresponding normal gene. The recombined gene is identified using a neomycin marker adjacent to the altered gene that make this ES cells resistant to a drug. Surviving ES cells are screened by southern blot or PCR. Cells from a selected ES cell clone carrying the altered gene are injected into mouse blastocysts, which are then surgically implanted into pseudopregnant mothers. Mice born from these are chimeras with a mixed cellular background partly from the blastocyst and partly from the ES cells with the altered gene. As the offspring is produced by ES cells derived from a mouse with the Agouti coat color and the donor blastocyst from black mice, the chimerism becomes evident. The degree of chimerism can be variable, ranging from a few percent to ca 80%. If the ES cells have contributed to the germ cells of the chimera, germline transmission to its offspring can generally be achieved. These will in turn be heterozygous for the altered gene. Further breeding of the
heterozygotes will then generate mice homozygous for the genetic alteration.

The chimera thus has five parents. Parents one and two gave rise to the blastocysts the ES cells are derived from, three and four to the blastocyst the ES cells are injected into and the fifth is mother they are transplanted into. The chimera consists of two cell types, the 129 of the ES cells and the cells of the blastocyst they are injected into. The chimera are mated with a suitable, inbred mouse strain, e.g. Balb/C or C57Bl to yield the F1 generation. Since the ES cells that give yield to sperm cells are derived from the the 129 mouse line, the progeny will represent a cross between this mouse line and the one they are bred against.

The blastocyst derived ES cells were of strain 129OlaHsd for TRα1, and of strain 129/Sv for TRβ. The chimeric mice created from these strains were bred with a mouse of strain BALB/c for TRα1 and C57Bl/6J for TRβ. The resulting TRα1/-β/- mice and their adherent wild-type mice thus have a mixed background of 129OlaHsd, BALB/c, 129/Sv and C57Bl/6J (Figure 4).

The knockout technique has hitherto been limited to mice only, due to the difficulty in generating functional ES cells or to achieve successful germ line transmission in other species (Muller, 1999). For other species,

![Image](image_url)

**Figure 4.** To the left a mouse lacking all known nuclear thyroid hormone receptors (TRα1/-β/-). To the right a normal (wild-type) mouse.
such as the rat, other successful approaches are now emerging (Zan et al., 2003).

**Species differences**

As we have examined bone growth in mice in Paper I and II and brain in Paper III it is important to note that some differences do exist in different species. In mice the long bone epiphyseal growth plate is sessated gradually throughout life, whereas it is definitely closed in humans after the sex hormone driven growth spurt (Kilborn et al., 2002; Silberberg and Silberberg, 1941). Also brain development differs among species. In mice much of the development of the CNS occurs postnatally, whereas in humans most is before birth (Howdeshell, 2002).
RESULTS and COMMENTS

TRs are nonessential for life (Paper I)

The starting point of this project was the crossing of a TRα1 knockout strain with a TRβ knockout strain. With the known importance of thyroid hormones in mind it was far from sure that any offspring, being devoid of all known thyroid hormone receptors, would live. Contrary to expectations, however, the TRα1-/-TRβ-/- pups survived far better than expected and a search for how these differed from normal mice followed. Besides a somewhat flattened face these TRα1-/-β-/- pups had no obvious peculiarities, but their differences to wild-type mice would soon show.

Any remaining TRs?

