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New Insights into Thyroid Hormone Action

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Abstract

Thyroid hormones (TH) are endocrine messengers essential for normal development and function of virtually every vertebrate. The hypothalamic-pituitary-thyroid axis is exquisitely modulated to maintain nearly constant TH (T4 and T3) concentrations in circulation. However peripheral tissues and the CNS control the intracellular availability of TH, suggesting that circulating concentrations of TH are not fully representative of what each cell type sees. Indeed, recent work in the field has identified that TH transporters, deiodinases and thyroid hormone receptor coregulators can strongly control tissue-specific sensitivity to a set amount of TH. Furthermore, the mechanism by which the thyroid hormone receptors regulate target gene expression can vary by gene, tissue and cellular context. This review will highlight novel insights into the machinery that controls the cellular response to TH, which include unique signaling cascades. These findings shed new light into the pathophysiology of human diseases caused by abnormal TH signaling.

Introduction

Thyroid hormones modulate gene expression in virtually every vertebrate tissue; their actions are finely tuned by a series of conserved pathways, which orchestrate the onset of crucial physiological processes for normal development, growth and energy metabolism. Since the cloning of the thyroid hormone receptors in 1986 three decades of intense research have enlightened our knowledge on the molecular basis of thyroid hormone action. In the present review we will address the new insights into thyroid hormone action that have been published within last four years.

The Hypothalamic-Pituitary-Thyroid axis

Thyroid hormones (TH) are essential for growth and development in virtually every vertebrate including humans. TH synthesis and secretion is finely modulated by the hypothalamic-pituitary-thyroid (HPT) axis. It is well appreciated that thyroid hormone production is governed by this central axis that begins in the paraventricular nucleus of the hypothalamus and proceeds through the pituitary before engaging the thyroid. Indeed,

Conflict of Interest

The authors declare that there are no conflicts of interest.

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human mutations have been described at every level of the axis and all cause hypothyroidism (Medici, Visser, Visser, & Peeters, 2015). Importantly, circulating thyroid hormones (TH) negatively feedback to the central components of the axis to regulate levels. Today, pituitary-secreted thyroid-stimulating hormone (TSH) remains the most important and universal biomarker of TH action in humans.

The hypothalamic section of the axis is represented by the thyrotropin-releasing hormone (TRH)-neurons in the paraventricular nucleus (PVN), which secrete TRH in response to a series of environmental and physiological stimuli. Interestingly, TRH neurons in the PVN co-secrete the neuropeptide cocaine- and amphetamine-related transcript (CART) but its role remains unclear (Hollenberg, 2008). Several other important neuronal populations exist in the PVN including corticotropin-releasing hormone (CRH), vasopressin and oxytocin neurons, which each have an important endocrine function. The TRH gene encodes a prepro-hormone that is ultimately processed to a tripeptide with the sequence of glu-his-pro (Nillni & Sevarino, 1999). Released into the median eminence, TRH induces the secretion of thyroid-stimulating hormone (TSH) from the anterior pituitary, which in turn accentuates the synthesis and secretion of thyroid hormones from the thyroid gland into the circulation (Figure 1). TRH released into the median eminence acts on the TRH-receptor located in the membrane of thyrotrophs, the TSH-producing cell in the pituitary. Thyrotrophs lose their ability to secrete TSH when disaggregated in cell culture, suggesting a functional role for the architecture of the anterior hypophysis of the pituitary and the possible participation of other signaling factors to modulate thyrotroph sensitivity to TRH *in vivo* (Bargi-Souza et al., 2015). The HPT axis plays the major role in maintaining the homeostasis of circulating TH levels as circulating T4 and T3 feed back to both the hypothalamus and the pituitary to regulate TRH and TSH production (see Negative Regulation by Thyroid Hormone).

Remarkably, the set point of the HPT axis differs between individuals (Fitzgerald & Bean, 2016). This is likely due to genetic factors. Genome-wide association studies (GWAS) analyzing different populations of euthyroid humans have identified polymorphisms associated with differences in circulating levels of TSH and T4 within the normal range of concentration. The loci associated with TSH levels are PDE8B, PDE10A, CAPZB, MAF/LOC440389, VEGFA, NR3C2, IGFBP5, NFIA, SOX9, PRDM11, FGF7, INSR, ABO, MIR1179, NRG1, MBIP, ITPK1, SASH1, GLIS3, whereas free T4 (FT4) was associated with DIO1, LHX3, FOXE1, AADAT, NETO1/FBXO15, LPCAT2/CAPNS2, (Malinowski et al., 2014; Porcu et al., 2013; Taylor et al., 2015; Zhan et al., 2014). The role of these polymorphisms in the modulation of TSH and FT4 are just starting to be elucidated, however many of these genes appear to have their actions in the thyroid. For example, polymorphisms in PDE8B, a phosphodiesterase expressed in the thyroid, are associated with high circulating levels of TSH together with slightly decreased FT4 levels (G. Roef et al., 2013). This is consistent with PDE8B regulating TSH action in regulating the synthesis of thyroid hormone (Figure 2). In thyroid tumor patients only, polymorphisms in another thyroid specific gene CAPZB were found to be associated with lower TSH levels (Feng et al., 2015). Polymorphisms in NRG1 and FOXE1 were associated with a risk of follicular adenoma (Rogounovitch et al., 2015). Polymorphisms in FOXE1 are also associated with congenital hypothyroidism (Carre et al., 2014). Some polymorphisms have little to no effect in patient care such as the type 1 deiodinase (DIO1), which does not change the dose of T4

required for TSH suppression in thyroidectomized patients (Santoro et al., 2014). Recently, hypothyroidism has been associated with both neurological disease and a longer life span. Levels of TSH have been associated with longevity, schizophrenia or bipolar disorder in two independent studies, suggesting that octogenarians and/or patients with schizophrenia have higher concentrations of circulating TSH (Jansen et al., 2015; Wysokinski & Kloszewska, 2014). Despite the fact that TSH has long been interpreted as a major marker of thyroid status it only reflects the effects of thyroid hormone signaling in the hypothalamus and pituitary, thus identifying novel markers of thyroid hormone action in the periphery becomes paramount.

