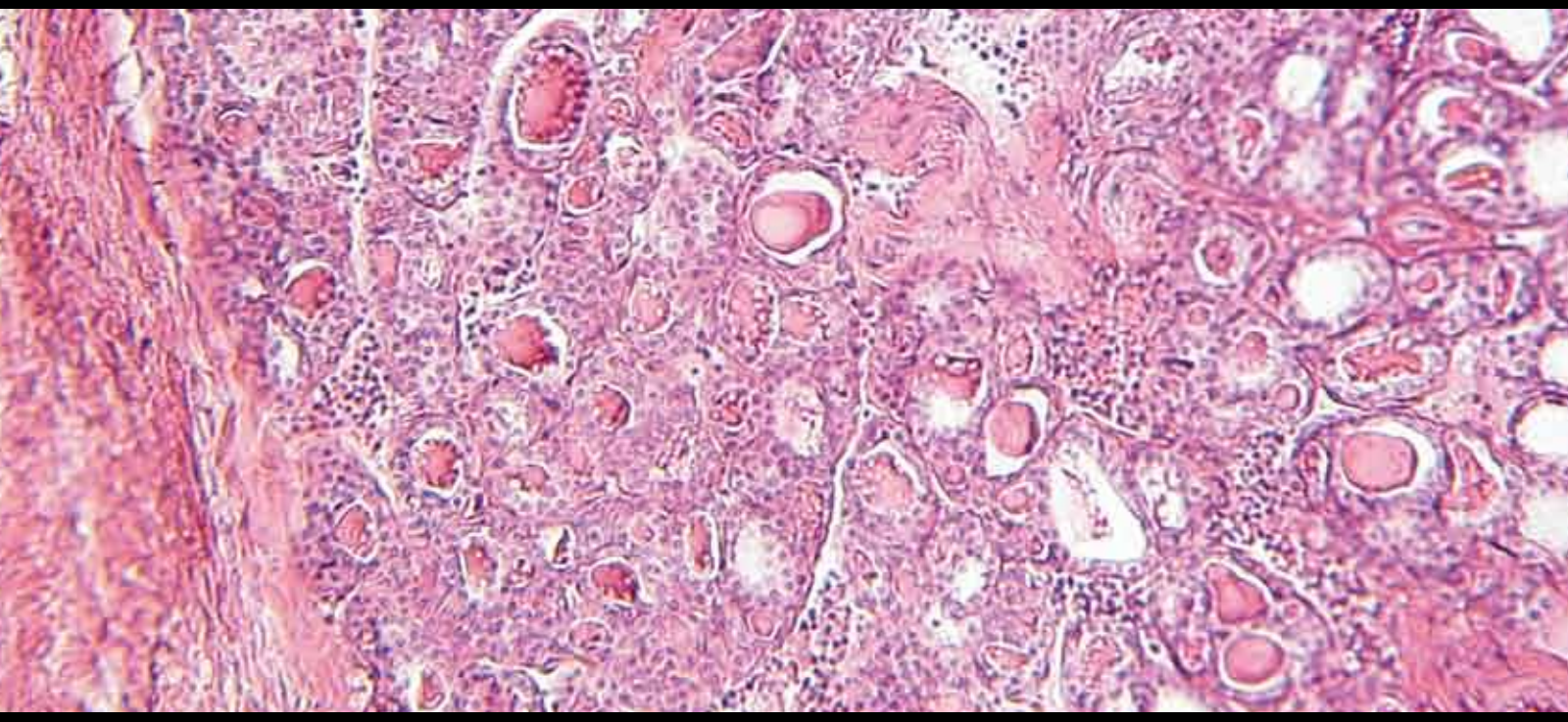
Journal of Thyroid Research

Thyroid Hormones and Their Receptors: From Development to Disease

Guest Editors: Michelina Plateroti, Juan Bernal, Samuel Refetoff, and Laurent Sachs



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Editorial

Thyroid Hormones and Their Receptors: From Development to Disease

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Thyroid hormone (TH) has a fundamental role in the development, growth, and metabolic homeostasis in all vertebrates by affecting the expression of a variety of genes. As a consequence, an altered thyroid status affects many organs and systems. TH action is mediated mainly by the TH receptors (TRs), which belong to the nuclear receptor superfamily of transcription factors. Given the rapidly accumulating data on the pleiotropic functions of the TH and TR, the aim of this special issue was to summarize the most recent advances in this field. In particular, investigators contributed with review articles that address the efforts to understand the function of the TH and TRs in development, growth, metabolism, and homeostasis. Moreover, reviews also focused on pathologies linked either to altered thyroid status or to gene alterations that modify hormone action.