Could we really be sure that these mice were actually devoid of all TRs? Hypothetically, a previously undiscovered “TRγ” could have sustained life in these mice. Gel mobility shifts (Paper I, Figure 1A and B) verified absence of proteins in nuclear extracts of vital organs (brain, lung, kidney, spleen, and heart) that could have bound to positive TREs with different structures, whereas the normal mice showed binding of nuclear extract proteins to these. Absence of T3 binding capacity was indicated by the failure of 125I-T3 to bind to nuclear extracts from brain or liver (Paper I, Figure 1C). Hypothetically, a “TRγ” could have been saturated by the high circulating T3 levels in the mutant mice. The mice were therefore made hypothyroid and brain and liver nuclear extracts were tested in saturation binding studies (Paper I, Figure 1D). Again, no signs of a high affinity, low capacity binding activity was detected. Furthermore, a “TRγ” would have been expected to exhibit gene repressing activity during hypothyroidism, thereby affecting the phenotype of the mice. If indeed the mice possessed an undiscovered TR, then the phenotype would have been expected to resemble that of the hypothyroid wild-type mice which decreased in weight (Paper I, Figure 4E). That there is no repressing undiscovered receptor is
supported by the observation that the knockout mice survived the hypothyroid state and did not lose weight. We thus concluded that there is no TR activity in the mice and that the mice are truly deficient of all genes for T3 binding receptors. Subsequently, a human genome database search revealed the existence of a total of 49 NR genes, and no additional NR gene with a homology to the previously known TRs (Robinson-Rechavi et al., 2001).

But is this the full picture? We observed no nuclear TR mediated T3 functions in the most important organs of the knockout mice, although we cannot exclude minor effects through a potential extra-receptor action. There are however no hyperthyroid effects evident in these mice that could compensate for the TR mediated pathways.

**A delicate existence**

The immediate conclusion that can be drawn from the viability of these mice, is that TRs are not essential for life. How then is life without TRs? There is a reduced chance of being born due both to a reduced fertility of the female and a reduced Mendelian frequency of true knockout offspring from a TRα1−/−β+/- mother, a breeding setup necessitated by the poor fertility of the TRα1−/−β−/- mothers. This is in line with that hypothyroidism affects female fertility more than male (Longcope, 1996). There is also an increased mortality risk: whereas all normal mice survive for 9 months, 30% of the knockouts do not. The reason for this increased mortality remains to be solved. In comparison, Pax8−/- mice, born without a functioning thyroid gland, die after weaning (Mansouri et al., 1998).

**Out of control**

In these mice the hypothalamus-pituitary-thyroid axis is hyperactive. The levels of thyroid hormone is extremely high, 11-60 times that in normal mice (Paper I, Figure 2A). In the newborns the thyroid glands were of normal size although some follicles were enlarged (Paper I, Figure 2A). At 5 weeks, i.e. puberty, the glands were enlarged and the
number of enlarged follicles was increased. At 2 months, i.e. in young adults, glands weighed 15 times more (Paper I, Figure 2A) than those of their wild-type counterparts despite the mutant mice weighing 30% less. Moreover the thyroid follicles were hyperproliferative and the colloids in a degenerative state (Paper I, Figure 2B-C). The level of TSH was increased 30- to 160-fold depending on age (Paper I, Figure 3A), and the TSH subunits, TSHα and TSHβ, were increased 3- and 26-fold respectively, as determined by northern blots (Paper I, Figure 3A). TSHβ is known to be more sensitive than TSHα to T3 regulation. From this we learn that TRα1 and TRβ have an overlapping role in regulating TSH production since TSHα and β mRNA were less than 3-fold increased in the TRα1-/- or TRβ-/- single knockouts. TSH is normally regulated by T3 in a negative feedback manner where T3 acts on nTRE in the pituitary to repress expression of the TSHα or TSHβ gene. It has been proposed that the nTREs of the TSH genes is activated by the absence of T3, i.e. by a ligand independend activation. This suggestion is not verified here: the serum levels of TSH were high at a constant unresponsive maximal level, regardless of whether the levels of TH were reduced or increased (Paper I, Figure 3D). This was the first in vivo evidence that an unliganded TR is not needed for activating TSH gene expression. A similar conclusion has also been drawn for another negatively regulated gene, the cardiac myosin heavy chain beta (MHCβ) (Mansen et al., 2001).