Thyroid Hormone Transporters

Thyroid hormones (T4 and T3) are primarily protein bound moieties in the circulation and are in equilibrium with free T4 and T3, which are biologically active (Figure 3). The ratio of protein bound T4 to FT4 is higher than T3, which partly explains its much longer half-life and role as a pro-hormone. In recent years, it has become clear that circulating T4 and T3 do not passively cross cell membranes such as those present in liver, thyroid follicular cells or astrocytes and neurons in the brain. Several T4 and T3 transport proteins have been identified including: 1. The monocarboxylate transporters MCT8 and MCT10; 2. The organic anion transporter OATP1 and 3: L-type amino acid transporter LAT. MCT8 has preference for T3, whereas T4 and rT3 are preferentially transported by OATP1. LAT transports both T4 and T3 but with relative lower affinity (Jayarama-Naidu et al., 2015; Schwabedissen et al., 2011).

The most clinically relevant thyroid hormone transporter is the MCT8 (Friesema, Visser, & Visser, 2010). Mutations in this transporter have been found in patients with the Allan-Herndon Dudley Syndrome (AHDS). AHDS is an X-linked disorder, which is heralded by potentially severe neurologic complications including mental retardation and movement disorders. Patients with AHDS also exhibit abnormal thyroid function tests that include a normal TSH with a low free T4 and high levels of circulating T3. It is very likely that the neuronal disorder is secondary to the inability for T3 to gain access to neurons during development. This was shown in a recent study of human brains from a fetus and child with AHDS. Both brains had anatomic and structural changes consistent with severe abnormalities of development concurrent with greatly diminished cerebral T3 levels (Lopez-Espindola et al., 2014). Also, the relationship of AHDS to a deficiency of T3 transport has been solidified by studies showing that human fetal and neonatal brain from patients with AHDS look remarkably similar to brain from humans with severe hypothyroidism (Gagliardi et al., 2015; J. H. Kim et al., 2015). The primary structure of MCT8 forms twelve trans-membrane subunits that are predicted to form a canal that facilitates the bidirectional transport of T3 in favor of its gradient of concentration. Interestingly, dimerization of MCT8 also appears to be necessary for its function (Fischer et al., 2014). The inner part of the canal contains residues of several amino acids, which appear to interact directly with the ligand. For example histidine 192 is required for T3-transport specificity (Braun, Lelios, Krause, & Schweizer, 2013) (Groeneweg, Lima de Souza, Visser, Peeters, & Visser, 2013), whereas mutation of Arg445 and/or Asp498 induced complete ablation of TH transport in cell lines. Both of these mutations have been found in patients with Allan-Herndon Dudley Syndrome

(Groeneweg et al., 2014). Transporters may also play a role in determining circulating levels of thyroid hormones. Indeed, MCT8 is expressed on the basolateral membrane of thyrocytes and appears to be necessary for T4 efflux from the gland (de Souza et al., 2015) (Trajkovic-Arsic et al., 2010). Furthermore, two SNPs in MCT8 have been associated with differences in circulating T3: the first SNP causes a missense mutation in serine107 to proline; the second is intronic and the effect on transporter expression is not yet understood (G. L. Roef et al., 2013).

Above all, TH transporters are key to TH action and function in humans. Their patterns of expression in stages of development and tissue-type modulate circulating and peripheral thyroid hormone availability. As discussed, mutations in the MCT8 gene highlight the necessity of transporters. In mice, mutation of the MCT8 recapitulates the thyroid function phenotype found in humans; however, the underlying cause of increased T3 and low T4 remains an open question. Remarkably MCT8 deficient mice are neurologically normal, most likely due to the co-expression of the OATP1 transporter during development. Mice that lack both MCT8 and OATP1 develop neurological disorders similar to the human disease (Mayerl et al., 2014). Human neurons express only MCT8 and do not have the redundancy of OATP1.

Studies in which the type 1 deiodinase (see Deiodinases section below) was ablated specifically in liver of MCT8KO mice did not prevent the increase in circulating T3 and low T4 (Wirth, Rijntjes, Meyer, Kohrle, & Schweizer, 2015), whereas the global ablation of Dio1 in MCT8KO mice normalized T3 and T4 in circulation. Thus, Dio1 activity in tissues other than liver, accounts for the pathologic thyroid status in MCT8KO mice (Liao et al., 2011).

As mentioned above, the relationship of AHDS to T3 transport deficiency has been highlighted by several studies in humans. Thus, the available mouse models of AHDS provide important pre-clinical utility in testing novel approaches and treatments. Indeed, therapeutic strategies to overcome the poor transport of TH in MCT8 deficiencies include the use of thyroid hormone analogs such as TRIAC, TETRAC and DITPA as the cellular uptake of these T3 analogs is MCT8-independent (de Vrieze et al., 2014) (Ferrara et al., 2014) (Mayerl et al., 2014). In addition to mouse, zebrafish have also been shown to be a suitable model to analyze the neurologic phenotype of MCT8 ablation (de Vrieze et al., 2014), (Vatine et al., 2012).

New insights have also determined that TH transport can be regulated. Indeed, the expression of MCT8 and OATP1 in the mouse brain was transiently diminished in response to an acute inflammatory challenge by lipopolysaccharide LPS (Wittmann et al., 2015). Moreover, studies in the developing brain of the chicken show that transporter mRNA expression mirrors the increasing intracellular T3/T4 ratio (Van Herck et al., 2015). Particularly, thyroid status modulates the expression of MCT8, MCT10 and OATP1 in the brain of teleosts. High levels of T3 down-regulated the expression of these transporters whereas methimazole induced hypothyroidism and increased transporter expression. Take together, this suggests that transporter genes are negatively regulated by thyroid hormone (Muzzio, Noyes, Stapleton, & Lema, 2014). At the protein level, long-term iodine deficiency increased the localization of MCT8 to the membrane of thyrocytes, and this effect was

prevented by treatment with T3 (Hu et al., 2014). In contrast, short term iodine overload down-regulated MCT8 mRNA but not LAT2 in the thyroid gland (de Souza et al., 2015).