We open this special issue with the paper by J. R. Tata, providing a historical perspective on the discovery of thyroid hormone, thyroid hormone receptors, and mechanisms of action. It also summarizes some of the current thinking on thyroid hormone action and proposes three models to explain the multiplicity of the thyroid hormone effects. Moreover, the paper highlights that the proposed models are not the ultimate mechanism of the action of thyroid hormone and that future studies will probably lead to the discovery of some fundamental principles of biological regulation and signalling as was done in the past. The paper by V. M. Darras et al. summarizes data available on thyroid hormone receptors in chicken and zebrafish, two major model systems for developmental biology. This contribution is of particular interest in reviewing the implication of TRs during development in nonmammalian species.

Two papers deal with the role of TH and TRs in the nervous system as they have addressed several aspects of TH action in the brain. Of particular interest is the regulation of T3 entry into neural cells. Brain T3 has dual sources, the circulation and local generation through T4 deiodination. The paper by P. Mohácsik et al. deals with mechanisms that regulate the synthesis of the nuclear receptor ligand T3 in the brain by the glial cells astrocytes and tanycytes as well as the cellular transport of T3 by specific membrane transporters. The paper by F. Chatonnet et al. is based on the work on cerebellar development carried out by the group of Frederic Flamant in Lyon. They address the multiple aspects of TH action in the brain, including the control of ligand production, the role of receptors, the regulated genes, the interactions among neural cells in TH actions, and the disruptions due to environmental contaminants.

Other papers focus on TH-alteration-dependent pathologies. TH acts in most body's tissues in a pleiotropic fashion. Two papers have addressed this issue focusing on several systems. The paper by M. Franco et al. is a general overview of the pleiotropic effects of TH on many different pathways and organ targets, based on studies on hypothyroidism. They review extensively the effect of hypothyroidism on mitochondria, ischemic reperfusion damage of the heart, renal physiology, vascular relaxation, and lipoprotein metabolism.

The paper by M. Hage et al. reviews the actions of TH on carbohydrate metabolism with emphasis on the relationship between diabetes and thyroid disorders. Finally, paper by C. Pantos et al. is a careful review of the pathophysiological mechanisms underlying the reactivation of a foetal phenotype in the damaged myocardium and the eventual implication/significance of a TH-based therapy.

We close our special issues with the paper by M. D. Rosen and M. L. Privalsky dealing with TR mutations in cancer. This review starts with a very elegant historical perspective and the authors describe the TR signalling and the effect of TR mutations in endocrine diseases.

Taken together, the contributors of this special issue clearly underscore the complexity of the TH action from a cellular and molecular point of view. As suggested by Professor J. R. Tata's conclusive remarks, they also point out that much has yet to be learned about how TH signalling regulates specific gene expression and diverse cellular functions from early development to cell death in both physiological and pathological situations.

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Review Article

Looking for the Mechanism of Action of Thyroid Hormone

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The mechanisms of action of thyroid hormone (TH), characterized by multiple physiological activities, proposed over the last 80 years are a reflection of the progression of our knowledge about eukaryotic signalling processes. The cumulative knowledge gained raises the question as to what is so special about the action of this hormone. The discovery in the 1980s that TH receptors belong to the family of nuclear transcription factors that regulate the expression of hormonal target genes was an important milestone. TH receptors are highly organized within the chromatin structure, which itself is modified by several chromosomal and nonchromosomal factors, in the presence and absence of the hormone. Recently, some investigators have suggested that TH acts via both genomic and nongenomic mechanisms and introduced the concept of networking within cellular complexes. While one cannot as yet precisely describe the mechanism of thyroid hormone action, I will attempt here to point out the present thinking and future directions to achieve this goal in the light of the historical background.

1. Introduction

Even before L-thyroxine (3,3′,5,5′-tetraiodothyronie or T_4) was identified by Kendall in 1919 as the active thyroid hormone in the thyroid gland, and later synthesized by Harington in 1926 [1], physiologists and biochemists had already proposed in the first two decades of the twentieth century several mechanisms of actions of the hormone (see [2]). Although the hormone was known to be endowed with multiple metabolic and developmental activities, most studies at first focused on the calorigenic action of the hormone, largely because of the clinical association between basal metabolic rate (BMR) and thyroid gland morphology and activity. The dramatic role of thyroid hormone in amphibian metamorphosis, demonstrated by Gudermatsch in 1911, was largely ignored for twenty years by those interested in explaining the biochemical mechanisms underlying thyroid hormone action.

It took nearly 35 years after thyroxine that 3,3′,5-triiodo-L-thyronine (T_3) was identified, almost simultaneously as a constituent of thyroglobulin in the thyroid gland and in human blood in the laboratories of Roche in Paris and Pitt-Rivers in London. Not long after that, several laboratories confirmed that T_3 is biologically more active than T_4 in several physiological assays, such as BMR, amphibian metamorphosis, lipid metabolism, and pituitary function [2, 3]. A larger part of T_3 in the blood and peripheral tissues of most vertebrates is derived from the partial deiodination of T_4 than was produced in the thyroid gland. This conversion has given rise to the commonly held view that T_3 is the biologically active thyroid hormone while T_4 is the prohormone, the enzyme responsible for the conversion, one of the deiodinases which remove I atoms from the inner and outer rings of T_4 and T_3. The deiodinases are considered to be important in the dynamics of TH activity [4]. For the purposes of this review, I shall consider T_3 as the biologically active thyroid hormone and T_4 as the pro-hormone, while the term TH will include any of their derivatives or conjugates with biological activity.