**TR effects on bone (Paper I & Paper II)**

**Reduced GH restrains growth**

The weight of newborn pups is reduced by about 10%, their growth spurt is delayed, and adults weigh almost 30% less than normal mice (Paper I, Figure 4A). Staining and x-ray showed they have shorter bones already from birth persisting to adult ages. Dual x-ray absorptiometry (DXA) and peripheral quantitative computerised tomography (pQCT) showed that mice have reduced bone dimensions and mineralisation and an increased risk of fragility. Moreover the growth plate is widened and disorganized and has delayed ossification (Paper I, Figure 5). Similar to
hypothyroid rodents, both GH and IGF-1 is reduced in serum (Paper I, Figure 4B) (Burstein et al., 1979).

**T₃ seems to act directly on bone**

The possibility still existed that part of the growth phenotype was due to the lack of TH action directly in bone tissue. Therefore GH levels needed to be normalized which would not only ameliorate the GH deficiency, but since the T₃ and GH pathways converge at the stimulation of IGF-1, also restore hepatic production of IGF-1.

The initial daily dose of GH (1.5 mg/kg) was insufficient for restoration of normal growth (Paper II, Figure 2A), and a higher dose (3.8 mg/kg) of GH was therefore given during the course of the experiment. GH is produced in a pulsative manner by the pituitary, and has a short half life in serum and the efficacy of it was estimated by the effect on the serum IGF-1 levels.

Treatment restored IGF-1 in the mutant mice whereas wild-type controls had elevated levels, suggesting that the GH treatment normalized the GH/IGF1 axis function. The higher dose of GH restored the growth rate and increased the length of the femur and the periosteal cortical circumference in the mutant mice but not in the wild-type controls. Importantly however, GH was unable to reverse the epiphyseal ossification. Both bone mineral density and content was aberrant and a wider epiphyseal plate was still persistant despite the GH treatment, indicative of a perturbed balance between hypertrophy and proliferation.

**Anomalous adipose tissue**

The TR deficient mice have a 60% reduction in white adipose tissue (WAT), a feature not unaltered by treatment with GH. It is possible that the lower WAT content is involved in the defective control of body temperature. On the other hand they have twice as much fat tissue in the bone marrow, as evidenced by an increased number of adipocytes and marker gene expression (Kindblom et al.). Bone marrow adipocytes
are derived from the same mesenchymal progenitor cell as osteoblasts and chondrocytes, and are regulated by GH. The function of bone marrow adipocytes is unclear. One suggested function is that it counterbalances these other two lineages and that GH deficiency drives this balance towards adipocytes (Gevers et al., 2002). The poor mineralisation of the TR deficient mice is accompanied by a reduction of a key regulator of osteoclastogenesis (receptor activator of NF-κb, RANKL) indicating that the TRs may be involved also in the NF-κb signaling pathway (Kindblom et al., manuscript).

**Testes maximus**

The TRα1/-β-/- have dramatically enlarged testes, weighing 35% more for untreated TR deficient related to normal mice per se, or 85% more if corrected for their smaller body weight. Correspondingly, hypothyroidism has been reported to increase testicular weight (Hardy et al., 1996; Hess et al., 1993).

**TR effects in brain (Paper III)**

**Many were called but few were chosen**

Since the role of TH in brain development has been extensively studied, the relatively unexplored T3 responsiveness of the adult brain prompted us to use microarrays to search for T3 regulated genes. Because of the limited knowledge of T3 target genes, and our present interest in TR dependence, 15 600 duplicate cDNA clones were used for screening expressed RNAs from both normal and TR deficient mice. The analysis aimed at finding genes with a rapid response to thyroid hormone, i.e. genes that would be direct targets for regulation by TRs. Therefore mRNA was prepared at 3 hours after induced hyperthyroidism. We used strict criteria for reproduction and significance: induction by 50% or repression by 33%. A surprisingly high number of T3 responsive genes were identified: 149.
The 149 genes were grouped in six classes according to their function. This showed that both suppressed and induced T3 target genes participate in wide range of functions in the brain, and which also (except for the ribosomal class) was due for the regulated target genes with a known neurological function.