The Deiodinases

Once thyroid hormones are synthesized they are subjected to further metabolism by a family of deiodinases. Deiodination is the process by which T4 is converted to its bioactive form T3, which can take place within the thyroid. However, TH levels are predominantly regulated by deiodinases in target tissues or cell types (St Germain, Galton, & Hernandez, 2009). Three iodothyronine deiodinases catalyze the stereospecific removal of iodine from T4 and T3 (Figure 3). The type 2 deiodinase (D2) activates T4 by outer-ring deiodination (ORD) to produce T3. The type 3 deiodinase (D3) deactivates T4 and T3 by inner-ring deiodination (IRD) to produce rT3 or 3',3-T2 respectively. The type 1 deiodinase (D1) catalyzes both ORD and IRD (Gereben, McAninch, Ribeiro, & Bianco, 2015). Recently, elucidation of the crystal structure of a portion of D3 (its globular domain) allowed us to understand that the deiodinases may recognize iodothyronines through a similar histidine-arginine clamp that is employed by both transporters such as the MCT8 and by the thyroid hormone receptors (Schweizer, Schlicker, Braun, Kohrle, & Steegborn, 2014).

As outlined the cell specific expression of deiodinases has the ability to determine the amount of T3 available. D3 activity is higher during late developmental and early postnatal stages, however it decreases to almost undetectable levels in most tissues after birth. In the cerebellum, mice that lack D3 mice show impaired foliation characterized by thinner external and internal germinal layers (EGL IGL) (Peeters et al., 2013). D2 and D3 play a role in the developing cochlea: D3 acts prior to the onset of hearing by preventing the premature response to TH and after birth D2 amplifies T3 levels to triggers the onset of auditory function (Ng et al., 2004; Ng et al., 2009). Silencing D3 during development has detrimental effects as observed in zebrafish, which results in delayed hatching, significantly smaller size, and decreased inflation of the swim bladder (Heijlen et al., 2014). Striking studies in different species show that reactivation of D3 expression occurs in early phases of tissue regeneration e.g. liver, muscle and fin. Notably, muscle satellite cell-specific ablation of D3 prevented muscle regeneration after cardiotoxin analogue III (CTX) injection due to apoptosis of satellite-cells (Dentice et al., 2014). Moreover, D3 activity has been shown to increase in a mouse model of myocardial infarction, which may be an adaptive response to decreased metabolism or a marker of tissue regeneration (Janssen et al., 2013). In both development and tissue regeneration, D3 activity is high and tissue concentrations of T3 are low suggesting that cellular differentiation occurs and/or prefers a hypothyroid environment.

The majority of human hypothyroidism is treated with L-thyroxine (T4) and thus requires deiodination to T3 for physiological activity. There has been significant interest in whether changes in deiodinase function could impair L-thyroxine therapy. A recently identified polymorphism in D2 segregates with decreased clinical effect of T4 therapy in humans (Panicker et al., 2009). This Ala92 change in the enzyme has been predicted to impair function of the enzyme. For example, patients with mild cognitive impairment carrying the Ala92-D2 SNP showed decreased levels of circulating T3 (Luo, Zhou, Zou, Keyim, & Dong, 2015). However, four SNPs identified in patients with primary hypothyroidism on T4-

treatment showed no effect on circulating TSH and fT3 (Al-azzam, Alkhateeb, Al-Azzeh, Alzoubi, & Khabour, 2013). To further address how the Ala92-D2 SNP affects D2 function, McAninch *et al* analyzed the expression of gene targets in human brain from individuals known to express this polymorphism. Surprisingly, the Ala92-D2 polymorphism modified the profile of gene expression in human brain without modifying the expression of TH-target genes suggesting that D2 could influence the expression of other genes through TH independent pathways (McAninch et al., 2015).

New insights into tissue-specific D2 activity have underlined the necessity for new biomarkers of TH action. D2 activity is modulated through T4-induced degradation via reversible ubiquitination, which is ER-localization specific and requires a D2 conserved lysine in the globular domain (Egri & Gereben, 2014). D2 ubiquitination is modulated by WD repeat and SOCS box containing 1 (WSB-1) in a tissue-specific manner. Within the brain, the hypothalamus D2 activity is not sensitive to T4, whereas the hippocampus and cortex showed increased D2 activity in astrocyte-specific WSB-1-KO mice (Werneck-de-Castro et al., 2015). Since most T3 in the brain is produced from T4, local T3 availability could be dependent upon neuronal sensitivity to D2 degradation. In the skeletal system, the expression of D2 is modified by SNPs and associated with osteoarthritis (OA). D2 up-regulation and D3 down-regulation causes locally increased levels of T3 in chondrocytes. This promotes the expression of the metalloproteinase ADAMTS5, which degrades the cartilage in joints (Nagase et al., 2013). The SNP rs225014 upstream of D2 has been associated with risk of osteoarthritis and carriers of this allele show defective epigenetic silencing of D2 in chondrocytes (Bomer et al., 2015). In contrast, D2 ablation in mice induced increased mineral content in bones with a normal cartilage phenotype (Waung, Bassett, & Williams, 2015). Further work will be required to delineate the role of deiodinases and T3 production in osteoarthritis.

D1 is the main deiodinase expressed in the thyroid gland and is highly upregulated during iodine deficiency (Lavado-Autric, Calvo, de Mena, de Escobar, & Obregon, 2013). Dio1 catalyzes both ORD and IRD with equimolar efficiency. Experimental evidence suggests that Dio1 functions as an iodine scavenger in the kidney whereas in the thyroid gland and liver it generates significant amounts of circulating T3 particularly in hyperthyroid patients. Insights from transgenic models show that Dio1 is a TR β 1-target (Forrest et al., 1996). Thus, the global ablation of TR β 1 downregulates Dio1. Dio1 is the most highly regulated T3-target, however the role of Dio1 on circulating T3 in euthyroidism remains controversial. Nevertheless SNPs in DIO1 found in several independent GWAS studies indicate that Dio1 plays a primary role in determining the set point of circulating T3 in euthyroid patients. Also, testosterone may play a role in Dio1 activity as observed in orchidectomized rats as the replacement with testosterone after surgery was shown to maintain normal Dio1 activity in liver (Sosic-Jurjevic et al., 2015).