A major advance in our understanding of the biochemical and molecular basis of the action of TH was the identification, cloning, and characterization of its receptors (TRs) in 1986 in the laboratories of Vennström [5] and Evans [6]. These receptors are members of a large multigenic family of transcription factors which are activated by hormonal or other signalling molecules. TRs are integral chromosomal proteins whose structure and chemical nature is modified by TH. The liganded receptor is such that they would modify

Table 1: Multiplicity of physiological and biochemical actions of thyroid hormone.

Growth and developmental actions	Metabolic actions
Rate of postnatal growth of many mammalian and avian tissues	Regulation of basal metabolic rate in endotherms
Functional and biochemical maturation of fetal brain and bone	Movement of water and Na^+ ions across cell membranes
	Calcium and phosphorus metabolism
Morphogenesis, gene switching, and cell death in amphibian larval metamorphosis	Regulation of metabolism of cholesterol and other lipids
Control of molting in birds	Nitrogen (urea, creatine) metabolism
Regulation of synthesis of mitochondrial respiratory enzymes and membranes	Control of oxidative phosphorylation and energy metabolism

the interaction between the receptor and its target genes, leading, directly or indirectly, to the physiological action of the hormone [7]. Much progress has been made in the last 20 years in enhancing our knowledge of chromosomal or chromatin structure, so that it is not surprising that a substantial amount has been learnt about the possible ways in which the structural and biochemical modifications of chromatin would modulate the action of thyroid hormone [8]. These advances have most recently led to exploring the processes of networking within and between cells and wider questions of systems biology for further exploration of the search for mechanism of hormone action [9, 10].

This paper is a brief review of some historical and current thinking and work relating to the mechanism of action of thyroid hormone covering the period of the last 80–90 years and gives a largely, but not uniquely, personal point of view of the author. It is not meant to be a comprehensive review of thyroid hormone action. If any of the readers' work or ideas are not cited below, it is not the author's intention to diminish the importance of their contributions to this field of research.

2. Multiple Effects but Unique Mechanism of Action?

Can one explain the multiple biological effects of thyroid hormone with a unique mechanism of action? First, let us consider the multiplicity of actions. Towards the end of the 19th century, physicians and surgeons in England, France, and Switzerland established the link between the thyroid gland and iodine deficiency disorders such as goitre, cretinism, and myxoedema in man. By the early 20th century, removal of the gland in experimental animals and grafting it back to reverse the effects of thyroidectomy corroborated the beneficial effects of administering ovine, bovine, and porcine thyroid powder or extracts for the treatment of patients suffering from myxoedema, cretinism and growth retardation in children, or other consequences of thyroid deficiency. The chance observation in Naples by Gudernatsch that feeding pieces or extracts of mammalian thyroid gland to frog tadpoles precociously induced metamorphosis emphasized the important role of thyroid hormone in regulating postembryonic growth and development in all vertebrates [11]. The establishment of L-thyroxine and triiodo-L-thyronine as the biologically active principles in the thyroid and peripheral tissues and the availability of synthetic T_4 and T_3, and many of their biologically active and inactive homologues, made it possible to establish, both qualitatively and quantitatively, the characteristics of the multiple hormonal activities.

In Table 1 are listed the major physiological actions of thyroid hormone, which can be loosely subdivided into two groups: (a) those that regulate metabolic functions and (b) those that control growth and development. It is immediately obvious that the biological responses to the hormone vary according to species, tissue, and developmental stage. Generally, the metabolic responses are most visible in endotherms, often manifested as basal metabolic rate (BMR), water retention lipid metabolism, and so forth. The hormonal effects on growth and development are particularly evident in ectotherms, and less during the postembryonic or perinatal period in mammals and other warm-blooded vertebrates. It should be emphasized that separation of the physiological and biochemical actions of TH into two categories, as in Table 1, does not imply a sharp boundary between the two, and that there can be some overlap determined by the variables mentioned above.

An even more striking example of the multiple responses to TH is seen when comparing tissue-specific responses to the hormone during amphibian metamorphosis, illustrated in Table 2. The process can be precociously induced by the administration of exogenous TH to immature tadpoles, which confirms the fact that the developmental programme is well in place before the larval thyroid gland is fully developed and can begin to secrete the hormone [12]. An important feature of the multiplicity of actions that emerges from Table 2 is that no two tissues or groups of cells exhibit the same hormonal responses, which range from *de novo* morphogenesis, as for limb and lung development, functional reprogramming, as for the brain, liver, and eye, total or partial tissue loss of tissue, as seen for the tadpole tail and gills. Similar processes are discernible, albeit less clearly and more attenuated, in other vertebrates and mammals during the perinatal period. Indeed, tissue-specific gene switching is central to hormonal signalling, not only as regards TH but also many steroid and protein hormones that regulate growth and development in general.