The genes were furthermore divided into 12 response patterns. One set of these comprising about half of the target genes, respond to T3 in normal mice (Figure 1C, e.g. response pattern 08). These are expected to include the genes directly regulated by TRs in a ligand dependent manner, thus reflecting genes typically modulated in hypo- and hyperthyroidism. A second set, about a third of the genes, respond to T3 in TR deficient mice (Figure 1C, e.g. response pattern 01). The reasons for that target genes in TR deficient mice are modulated by injections of T3 can at present only be hypothesised. It may be an in vivo indication of any of the alternative pathways (Losel and Wehling, 2003). A third and last set cover the target genes that are insensitive to ligand but modified in regard to TR presence (Figure 1C, response pattern 02 and 09 only). The T3 independent TR regulation of these genes could derive from metabolic or developmental differences between normal and TR deficient mice. Another speculation is that interplayers such as heterodimerisation partners, corepressors, and coactivators in the absence of TRs influence the other reactions they are involved in.

**Other collaborations**

**TRs in heart**

TH has many effects on the heart and the vascular system. Hyperthyroid patients have increased heart rate, cardiac output, and blood volume, whereas in hypothyroid patients the condition is reversed (Klein and Ojamaa, 2001). TH directly or indirectly regulates target genes in the heart. The myosin heavy chains α and β (MHCα and β), whose relative expression take part in determining cardiac contractility, are however directly regulated: T3 increases transcription of MHCα via two TREs and decreases that of MHCβ via one nTRE (Dillmann, 1990).
TRα1/-β/- mice have a reduced heart rate. They also have an EKG with extended repolarisation. This is similar to what seen in hypothyroidism and in the of TRα1-/- mice (Johansson et al., 1998). The reduced heart rate in the TRα1-/- mice could be elevated by T3 administration, but not in the TRβ-/- mice, indicating that TRα1 has a major role in maintaining the basal heart rate and that TRβ may mediate TH stimulation of heart rate (Johansson et al., 1999). This view is supported by the evidence that mice lacking both TRα1 and TRα2 also have a reduced heart rate (Gloss et al., 2001). Furthermore, the TRα1-/-β/- mice have highly elevated levels (by 50-fold) of MHCβ, similar to TRα1-/- mice or hypothyroid mice but not TRβ-/- mice, indicating that TRα1 has the primary role in the negative regulation of the MHCβ gene (Mansen et al., 2001).

**TRs in skeletal muscle**

T3 is an important regulator of skeletal muscle development and adult function (Finkelstein et al., 1991; Gambke et al., 1983). Reminiscent of bone remodeling, proteins of skeletal muscle are subjected to continuous synthesis and breakdown. Both hyper- and hypothyroid patients display skeletal muscle weakness and increased fatigability (Rooyackers and Nair, 1997). Among skeletal muscle types, slow-twitch muscle is more sensitive to TH than fast-twitch muscle (Larsson et al., 1994). TH exerts its effects on genes coding for contractile proteins including the group of ten members of the multigene family MHC, that all respond to TH. Both fast and slow-twitch muscles are composed of fast and slow MHC isoforms. An increase in TH causes a shift in myosin isoenzyme distribution toward faster myosin types, whereas a reduced level of TH has the opposite effect (Caiozzo et al., 1993).

Deficiency of all TRs have more pronounced effects on slow-twitch muscles (solus) than on fast-twitch (extensor digitorum longus, EDL). In the TRα1/-β/- mice there is a major switch towards slower MHC isoforms in the slow-twitch muscles, and a minor switch in the fast-twitch muscles (Yu et al., 2000). Also in these mice, contraction and relaxation speed was reduced in the slow-twitch muscles, but not in the fast-twitch muscles (Johansson et al., 2003). Both the contractile properties and the myosin composition switch is similar to hypothyroid
mice and are less affected in mice deficient in only a single TR. (Johansson et al., 2003; Yu et al., 2000). This indicates that the TRs mediate the effects observed and that there is a functional overlap between the two TRs.