Thyroid Hormone Receptors

T3 action is completely dependent upon its cognate receptors, the thyroid hormone receptor isoforms (TRs) (Figure 4). Members of the nuclear receptor superfamily, the TRs are ligand-activated transcription factors that interact with T3 via a C-terminal ligand-binding domain

(LBD) (Figure 5). Conformational change in the LBD dictates action of the TR based upon its recruitment of coregulator proteins, which mediate epigenetic change (see below). There are three T3-binding isoforms: TR β 1 and TR β 2 are alternatively spliced products of a single gene while TR α 1 is encoded for by a separate gene but shares a very high degree of structural homology with the TR β isoforms. TR α 2 has a separate C-terminal extension and does not bind T3. The functional role of each TR isoform appears to be most dictated by its tissue-specific expression as shown by multiple mouse models where individual receptor isoforms have been knocked-out (Brent, 2012).

Resistance to thyroid hormone (RTH) syndrome has gleaned significant insight into the role of TR isoforms in humans. RTH is due to mutations in the TR isoforms that cause distinct syndromes based on the tissue distribution of the receptor. For example mutations in the TR β isoforms lead to the syndrome of resistance to thyroid hormone beta (RTH β), which is characterized by inappropriately elevated levels of TSH in the face of high levels of T4 and T3 due to defective TH feedback at the level of the pituitary and hypothalamus (Forrest et al., 1996). Additionally, there is significant RTH in the liver while tissues such as the heart are in fact hyperthyroid. This variable phenotype is due to the presence of the TR β isoforms in the pituitary, hypothalamus and liver while TR α is primarily expressed in other tissues including the heart. Thus, TR α expressing tissues see the high TH levels and become functionally hyperthyroid.

In contrast, RTH in patients due to TR α mutations has a phenotype that includes short stature with constipation, bradycardia and mild intellectual impairment due to the primary presence of TR α in the GI tract, heart, bone and many neuronal subtypes in the brain (Schoenmakers et al., 2013). Importantly, RTH secondary to TR α mutations were not easily identified because only mild abnormalities were found in thyroid function tests unlike RTH β . Still more cases of RTH due to TR α are likely to be identified given the potential for some mutations to cause high-normal T3 levels with low-normal T4 levels and low reverse T3 levels. Similar to RTH due to TR β , there is a phenotypic spectrum in humans with TR α mutations depending upon the severity of the mutation in context of impairing T3-binding. Finally, as will be discussed later, the deleterious function of mutant TRs in both RTH α and RTH β is likely due to excessive recruitment of nuclear corepressors and ultimately deacetylation and repression of TR target genes (C. Moran & Chatterjee, 2016; Carla. Moran & Chatterjee, 2015; Schoenmakers et al., 2013; Vlaeminck-Guillem, Espiard, Flamant, & Wemeau, 2015).

To better understand how the TR isoforms mediate their transcriptional properties investigators now use novel approaches to characterize their role on a genome wide level (Table 1). Studies in cell culture using HeLa or HepG2 cells stably expressing TR α 1 or TR β 1 have shown that both TRs can modulate the same genes in the same fashion, however TR isoform specific signaling patterns were observed on some target genes (Lin et al., 2013). Similarly, specific sets of genes had a receptor-selective response to T3 in the neural cell line C17.2 stably expressing TR β 1 or TR α 1 (Chatonnet, Guyot, Benoit, & Flamant, 2013). In this study, each isoform had a separate cistrome indicating that some of the differences in gene expression could be secondary to differential binding properties of the individual

receptors. Further analysis of the isoform-specific effects of the individual TRs will require genetic swapping experiments *in vivo*.

To determine how the TRs function *in vivo* investigators have now characterized the binding sites of the TR β isoform in the mouse liver using a variety of techniques. Mice expressing a biotin-tagged TR β 1 in the liver showed enrichment of binding sites mostly in intergenic regions but also demonstrated binding sites in proximal regions or introns of target genes (Ramadoss et al., 2014). Likewise, in HepG2 cells that stably express TR β , the TR bound to clusters of binding sites in intergenic, promoter and upstream regions of responsive genes (Ayers et al., 2014). Of note, these studies used motif enrichment analysis and determined that a DR+4 motif was the most common target for the TR (Ramadoss et al., 2014) (Chatonnet et al., 2013; Grontved et al., 2015). Finally, genome wide Chip-seq and DNase hypersensitivity analysis showed extensive chromatin remodeling in response to T3 stimulation, which indicates ligand-induced recruitment of TR β 1 to binding sites. Ultimately, this suggested that not all TRs are bound to DNA in the absence of ligand as proposed by the current consensus model (Ramadoss et al., 2014). This notion is supported by data showing that subcellular localization of the TR is facilitated by a conserved nuclear localization sequence that is harbored in the helix 3 and 6 of the LBD (Mavinakere, Powers, Subramanian, Roggero, & Allison, 2012). Recently it has been shown that the importin α 1, β 1 and 7 translocate TR α 1 from the cytosol into the nucleus (Roggero et al., 2016).

These genome wide binding studies have also established that RXR α ChIP peaks overlap with TR β 1 ChIP peaks in both the hypothyroid and hyperthyroid state. This further reinforces the notion that TR β 1 functions as heterodimer with RXR α (Ramadoss et al., 2014) (Grontved et al., 2015). Notably, these studies also suggest that TR homodimers or monomers could be bound to TREs *in vivo*, which strengthens previous *in vitro* studies that showed gene repression is preferentially mediated by TR homodimers (Machado et al., 2009; Makowski, Brzostek, Cohen, & Hollenberg, 2003). In contrast to positive targets, negative targets of T3 were not as commonly associated with TR β 1 binding peaks, suggesting that many T3-negative targets do not bind TR β 1 directly (Grontved et al., 2015). However in some instances the ligand-bound TR β 1 was recruited to certain negative T3 targets. This implies that there could be a role for direct TR binding in some instances of negative regulation (J. Y. Kim, Son, Kim, & Lee, 2010) (Ramadoss et al., 2014). While genome-wide studies are lacking *in vivo* data for TR α 1, Dudazy-Gralla *et al* took advantage of a mouse model that expresses a TR α 1-GFP fusion protein from the endogenous locus. *In vivo* ChIP assays using this model confirmed TR binding sites in known T3-regulated genes in the brain such as Hairless and identify a novel T3-regulated gene (RNF166) via its ability to recruit TR α 1 to its 3' untranslated region (Dudazy Gralla et al., 2013).