Early investigators were intrigued by how the relatively simple, but quite unique, molecule of a hormone as TH

Table 2: Morphological and biochemical responses to thyroid hormone during amphibian metamorphosis.

Tissue	Response	
	Morphological	Biochemical
Brain	Restructuring, axon guidance, axon growth, cell proliferation, and death	Cell division, apoptosis, and new protein synthesis
Liver	Restructuring, functional differentiation	Induction of urea cycle enzymes and albumin; larval to adult haemoglobin gene switching
Eye	Repositioning; new retinal neurones and connections; lens structure	Visual pigment transformation (porphyropsin → rhodopsin); β-crystallin induction
Skin	Restructuring; skin granular gland formation; keratinization and hardening; apoptosis	Induction of collagen, 63 kDa (adult) keratin and magainin; induction of collagenase
Limb bud, lung	Do novo formation of bone, skin, muscle, nerves, and so forth.	Cell proliferation and differentiation; chondrogenesis
Tail, gills	Complete regression	Programmed cell death induction and activation of lytic enzymes (collagenase, nucleases, phosphatases, and matrix metalloproteinases); lysosome proliferation
Pancreas, Intestine	Major tissue restructuring	Reprogramming of phenotype, induction of proteases, fatty acid binding protein, and stromelysin-3
Immune system	Redistribution of cell populations	Altered immune system and appearance of new immunocompetent components
Muscle	Growth and differentiation; apoptosis	Induction of myosin heavy chain

See [11, 34] for details.

orchestrated such diverse effects. Is there a single mechanism of action that governs this diversity of responses or is each manifestation of hormonal activity the result of a different mechanism? At first, physiologists and biochemists attempted to explain the mechanisms underlying their actions as being common to all hormones, but as increasing numbers of hormones became available as pure substances, and as the biochemical and physiological endpoints were better characterized, there was a move away from a generic mechanism of hormone action. Furthermore, as our understanding of complex whole-body physiological processes progressed from biochemical mechanisms, such as enzymology, to receptor analysis, to structural cell biology, to genetic networks, so did the explanations for hormone action continue to evolve.

3. Early Studies on the Mechanism of Action of TH

Table 3 summarizes the progression of some major ideas concerning the mechanism of thyroid hormone action following the synthesis of thyroxine, that is, over the fifty years until around 1980. By the early 1930s, it was thought, the activation or inhibition of a given enzyme resulting from the interaction between the hormone and the enzyme could explain the physiological action of the hormone [2]. For example, the stimulation by T_4 or T_3 of oxygen consumption by isolated tissues or that of BMR *in vivo* was explained on the basis of a direct interaction between the hormone and some dehydrogenases, the phenomenon often termed allosteric or conformational changes in the enzyme. These direct hormone-enzyme interaction models were eventually discarded because of serious methodological discrepancies, for example, the concentrations of hormone used *in vitro* would be several orders of magnitude higher than it would ever occur *in vivo,* or that biologically inactive analogues of the hormone would be often more active than the natural hormone in direct interactions.

The realization that the regulation of biochemical functions *in vivo* had to be considered in the context of complex cellular structures led to models which took into account the structural organization of physiological activities. Major advances, both technical and conceptual, in the 1950s and early 1960s introduced the idea that the cell membrane, the mitochondrion, the protein synthesizing machinery, and the cell nucleus constituted valid targets for hormones, vitamins, drugs, and other biologically active molecules [13]. Although already in the 1940s Levine had demonstrated that insulin controlled sugar metabolism by interacting with its transport machinery residing in the cell membrane, it was the discovery of cyclic AMP (cAMP) by Sutherland and Rall in 1956 as a "second messenger" of adrenaline and glucagon followed by the equally important discovery that adenylyl cyclase was located in the plasma membrane firmly

Table 3: Milestones in the search for mechanism of thyroid hormone action.