**TRs in thermogenesis**

TH has a pronounced physiological influence over metabolism thermogenesis and body temperature (Lanni et al., 2003). Hypothyroid patients are cold sensitive and hyperthyroid patients feel overheated.

Thyroid hormone is involved in both basal and adaptive thermogenesis (Lanni et al., 2003). In basal thermogenesis the basal metabolic rate is raised by elevated consumption of oxygen and adenosine triphosphate (ATP) within cells leading to increased heat production throughout the body. In small mammals, mice and newborns, adaptive thermogenesis is important for survival in a cold environment. In this, the uncoupling protein UCP1 dissipates energy in brown adipose tissue (BAT) by uncoupling the proton electrochemical gradient from the ATP production within the inner membrane of mitochondria. Rodents lacking either T3 or UCP are all cold sensitive (Enerback et al., 1997; Sellers, 1950).

The body temperature of the TRα1-/-β-/- mice is reduced by 0.4 °C, which is similar to the reduction for TRα1-/- mice, whereas the TRβ-/- mice are normal. The body temperature of the TRα1 -/- mice could be elevated by T3 injections, suggesting that TRα1 is involved in setting the basal body temperature (Johansson et al., 1999; Wikstrom et al., 1998). In comparison to the TR deficient mice, hypothyroid rats have a 2-4 °C reduction in body temperature (Gordon, 1997).

Moreover the basal metabolic rate of the TRα1-/-β-/- mice is reduced by 30%, they have a 4 °C reduction in the lower critical temperature of their thermoneutral zone, and they are cold insensitive: at 4 °C their body temperature drop to critical levels within hours (Golozoubouva, manuscript). The reason for this is unclear since the mice are able to induce adaptive thermogenesis with UCP1. However, the TR deficiency seems to cause an impairment in lipolytic action. At fasting the mice are
inappropriately normoglycemic, which may be a compensating mechanism for lower fat mobilization (Tinnikov, manuscript).
GENERAL DISCUSSION

TRα1-/-β-/- mice vs other TR deficient mice

In parallel with the TRα1-/-β-/- mice described in this thesis, other mouse strains carrying targeted TR gene mutations have been described. The Samarut laboratory in France established a TRα-/-β-/- mouse line that has a phenotype distinct from that of our TRα1-/-β-/- mice. They employed a strategy yielding a strain deficient in both TRα1 and TRα2 but with elevated expression of Δα1 and Δα2 (Fraichard et al., 1997). In contrast to the TRα1/- mice by Wikström et al., the French TRα-/- mice exhibit thyroid gland misdevelopment, severe hypothyroidism, aberrant development of bone and intestine, and premature death. The dissimilarities in phenotype were suggested to be due to the remaining TRα2, Δα1, or Δα2. However, ablation of these gene products yield viable mice (Plateroti et al., 2001; Salto et al., 2001). Thus the compound TRα-/-β/-/- mice also expressed the truncated TRα forms, leading to a lethal phenotype. In their second approach to generate TR deficient mice, a larger deletion was introduced into the TRα locus, also removing the expression of the truncated forms (Gauthier et al., 2001). When crossed to their TRβ-/- strain (Gauthier et al., 1999), this yielded mice (the so called TRα0/0β-/- mice ) with a phenotype virtually indistinguishable from that of the TRα1-/-β-/- mice (Gauthier et al., 2001). The authors have therefore suggested that their original deletion of sequences in the TRα gene caused a deleterious overexpression of the Δα1 or Δα2 isoform.