The ability to technically perform ChIP-seq *in vivo* has certainly emphasized the importance of the genomic actions of the TR isoforms. Still, it is clear that the TRs can function in a non-genomic fashion. Indeed many actions of T3 may occur too quickly to be mediated by enhanced or repressed target gene expression. Martin *et al* have identified two tyrosine motifs in the second zinc finger of TR β 1 that are not present in TR α 1. In the absence of T3 the TR β 1 isoform can bind the regulatory subunit of PI3K. The addition of ligand dissociates this interaction and causes an increase in PI3K signaling. Remarkably, mutating

one of these tyrosines in the TR β 1 isoform *in vivo* using a knock-in strategy did not impair classical nuclear TR β 1 signaling in context of thyroid function or regulation of genomic targets. When hippocampal T3-signaling was examined in this mutation, there were significant impairments in synaptic strength and plasticity demonstrating a developmental defect in PI3K signaling likely caused by the absence of TR β 1 (Martin et al., 2014). A role for TR β 1 signaling via PI3K has also been demonstrated in the thyroid where a mutant TR β 1 can activate growth via PI3K (Furuya, Lu, Guigon, & Cheng, 2009).

Post-translational modifications have been shown to play a major role in modulation of transcription factor activity including TRs. Both TR isoforms can undergo sumoylation via separate pathways but on specific lysine residues. Importantly, sumoylation appears to be critical for the release of corepressors and the recruitment of coactivators and is required for a normal response to hormone stimulation. In this context, TR- sumoylation is needed for normal differentiation and growth of a human preadipocyte cell line, 3T3L1 cells. Targeted mutation of several lysine residues known to be sumoylated in human TRB1 impairs growth, differentiation and lipid storage of these cells (Y. Y. Liu, Kogai, Schultz, Mody, & Brent, 2012). Moreover, this phenotype was attenuated by blocking the TR's interaction with the nuclear receptor corepressor, which indicates that TR-sumoylation is needed for a normal interaction with coregulators (see Coregulators section below) (Y.-Y. Liu et al., 2015)

While these signaling and genomic strategies are beginning to illuminate novel functions of thyroid hormone receptors *in vivo*, mouse models are elucidating their isoform-specific effects. Using a unique conditionally – targeted *thrb* mouse model, Selma-Ruby *et al* have demonstrated a role for TR β directly in thyroid follicular cells. Thyrocyte-specific deletion of TR β in mice leads to increased circulating levels of T4 and rT3, low TSH concentration and normal free T3 levels (Selmi-Ruby et al., 2014). Clearly, mice that lack all TRs develop follicular cells. Thus, it is likely that the TRs play little role in follicular cell development. Instead TRs may provide another layer of feedback to the HPT axis. In addition to the thyroid follicular cell, evidence exists for TR β expression in the adrenal gland. Huang *et al* used a mouse model that marked TR β 1 expression with β -galactosidase and saw expression in a region of the adrenal cortex that expresses 20- α -hydroxysteroid dehydrogenase. Interestingly, this region in mouse regresses early in life in males but not in females. This differentiation process appears to be T3-dependent via TR β 1. Whether there is role for T3-signaling in adrenal function in humans remains unknown (Huang, Kraft, Moy, Ng, & Forrest, 2015).

As discussed previously, TH has profound impact on the developing brain. While neurons express both TR isoforms, resolving their specific roles in individual neurons has been difficult. To overcome this Fauquier et al have developed a mouse model where a dominant negative TR α 1 allele can be expressed in a cell-specific manner. Using the cerebellum, the investigators induced the mutant TR in a variety of cell-types. Remarkably, its expression in Purkinje cells and Bergman cells was able to affect all cerebellum development suggesting that TH-signaling can control cell function indirectly by regulating the environment or other signaling pathways (Fauquier et al., 2014). Using a different TR α 1 mutant mouse model, Mittag et al demonstrated another example of the neuronal effects of TR α 1. A select group of TR α 1 expressing neurons in the anterior hypothalamus exerts specific control over blood

pressure and heart rate. This indicates that TR α 1 effects in the cardiovascular system may be also controlled indirectly via the CNS (Mittag et al., 2013). Finally, this same mouse model has demonstrated a unique role for TR α 1 in mediating endothelial vasoconstriction in the tail vein of mice. This work has profound implications for energy expenditure studies using animal models. In this particular study, enhanced heat loss from the tail was compensated for by an increase in energy expenditure. This underlines the importance of accounting for tail heat loss in animal studies. If not done, it could be incorrectly assumed that the model has a primary increase in energy expenditure (Warner et al., 2013).

Coregulators

The classic view of TH action on positively regulated TR targets is that gene expression is mediated at the genomic level by the TRs either in the presence or absence of T3. In the absence of T3 or when present in low abundance, the TRs recruit a multiprotein complex that includes the nuclear corepressors, NCoR1 and SMRT, which in turn recruit HDAC3 and other proteins to mediate transcriptional repression via histone deacetylation (Sun et al., 2013; You et al., 2013). The presence of T3 leads to dismissal of the corepressor complex and recruitment of group of coregulators that include the SRC family of coactivators as well as p300/CBP and other transcriptional activators that lead to histone acetylation and transcriptional activation (Astapova, 2016) (Figure 4). However, recent findings have challenged this model and the exact mechanisms of transcriptional repression in hypothyroidism and activation in hyperthyroidism remain unclear.