Year/Period	Milestone
1905	Starling introduces the word hormone and the concept of chemical messengers
1911	Mammalian thyroid extracts shown to induce amphibian metamorphosis
1919	Thyroxine and cortisone extracted and chemically characterized by Kendall
1920–1935	Effects of thyroid hormone on tissue and whole body respiration and metabolic functions
1925–45	Isolation and characterization of pituitary protein hormones
1935–50	Hormone-enzyme interactions thought to explain hormone action
1941–55	Insulin and other hormones shown to regulate transport processes
1955-62	Thyroxine thought to act by uncoupling oxidative phosphorylation
1956	Discovery of cyclic AMP by Sutherland and the concept of "second messenger"
1960	Ecdysteroids induce chromosomal puffing during insect development—first indication of hormone action at the nucleus
1962	Oestradiol shown to bind to nuclear proteins. First, indication of nuclear receptors
1962–66	Steroid and thyroid hormones and retinoids selectively regulate protein synthesis and transcription
1975–85	Protein hormone receptors located in cell membranes identified as homologues of c-erbB oncogene; protein phosphorylation cascades identified
1979–89	Steroid/thyroid/retinoid receptors cloned as a large family of c-erbA-related transcription factors interacting with target genes and modifying chromatin structure
1990s	Crystal structures for many hormone receptors and partners. Transgenesis and mutagenesis of receptors *in vivo*
1996	Coactivators and corepressors modulate gene expression by TR and other nuclear receptors
1998	Phosphorylation, acetylation, and methylation of TR and other nuclear receptors, histones, and chromosomal proteins
2002–2010	Convergence of hormonal signals via membrane and nuclear receptors. Emergence of concepts of systems biology, bioinformatics and gene, and metabolic networking applicable to hormone action

established the view that the cell membrane is a major site of action for hormones [14].

At almost the same time as cyclic AMP was discovered, Knox demonstrated that glucocorticoids regulate hepatic metabolism by selectively enhancing the synthesis of the enzyme tyrosine aminotransferase [15]. New methods to study cell-free protein synthesis and the availability of specific transcription inhibitors allowed a more precise analysis of how growth and developmental hormones influenced protein synthesis in their respective target cells. The resulting observations that all steroid and thyroid hormones administered *in vivo* affect the protein synthesizing machinery *in vitro* soon shifted the focus on transcriptional control [16]. In the mid-1960s, several investigators were able to reproduce the transcriptional effects of steroid and thyroid hormones in cell-free transcription systems using isolated nuclei and nuclear extracts from target tissues following hormonal administration *in vivo*. Kinetics of labelling of nuclear RNA further revealed that all steroid and thyroid hormones strongly influence the formation and turnover of messenger RNA.

In 1961, Jensen had made the important observation that highly radioactive estradiol very rapidly (within minutes) accumulated in the nucleus of its target tissues (see [17]). This and subsequent investigations laid the foundation of the concept of nuclear receptors for steroid and thyroid hormones, retinoids, vitamin D_3, and so forth. A decade later, Oppenheimer carried out a similar investigation on the selective nuclear localisation of radioactive T_3 in rat liver [18]. The main significance of this finding is that the accumulation of the hormone anticipated by several hours the stimulation of transcription, translation, and cellular respiratory responses to this hormone in other cellular organelles of the same tissue under identical conditions. This is schematically illustrated in Figure 1. What is particularly important in this kind of time-course representation of different cellular responses is that inhibition of transcription abolishes the subsequent, different responses and biological activities of thyroid hormone in different mammalian and amphibian biochemical functions, such as protein synthesis, respiration, morphogenesis, and cell death [3, 19]. Although the binding of TH and steroid hormones to target cell nuclei exhibited many of the functional characteristics of receptors, one had to wait till the technologies of gene cloning had been well established to make the next important step of establishing the nature of nuclear receptors, their organization in cellular structures, and their functional significance.

4. Later Studies on the Mechanism of Action of TH

The first indication that hormonal signals regulate transcription was, however, provided by gene puffing in polytenic chromosomes in larval salivary glands of insects, such as *Drosophila* and *Chironomus*, during their metamorphosis induced by the molting hormone ecdysone [20, 21]. These puffs, which contain newly synthesised RNA from sequential activation of specific genes, can be precociously induced by incubating the target cells with ecdysteroids. Kinetics of labelling of nuclear RNA revealed that all steroid and thyroid hormones strongly influenced the formation and turnover of messenger RNA. In the mid-1960s, several investigators were able to reproduce the effects of *in vivo* administration of