Common and diverse functions of TRα1 and TRβ

In the absence of only TRα1 or TRβ the full phenotype is masked due to overlap of receptor function. The TRα1-/-β-/- mice have both exacerbated phenotypes compared to the single TR deficient mice, and an additional array of phenotypes. Since the TR isoforms appear to have redundant functions in any given tissue the question arises: is there a
TR isoform specificity in regulation of distinct function, or is it so that the most abundant TR isoform in a tissue has the greatest role in regulating target gene expression? The TRα1/-β/- mice are distinct from both the TRα1 and TRβ deficient mice in the HPT axis, regulation of GH and growth, and bone development. In the pituitary, deficiency for TRα1 results in only a mild reduction in serum TSH in male mice only, whereas deletion of TRβ results in a moderate elevation in TSH levels (ca 3-fold). That a complete dysregulation of the TSHβ gene is seen only in the TRα1/-β/- mice indicates that in the absence of TRβ, TRα1 can suppress TSH expression, albeit not sufficiently to normal levels. Mice deficient for only one TR have neither an overt growth phenotype nor reductions in pituitary GH mRNA levels. TRα1 and TRβ thus have overlapping functions in regulation of the GH gene and in controlling body growth. Similarly, growth and bone abnormalities are only seen in the TRα1/-β/- mice. Thus, an extended range of functions are controlled by common pathways in which TRα1 and TRβ interact with, or can substitute for, each other.

This suggests that the abundance of a given TR in a tissue is a major determinant of the TR isoform specificity. A similar finding on the importance of abundance concerns hearing. TRβ/- mice have a severe hearing impairment (Forrest et al., 1996a) whereas TRα1 mice are normal (Rusch et al., 1998). The TRα1/-β/- mice however exhibited a complete hearing loss accompanied by a misdevelopment of the tectorial membrane in the cochlea (Rusch et al., 2001). Superficially, this would indicate that TRα1 and TRβ have distinct function in the development of hearing. However, it was known that TRα1 was expressed at very low levels in the cochlea during development. The hypothesis thus arose: can hearing be rescued in TRβ deficient mice if TRα1 expression is increased to levels similar to those for TRβ? Therefore, the TRα2/-β/- mice were developed, expressing TRα1 at 3- to 6-fold elevated levels. Subsequent analyses showed that not only was the hearing restored completely despite the absence of TRβ, but the regulation of TSH production was also normalized (Ng et al., 2001c; Salto et al., 2001).

The ability of TRα1 to substitute for TRβ in the liver has also been addressed. Here, a hallmark of TRβ action is its ability to lower serum cholesterol levels (Gullberg et al., 2002). TRβ/- as well as TRα1/-β/- mice were completely unable to perform this function whereas TRα1/-
mice were normal. Surprisingly, the TRα2/-β/- mice (which overexpress TRα1 3- to 6-fold in the liver) were also unable to lower serum cholesterol as a response to T3 (Gullberg et al., 2002). At first, this situation suggested an isofom specificity in lowering serum cholesterol. However, a close examination of the expression patterns of TRα1 and TRβ in the liver demonstrate that whereas TRα1 is present over a wide zone of the functional liver unit, expression of TRβ is confined to a narrow zone around the portal vein. Furthermore, the genetic trick employed with the TRα2/-β/- mice failed to attain levels of TRα1 expression in the periportal zone comparable to those for TRβ in wt mice (Gullberg et al., manuscript). A similar microheterogeneity of TR expression is likely to occur in the various regions of the brain. Thus our data suggest that the isofom specificity lies not primarily in the promotors of the TR target genes, but rather in the promotors of the receptors themselves. According to this hypothesis, the promotors for the TRs govern not only the levels of the expression of a particular TR isoform in a given tissue, but also the temporal window in which a receptor may be expressed in a given tissue. This does not however exclude that intrinsic structural differences between the TRs, as seen primarily in their N-terminal domains, have important functions in fine tuning the physiological response to T3.