To understand the role of corepressors and coactivators in TH action, Vella *et al* developed a model disrupting both NCoR1 and SRC-1 action. An SRC-1 knockout mouse was crossed to a mouse expressing a mutant NCoR1 allele, NCOR ID, that cannot interact with the TR (Figure 6) (Astapova et al., 2008). Previous work had already established that NCoR1 and SRC-1 played specific roles in TH action in the liver (Vella et al., 2014). In original studies characterizing expression of NCOR ID, NCoR1 played a key role in mediating sensitivity to the hormone such that in its absence there was increased sensitivity to T3 with enhanced activation of classic T3-target genes (Astapova et al., 2008). In contrast, deletion of SRC-1 led to a decreased response in context of gene activation to a set level of T3 (Takeuchi et al., 2002). Taken together these data demonstrate that the balance between corepressors and coactivators determines the target set point of gene expression (Vella et al., 2014). Interestingly, expression of NCOR ID and deletion of SRC-1 together led to the re-establishment of normal T3-sensitivity as this allowed for the recruitment of SRC-2 to target genes. Overall this model suggests that cellular levels of both TR-specific corepressors and coactivators determine T3-sensitivity. This is supported further by the discovery of splice variants of corepressors that have variable interaction with the TRs (Snyder, Goodson, Schroeder, & Privalsky, 2015). Furthermore, corepressor specificity does exist as SMRT plays little role in modulating TH action both in the liver and systemically in context of the thyroid axis (Shimizu et al., 2015). Thus, T3 action in a target cell can be functionally determined by the amount of NCoR1 present in conjunction with coactivators present. Further support for the notion of corepressor specificity comes from experiments where the hypomorphic NCOR ID allele has been crossed into mice that express mutant TR β or TR α alleles (PV mutation). The TR β and TR α PV mutations are akin to those found in human

syndromes of RTH where the mutant TR constitutively bound to NCoR1 (Yen, 2003). In both cases the presence of the NCoR ID allele improves the RTH phenotype. In the TR β PV mice, NCoR ID reverses and normalizes the HPT axis abnormalities. In TR α PV mice, presence of the NCoR ID allele allows previously infertile mothers to produce live pups. While these models suggest that the constitutive recruitment of NCoR1 by mutant TRs is responsible for the phenotype of RTH, much work remains to further characterize the mechanism of action of both the TRs and NCoR1 in the tissues studied (Fozzatti, Park, Zhao, Willingham, & Cheng, 2013; Laura. Fozzatti et al., 2013).

Despite the established roles of NCoR1/SMRT and the SRC family in context of TH action in the liver and the HPT axis, there are numerous other potential coregulators that may play a role in TH action. Each of the T3-binding TR isoforms has a distinct amino-terminus or A/B domain, which have been shown to potentially recruit a host of distinct proteins and suggest that isoform specificity could be dictated by coregulator recruitment (Yi et al., 2015). Moreover, mass spectrometry studies have demonstrated that TRs display isoform specific interaction with a wide set of nuclear proteins that is potentially dependent on the AF1 domain, which has the highest variability amongst the domains of the TR isoforms (Hahm & Privalsky, 2013; Hahm, Schroeder, & Privalsky, 2014). Additional coregulators that may play a role in T3 action include the histone deacetylase Sirt1 and the mediator subunit Med1 (Fondell, 2013; Suh et al., 2013). It is expected that multiple T3-responsive tissues will allow for their own code of regulators, which then mediate T3 action. For example the set of co-regulators that may act in the heart is not known as experiments that express NCoR ID or delete SRC-1 globally have little effect on the response of T3-target genes in the heart (Vella et al., 2014).

Even though the recruitment of coregulators is paramount for TH action, the mechanism by which they mediate their effects is key to understanding the molecular components of TH action. Current thought involves TR isoforms binding NCoR1/SMRT, which then recruits the active histone deacetylase HDAC3. However, in mouse models where the NCoR1 or SMRT domain required for the deacetylase function of HDAC3 is mutated the activation of gene targets in the liver is much less than that seen in HDAC3 KO mice or in mice that lack both NCoR1 and SMRT function (Sun et al., 2013) (Shimizu et al., 2015). This implies that NCoR1/HDAC3 function may require more than deacetylation to silence gene expression in hypothyroidism. Indeed, deletion of HDAC3 or NCoR1/SMRT in murine liver cells in culture or *in vivo* leads to the strong activation of gene targets important in lipogenesis. Many of these genes are direct TH targets. In addition, HDAC3 deletion leads to activation of histone acetylation on these targets. However, the re-expression of a modified HDAC3 that can no longer deacetylate restores repression of these genes independently of changes in histone acetylation (Sun et al., 2013). This suggests that HDAC3 represses gene expression independently of histone acetylation, though this needs to be established directly on thyroid hormone targets. Taken together these data suggest that while NCoR1 and HDAC3 are necessary for the repressive effects of the TR, the exact molecular mechanism remains unclear. This is further complicated by the fact that the TR has the ability to activate certain targets such as TRH in the hypothalamus or FBXO21 in the liver in the hypothyroid state (Joseph-Bravo, Jaimes-Hoy, & Charli, 2015) (Astapova et al., 2008). The role of coregulators in so-called ligand-independent activation is not understood at all.

Unique Paradigms of TH Action – Negative Regulation by TH

As discussed extensively, TH is the major regulator of the set point of the HPT axis and it exerts its biologic effects through feedback inhibition at the level of the hypothalamus and pituitary. In addition, TH down regulates more genes in the liver in the presence of T3 that it activates (Ramadoss et al., 2014; Sasaki et al., 1999). While positive regulation by T3 is well explained by the models described above negative regulation remains a paradox. The expression of TRH in the paraventricular nucleus of the hypothalamus and the TSH subunit genes in the thyrotroph of the pituitary are regulated at the level of transcription mainly through TR β 2 though more recent data suggests that TR β 1 also plays a role (Ng et al., 2015). In addition, it has recently been observed that the ablation of TR α 2, a non-T3 binding receptor in hypothalamus of mice, resulted in the overexpression of TR α 1. These mice had a phenotype of low of free T3 and T4 levels and normal TSH suggesting a role of this previously neglected TR isoform in the regulation of HPT axis. Normally, TR α 2 acts as a dominant inhibitor of T3-binding TR isoforms by competing for DNA binding sites. *In vivo* experiments of using siRNA to ablate or overexpress TR α 2 show abrogated T3 action on TH-reporter genes in the hypothalamus consistent with the hypothesis that TR α 2 can block WT receptor function (Guissouma et al., 2014). Further involvement of TR α isoforms in the regulation of the HPT axis can be garnered from RTH patients with mutations in TR α . Some patients have a subnormal free T4/T3 ratio with a normal or low TSH, though this may depend on the mutation present (C. Moran & Chatterjee, 2016; Carla. Moran et al., 2014). Taken together, these findings suggest that both THRB and THRA are required for normal set point of the HPT axis and negative regulation by T3.