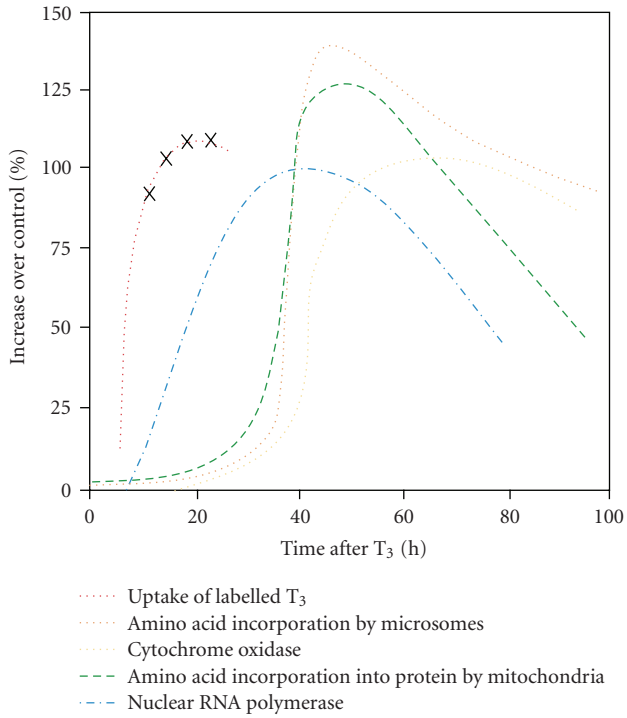

FIGURE 1: An idealized representation of the time course of response of some activities of nuclei, mitochondria, and microsomes from livers of thyroidectomized rats after a single injection of 20 μg T_3/100 g. body wt. The stimulatory effects are expressed as % increase in specific activity in the different subcellular fractions from T_3-injected animals over control animals. The main features are the following: (a) mitochondrial respiration (here expressed as cytochrome oxidase activity) reached a peak after amino acid incorporation into protein by microsomes and mitochondria; (b) the increase in protein synthetic capacity of the two organelles was coupled, following a relatively long lag period after hormone administration; (c) nuclear RNA polymerase activity was enhanced several hours before cytoplasmic protein synthesis and BMR. The time course of nuclear accumulation of T_3 ($\times \cdots \times$; [18]) is superimposed on that of the hormonal effects on mitochondrial, transcriptional, and protein synthetic activities (Data assembled from [22–24]).

the hormone in cell-free transcription systems using isolated nuclei and nuclear extracts from target tissues of steroids and TH [16].

When Jensen and colleagues tracked the sex hormone oestradiol-17β of high specific radioactivity to female accessory sexual tissues, such as uterus and vagina, it was found to accumulate in the nucleus as a complex with a protein which fulfilled the criteria for a receptor [17, 25]. Following the work from the laboratories of Chambon, Evans, and Vennstrom in the 1980s on the cloning of ER, TR, and GR, more than 35 nuclear receptors have been cloned, sequenced, and obtained as pure recombinant proteins, including several termed as "orphan" receptors, that is, whose ligands remain unknown. All nuclear receptors, which are products of the oncogene v-*erb*A, function as ligand-activated zinc-finger transcription factors with a modular structure comprising six domains [7, 26–28]. Nuclear hormone receptors can be subdivided into two groups, according to whether they form cytoplasmic complexes with hsp90 and are active as monomer and homodimer or as heterodimers. All vertebrate steroid hormone receptors belong to the first category, while the liganded receptors for retinoic acid, TR, vitamin D_3 (VDR), and peroxisome proliferator (PPAR) function as heterodimers with RXR, the 9-cis-retinoic acid receptor. This group of receptors exist as multiple isoforms, the multiplicity residing in the N-terminus of the receptor, which is a well-established characteristic of TRs present as TRα and TRβ, often exhibiting quite distinct functions.

An interesting question arises as to how the high degree of target gene specificity for a given hormone and its receptor is achieved within the above group of nuclear receptors comprising TR. The answer lies in the highly precise spacing of nucleotide repeats in the hormone response element (HRE) of the promoter of the target gene and the DNA-binding domain (DBD) of the receptor which recognises it. Interestingly, the HREs of the nonsteroid receptors, that is, TR, RAR, RXR, VDR, and PPAR, all share the same AGGTCA hexad but are organised as direct repeats (DRs) separated by one to five nucleotides. This arrangement of HREs explains the fine discrimination of target genes by the heterodimers formed by each of these receptors with RXR and which confers an extraordinary hormonal specificity. It is a most remarkable biochemical example of selective transcriptional regulation, confirmed for TR by NMR spectroscopy and X-ray crystal structure analysis [29]. Another interesting feature of TRs is that in the absence of its ligand (TH) it acts as a strong inhibitor of transcription, a property that is reversed upon the addition of TH [30]. In fact, Roeder and his colleagues have exploited this unique characteristic of TR to dissect biochemically the individual steps involved in eukaryotic gene transcription. An ever-increasing number of nonreceptor proteins are now known to interact with nuclear receptors and transcription factors, and that these complexes may function synergistically or in a mutually antagonistic manner [16]. For example, large protein molecules termed CBP (CREB binding protein) and p300 are thought to form bridges between nuclear hormone receptors, including TR, and other transcription factors. This conclusion was arrived at by the unexpected observation that nuclear receptors inhibit the activity of the nonreceptor transcription factor AP-1 by competing for limited amounts of CBP/p300 normally present in cells. Other important elements of the complex are the p160 nuclear receptor co-activator and the 270 kDa nuclear receptor corepressor (N-CoR), which have been purified and their functions tested. Evans and collaborators undertook a structural and thermodynamic analysis of the interaction domains of CBP and the p160 coactivators of TR and RAR to elucidate the assembly of the multiprotein activation complex. They describe a mechanism of mutual synergistic folding whereby the co-activators recruit CBP/p300 to facilitate the transmission of the hormonal signal to the transcriptional machinery [29]. Indeed, these examples of protein-protein interactions may just be the tip of an iceberg, and future studies on how TR function can be modified by other nuclear and extranuclear entities may turn out to be quite rewarding.