Deficiency of T3 vs deficiency of TR

Both congenital and adult hypothyroidism has debilitating consequences. It was therefore unclear at the outset of this project if the creation of a mouse strain devoid of all known thyroid hormone receptor genes would be possible at all. Such a mouse would hypothetically present a severely hypothyroid phenotype, and was at the outset of the project expected to be aborted, stillborn, or to die shortly after birth. The phenotypes of TRα1/- and TRβ/- mice are remarkably limited as these represent only a few of the symptoms of hypothyroidism. Would a mouse, generated by crossing the two single knockout strains, better reflect the symptoms of congenital hypothyroidism and/or endemic cretinism? It is therefore remarkable that TRα1/-β/- mice that are devoid of all TRs from conception thrive relatively well. This indicates that nuclear TRs in general are nonessential for life.
What is the explanation for the milder phenotype caused by absence of TRs as compared to loss of thyroid hormone? Several possibilities have been considered, including the existence of hypothetical, as yet unidentified TRs. No evidence was however found for such a TR in the TRα1-/-β-/- mice. Tissue nuclear preparations exhibit no specific T3 binding capacity and contain no detectable TR proteins that bind T3 responsive elements, making it unlikely that there are novel nuclear TRs, at least in major tissues. Non-TR-mediated actions of thyroid hormone may also help explain the mild phenotype. TRα1-/-β-/- mice have an excess of thyroid hormones but show no signs of conventional T3 response: there are no thyrotoxic symptoms of hyperactivity, tachycardia or hyperstimulated metabolism. Nor does the induction of hypothyroidism alter major functions that would normally be T3-dependent. Thus, while extra-receptor mechanisms presumably remain intact in TRα1-/-β-/- mice, they seem unlikely to modulate the phenotype in a major way.

Functions of unliganded TRs may cause the insults seen in hypothyroidism. In vitro, in the absence of T3, TRs bind to target DNA, in association with corepressors to suppress basal transcription of reporter genes that would be activated by the T3-bound TR. Although the TR deficient mice have very high levels of TH they are hypothyroid at the tissue level. The comparison between the TR deficient mice and congenitally hypothyroid rodents indicates a fundamental difference between the phenotype caused by TR deficiency and hypothyroidism caused by unliganded TRs.

What are the differences between living without the hormone T3, and living without its receptor, TR? Several characteristics of the TRα1-/-TRβ-/- mice differ radically from that seen in hypothyroid mice. The data on the HPT axis show similarities with and differences from what is seen in a congenitally hypothyroid wild-type mouse. Both express similar, high levels of TSH as indications of hypothyroidism. Only the mutant mice, however, show both elevated serum levels of TH as well as goitre. Our data thus illustrate well the role of the ligand bound TR in suppressing TSH and therefore also its role in the HPT axis. Hypothyroidism in e.g. adult rodents results in weight loss and a severe impairment in the control of body temperature (Gordon, 1997). The TRα1-/-β-/- mice however do not loose weight when treated to achieve
hypothyroid T3 levels, in contrast to what found in the wt controls. Moreover, their body temperature is reduced by only 0.4 °C (Johansson et al., 1999), in contrast to what seen in wild-type mice (Gordon, 1997; Mansouri et al., 1998). This indicates the ability of the unliganded TR to act as a potent transcriptional regulator.

The difference between TR deficiency and hypothyroidism is further emphasised by comparing the Pax8-/- mice with the compound Pax8-/- TRα0/0β-/- mice: the former exhibit all the hallmarks of severe hypothyroidism, whereas the latter are virtually indistinguishable from our TRα1/-β/- mice. Unliganded TRα1 is the likely cause of the adverse effects of hypothyroidism in the Pax8-/- animals (Flamant et al., 2002; Mansouri et al., 1998).