While the TRs are required for negative regulation, the mechanism by which it is mediated is not clear. Genome wide ChIP studies in the liver (see Table 1) demonstrate that negatively regulated targets are much less likely to have TR-binding sites in the region surrounding them suggesting that negative regulation could be indirect. In contrast, direct evidence of TR β 2 binding to the TSH β promoter has been obtained in a pituitary cell line (Chiamolera et al., 2012). Thus, it is likely that both direct and indirect mechanisms will explain how the TRs are able to mediate negative regulation. The role of the coregulators also remains enigmatic in negative regulation. Paradoxically, deletion of SRC-1 causes a RTH syndrome similar to that seen with a mutated TR β isoform with inappropriate TSH secretion in the setting of elevated TH levels (Weiss et al., 1999). This phenotype suggests that SRC-1 plays a direct role in T3-mediated repression. Indeed, co-expression of NCoR ID in SRC-1 $-/-$ mice normalizes the HPT axis and re-establishes normal negative regulation of the TSH β subunit gene (Vella et al., 2014). Thus, in the pituitary the coregulators appear to play paradoxical roles in negative regulation of the TSH β gene by T3 (Costa-e-Sousa & Hollenberg, 2012). Whether the co-regulators have similar effects on TRH expression in the PVN remains unclear. Indeed many uncertainties remain in context of T3-mediated regulation of TRH. For example it is not clear how T3 accesses the PVN as local T3 is primarily derived in the region of the tanocytes at the base of the hypothalamus (Hanon et al., 2008; Watanabe et al., 2007). Thus, it remains possible that T3 may act indirectly on the TRH neurons in the PVH (Hollenberg, 2008). Remarkably T3 has no effect on TRH gene expression in neurons in the lateral hypothalamus, which is in much closer proximity to the tanocytes where T3 is produced (Sugrue, Vella, Morales, Lopez, & Hollenberg, 2010).

While SRC-1 and NCoR1 clearly play a role in the regulation of TSH β gene expression and the set point of the HPT axis, the deletion of SRC-1 or NCoR1 has no effect on negative T3 targets such as Gsta1 or Fbxo21 in the liver (Vella et al., 2014). Thus, tissue specific and coregulator specific mechanisms must be in place to allow for negative regulation by T3 in a target cell. Further studies on epigenetic marks and their role in negative regulation are required in order to better understand the molecular mechanisms in play in negative regulation.

Summary and Conclusions

Over the last number of years work on thyroid hormone action has firmly established that the intracellular actions of the hormone are quite discernible from circulating levels. While the HPT axis moves to keep circulating TH levels within a tight range based on a genetic set point with environmental cues, the intracellular availability of T3 is based on a myriad of other determinants. We now realize that a multitude of transporters mediate TH access to distinct cell types and that these transporters can be regulated themselves. Next, the deiodinases play a distinct role in regulating cell-specific levels of T3 that can reach the nucleus. They, too, are regulated in pathophysiologic states at both the level of transcription and translation. Finally, the intranuclear milieu of TRs and co-regulators is highly varied depending upon the cell-type studied. This variability undoubtedly leads to changes in response to a set level of T3. Remarkably, the model of an unliganded versus a liganded TR in context of co-regulator function is no longer valid. Clearly the TRs have the ability to bind to DNA in the presence of T3 or leave in its absence. Yet how this is regulated remains unclear. Despite the advances made, a unifying model of negative regulation by TH and the TRs remains elusive. It is likely that numerous models will be required to explain this phenomenon. Although questions remain, it is also clear that our ability to better understand tissue-specific thyroid hormone action at the molecular level will lead to better biomarkers in humans so that therapy can be better tailored in different pathophysiologic states.

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Abbreviations

TH	Thyroid hormones
T4	thyroxine
T3	Triiodothyronine
CNS	central nervous system
HPT	hypothalamic-pituitary-thyroid axis
TRH	thyrotropin-releasing hormone
PVN	paraventricular nucleus

CART	Cocaine- and amphetamine-regulated transcript
CRH	corticotrophin releasing hormone
TSH	thyroid stimulating hormone
GWAS	(genome-wide association studies)
PDE8B	Phosphodiesterase 8B
PDE10A	Phosphodiesterase 10A
CAPZB	Capping Actin Protein Of Muscle Z-Line Beta Subunit
MAF/LOC440389	V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog
VEGFA	Vascular Endothelial Growth Factor A
NR3C2	Nuclear Receptor Subfamily 3 Group C Member 2
IGFBP5	Insulin Like Growth Factor Binding Protein 5
NFIA	Nuclear Factor I A
SOX9	SRY-Box 9
PRDM11	PR Domain 11
FGF7	Fibroblast Growth Factor 7
INSR	Insulin Receptor
ABO	ABO Blood Group
MIR1179	MicroRNA 1179
NRG1	Neuregulin 1
MBIP	MAP3K12 Binding Inhibitory Protein 1
ITPK1	Inositol-Tetrakisphosphate 1-Kinase
SASH1	SAM And SH3 Domain Containing 1
GLIS3	GLIS Family Zinc Finger 3
DIO1	Deiodinase, Iodothyronine Type I
LHX3	LIM Homeobox 3
FOXE1	Forkhead Box E1
AADAT	Aminoadipate Aminotransferase
NETO1/FBXO15	Neuropilin And Tolloid Like 1/F-Box Protein 15