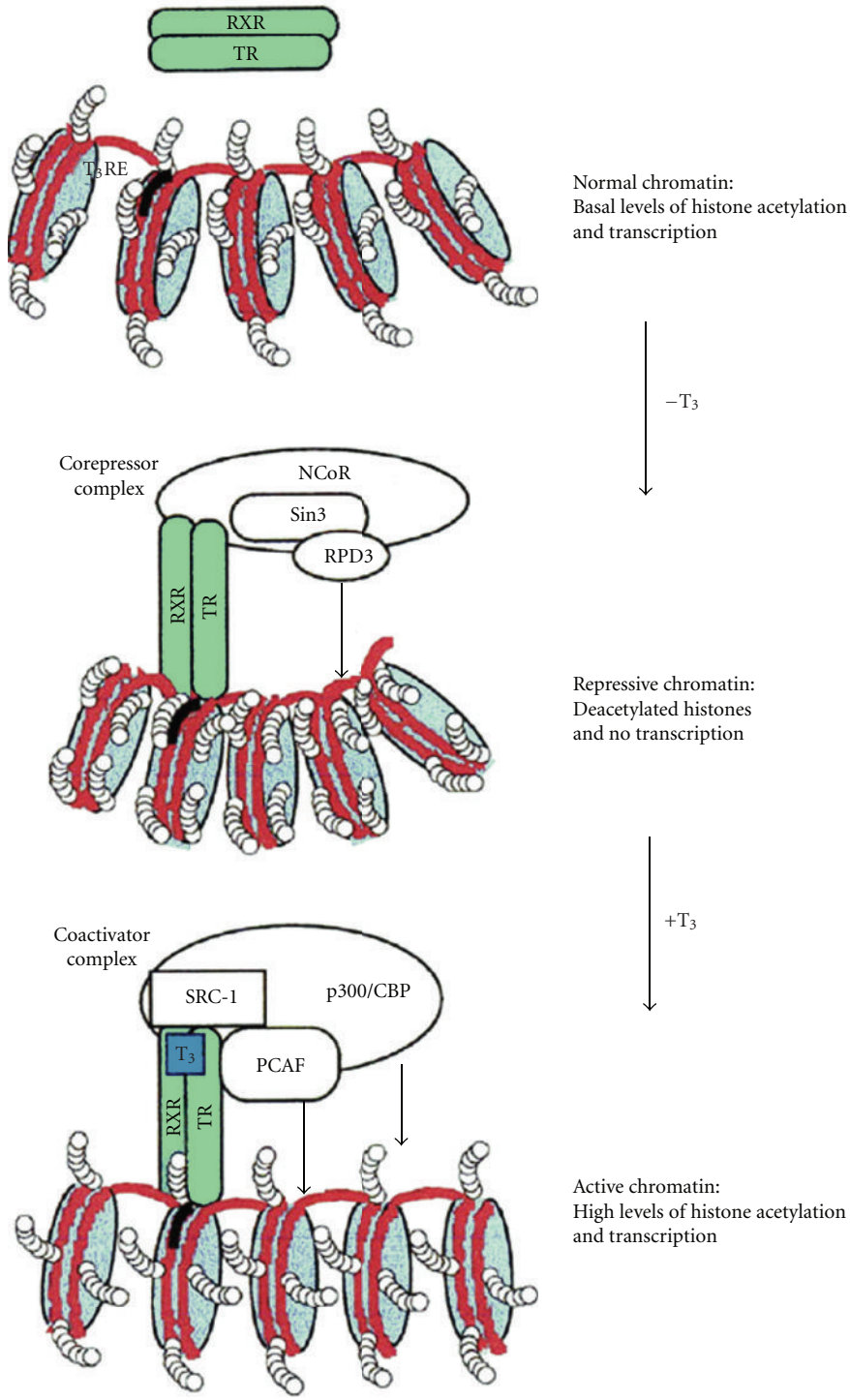

Figure 2: A representation by Wolffe [8] of how a ligand-activated nuclear receptor could modify the higher-order structure of chromatin. The packaging of DNA into chromatin is visualized in three transcriptionally active states: normal, repressive, and active. In this example, the region of chromatin chosen contains the thyroid hormone receptor (TR)/RXR heterodimer, with or without its ligand triiodothyronine (T_3), bound to the thyroid responsive element (TRE) in the target gene. In normal chromatin, histone acetylation is at its basal level and so is the transcriptional activity. In the absence of T_3 (as during early stages of development), chromatin exists in its condensed and transcriptionally repressive form whereby the histones are in a largely deacetylated state with no transcription of the TR's target gene. In the presence of T_3, the chromatin is now active with elevated levels of histone acetylation and transcription. The other components are proteins that form "corepressor" and "coactivator" complexes with complexes with the TR/RXR receptor heterodimer. For more details, see [8].