**Deficiency of T3 vs deficiency of GH**

T3 is known to stimulate chondrocyte maturation in vitro (Ohlsson et al., 1992) as well as their hyperproliferation in the growth plate (Lewinson et al., 1989). The growth phenotype for the TRα1/-β/- mice involves both impaired maturation of the epiphysis and retarded elongation. The bone phenotype of the mutant mice resembles that seen in hypothyroid mice although the Pax8-/- mice have a more severe bone morphology as compared to TR deficient mice (Flamant et al., 2002).

Thus the GH treatment reversed the growth phenotype in terms of bone dimensions and body weight. On the other hand, the effects on cartilage, bone ossification as well as bone mineral density and content were unaffected by GH treatment. A wider epiphyseal plate was still persistent despite the GH treatment. This indicates that the cause of the growth and bone alterations in the mutant mice can be mechanistically separated into two pathways. The growth deficiencies appear to be due to the failure of the mice to properly produce GH and therefore also IGF-1, as revealed by the normalisation by GH treatment. On the other hand, the effects on cartilage and bone mineralization were unaffected by GH treatment, lending further support to the direct role of T3 on bone. It has recently been suggested that T3 enhances a pathway in which the fibroblast growth factor and one of its receptors
(FGFR-1) mediate osteoblast activity and bone metabolism (Stevens et al., 2003).

By comparison to mice deficient in only a single TR, that have neither overt growth phenotypes nor reductions in pituitary GH mRNA levels, our results suggest that the TRs have overlapping roles in control of body growth and regulation of the GH gene expression. Thus, these receptors act together in mediating both the indirect effects via GH and the proposed direct effects of T3 on bone.

Various responses to T3 in adult brain

Mice that lack a TR gene present neurological alterations. TRα1 deficiency gives behavioral alterations (anxiety) associated with dysregulation of hippocampal function (Guadano-Ferraz et al., 2003). Deficiency for TRβ1 leads to a susceptibility for audiogenic seizures (Ng et al., 2001b; Rusch et al., 1998) despite impaired hearing (Forrest et al., 1996a). TRβ2 is required for development of color vision and its deletion result in both perturbed levels and distribution of photoreceptor levels (Ng et al., 2001a).

A microarray analysis is an unbiased and hence exciting method for detecting changes in expression levels for a large number of candidate genes e.g. as a response to a hormone in a given tissue. How sensitive the brain is to T3 levels is an ongoing issue. A recent report illustrated difficulties in inducing hyperthyroidism and concluded that the brain strongly defends its T3 levels, more than other tissues (Broedel et al., 2003). In contrast, that T3 injections increase pituitary GH levels in 2 hours (Tinnikov et al., 2002) as well as the present results indeed underscore that the adult brain is as T3 sensitive as observations from the clinics indicate (Bauer and Whybrow, 2001; Fukui et al., 2001; Sher, 2001).

Still, few genes showed more than 3-fold change in expression levels. Adult brain genes may in general be only moderately regulated by T3, or the brain is not critically dependent on the hormone. Also, 3 hours may be too short for higher expression levels to be achieved. On the other
hand, as the brain is a highly complex organ with temporal and spatial differences in expression, a gene responding to T\textsubscript{3} with a high expression level in a particular region will most likely undergo a dilution effect as the whole brain is screened. Therefore the detected genes may be those affected in the whole brain or those with extreme alterations in expression levels in certain regions.
SUMMARY

Living without TRs is less harmful than living without T3, as shown by comparing mice lacking all TRs to animals or patients deficient in T3. This underscores the importance of repression that unliganded TRs exert on their target genes.

The use of several TR isoforms contributes to diversity and fine tuning of the effects of T3. Nevertheless, our data also demonstrate that TRs can substitute for each other in a variety of tissues.

T3 seems to have direct, TR mediated effects on bone development. The lack of TRs also causes a reduction in GH, the primary but indirect reason for a retarded bone development.

Many genes in the adult brain respond to T3, of which a large fraction (59/149) are independent of TR.
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