LPCAT2/CAPNS2	Lysophosphatidylcholine Acyltransferase 2/Calpain Small Subunit 2
FT4	free thyroxin
MCT8	monocarboxylate transporter 8
MCT10	monocarboxylate transporter 10
OATP1	organic anion transporter
LAT	L-type amino acid transporter
AHS	Allan-hendon dudley syndrome
SNP	single nucleotide polymorphism
TRIAC	3,5,3'-Triiodothyroacetic acid
TETRAC	Tetraiodothyroacetic acid
DITPA	3,5-Diiodothyropropionic Acid
LPS	lipopolysaccharide
LAT2	L-type amino acid transporter 2
D2	type 2 deiodinase
D3	type 3 deiodinase
IRD	inner-ring deiodination
ORD	outer ring deiodination
D1	type 1 deiodinase
EGL	external germinal layers
IGL	internal germinal layers
CTX	cardiotoxin analogue III
WSB-1	WD Repeat And SOCS Box Containing 1
OA	osteoarthritis
ADAMTS5	ADAM Metallopeptidase With Thrombospondin Type 1 Motif 5
TR	thyroid hormone receptor
LBD	ligand binding domain
TRB1	thyroid hormone receptor beta 1
TRB2	thyroid hormone receptor beta 2

TRa1	thyroid hormone receptor alpha 1
RTHB	resistance to thyroid hormone beta
RTH	resistance to thyroid hormones
GI	gastrointestinal
RXRα	retinoic X receptor alpha
GFP	green fluorescent protein
RNF166	Ring Finger Protein 166
PI3K	Fosfoinositol 3-quinasa
NCoR1	Nuclear receptor corepressor 1
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
HDAC3	Histone Deacetylase 3
SRC-1	steroid receptor coactivator 1
CBP	CREB-binding protein
NCOR ID	Nuclear receptor corepressor 1 that lacks nuclear receptor interaction domains
SRC-2	steroid receptor coactivator 2
FBXO21	F-Box Protein 21
Gsta1	Glutathione S-Transferase Alpha 1

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Figure 1.

The hypothalamus-pituitary-thyroid (HPT) axis maintains thyroid hormone homeostasis. The PVN neurons secrete TRH in response to low circulating T3/T4 ratio. In turn, TRH signaling in the pituitary stimulates the secretion of TSH, which triggers the release of T4 and T3 from the thyroid gland into the bloodstream. T3 negative feedback upon expression of TRH and TSH genes in the PVN and the pituitary respectively keeps the T3/T4 ratio virtually constant in circulation.

Figure 2. The thyroid gland secretes T3 and T4 into the circulation

The thyroid follicular cells concentrate iodine within the colloid by using NIS/Pendrin transporters, which captures circulating I^- into the cytoplasm, followed by secretion to the colloid respectively. In the colloid the I^- is organified and coupled to thyroglobulin-tyrosine residues by TPO catalyzed oxidation, which requires of H_2O_2 generated by DUOX as cofactor; thus generating DIT and MIT, which form T4 and T3. TSH stimulation promotes endocytosis of the T4/T3-containing thyroglobulin, which releases the hormones after proteolysis in the cytosol. TSH receptor is coupled to G-protein and increases cAMP, which in turn is hydrolyzed by PDE8B. The MCT8 appears necessary for the transport of T4 from the thyroid gland while it and other transporters may play a role in T3 transport from the gland.

Figure 3. T3 modulates gene expression in virtually every vertebrate

T4/T3 circulates attached to serum proteins including thyroxine binding globulin (TBG) transthyretin (TTR), and albumin. A small fraction of circulating T4 is free to be transported into the cytoplasm, where it is activated to T3 by outer-ring deiodination catalyzed by Dio1 or Dio2. The resulting T3 is thought to be diffused into the nucleus to bind the thyroid hormone receptors (TR). Upon binding to the thyroid hormone receptors, T3 modulates the rate of mRNA synthesis and ultimately the protein levels of thousands of genes in virtually every cell.

Figure 4. T3 binds to the thyroid hormone receptors to modulate gene expression

In the absence of ligand the TR recruits NCoR1, which forms a multiprotein corepressor complex including Tbl1 and HDAC3. Binding of T3 induces the dismissal of the corepressor complex and allows the recruitment of coactivators (SRC1/CBP, CARM1/PRMT1), which include histone acetyl and methyl transferase activity thus facilitating the activation of the general transcription machinery and mRNA synthesis.

Figure 5. Schematic representation of the thyroid hormone receptor isoforms

TRs are encoded by the THRA and THRB genes, which produce multiple isoforms, Depicted are the major isoforms that modulated the actions of T3, with exception of TRα2 which does not bind T3 but acts as negative dominant by competing for the TRE with other TRs. TR isoforms share high sequence homology within their functional domains.

Figure 6. Corepressors and coactivators harbor nuclear receptor interacting domains

NCoR1 and SMRT harbor three nuclear receptor interacting domains; N3 and N2 interact preferentially with unliganded TR, whereas SRC harbors an RID that interacts with the TR upon adoption of liganded conformation.

TRB1 chip-seq studies

Table 1

Reference	Gronved, L. et al 2015	Ramados, P. et al 2014	Ayers, S. et al 2014	Chatonnet, E. et al 2013
Model	mouse liver	mouse liver	cell culture	cell culture
Detection method	Monoclonal AB C1	biotin-streptavidin	biotin-streptavidin	biotin-streptavidin
Findings				
<i>DR4 is the most common TRE in positive targets</i>	☑	☑	☑	☑
<i>DR0 is associated with negative targets</i>		☑		
<i>T3 induced recruitment of TR to TRE</i>	☑	☑	☑	
<i>T3 independent interaction between TR and TRE</i>	☑	☑	☑	
<i>Majority of T3 binding sites found in intragenic regions</i>	☑	☑	☑	☑
<i>Little TRB binding sites near negative target genes</i>	☑	☑		
<i>RXRalpha heterodimer partner</i>	Not done	☑	Not done	Not done
<i>TR isoform specific binding sites</i>	Not done	Not done	Not done	☑
<i>Motif enrichment analysis for TF other than TR</i>		☑	☑	
<i>T3 regulated DNase hypersensitive sites</i>	☑	☑	Not done	Not done