The significance of protein-protein interactions, in the context of regulation of transcription by TH via TR, has been extended to cell structure by Wolffe [8]. As described schematically in Figure 2, changes in chromatin structure, induced by histone modifications, in the presence and absence of T_3 are thought to underly the hormonal regulation of expression of specific genes (details in legand to Figure 2).

5. Expression of TR Genes

The expression of the two TH receptor genes TRα and TRβ in *Xenopus* tadpoles is under developmental control [31]. Very small amounts of both TR transcripts can be detected in unfertilized eggs and early embryos. A substantial increase, particularly of TRα mRNA, occurs at around stage 44, which, quite significantly, is when the *Xenopus* tadpole first exhibits competence to respond to exogenous thyroid hormone. At this stage of development, several tissues which are programmed to undergo major changes later during metamorphosis show high concentrations of TR mRNA, such as brain, liver, limb buds, small intestine, and tail. After stage 54 and until the completion of metamorphosis, there is good correlation between the accumulation of TR transcripts and the circulating level of thyroid hormone in Xenopus tadpoles [32, 33]. The relative amounts of TRα and β mRNAs vary according to the tissue of the tadpole and also according to the progression of metamorphosis. There is a possibility, not yet firmly proven, that the multiplicity of TH actions may in part arise from the two TR isoforms acting on different cellular or biochemical targets. Several studies from the laboratories of Brown, Shi, and Tata have established that administration of exogenous T_3 to premetamorphic stages of *Xenopus* tadpoles causes a substantial induction of TR mRNA [12, 34, 35]. The same phenomenon of "autoinduction" of TR mRNA is observed for *Xenopus* tadpole tails following exposure to TH *in vivo* and in organ culture, a tissue programmed not for further morphogenesis but for cell death and total regression [19]. In all these studies, the extent of "autoinduction" is more marked for TRβ mRNA than for TRα. Also, the upregulation of TRβ mRNA can be seen as early as 4 h after the exposure of these premetamorphic tadpoles to exogenous T_3, which is among the most rapid biochemical responses of *Xenopus* tadpoles to the hormone. The exact period preceding the autoinduction of TRβ receptor can vary according to the experimental conditions in different laboratories and can vary according to the stage of development of the tadpole or whether one adds the hormone to the whole organism, isolated tissues, or cell cultures. There is also the possibility that some other biochemical response may occur before the upregulation of the receptor. It has been suggested that such an earlier responsive element, as for example BTEB1, may be involved in the autoinduction of the TH receptor [36]. Nevertheless, it is important to consider in this context that TRβ is a direct-response gene, namely, that its upregulation occurs in the absence of protein synthesis and that the promoter in its gene comprises a fully responsive "thyroid-responsive element" [34, 35].

What is the likely mechanism of autoinduction of TRs? The most simple mechanism to explain the phenomenon of auto-regulation would be a direct interaction between TR proteins and the promoters of the genes encoding them [34]. It is significant, as already mentioned, that the promoter of the *Xenopus* TRβ gene has two TREs of the more common DR+4 (direct repeat +4) type, and that transfection of *Xenopus* XTC-2 and XL-2 cells, which express both TRα and β overexpression of unliganded TRα, and β in these *Xenopus* cell lines caused a substantial suppression of basal transcriptional activity. Under the conditions of transcriptional suppression, the addition of T_3 to *Xenopus* cells, cotransfected with the full-length TRβ promoter, produces up to 20-fold enhancement of TR gene transcription. Furthermore, these studies have shown that TR-RXR heterodimers, which are the natural form of functional TRs, but not TR monomers or homodimers, specifically interact with the DR+4 TREs of TRβ gene promoter [37]. These studies strongly support the idea of a direct interaction between the thyroid hormone receptor and the promoter of its own gene as the most likely mechanism underlying *Xenopus* TR "autoinduction." An indirect approach to understanding the significance of receptor autoinduction would be to look for an intimate association between TR upregulation and the expression of a TH target gene. One such study involved the simultaneous measurement of the expression in premetamorphic *Xenopus* tadpoles of TRβ and Xenopus 63 kDa keratin gene, which is only induced by TH during metamorphosis [38]. In stage 52 *Xenopus* tadpoles (which is before they show signs of metamorphosis), T_3 strongly induced simultaneously the accumulation of TRβ transcripts tadpoles *de novo* transcription of the adult keratin gene. The same was true if TR upregulation by T_3 was inhibited [38]. While such experiments do not establish a direct cause-effect relationship, they strongly suggest the intimate relationship between autoinduction of TR and the activation of the hormone's target genes.

Of wider significance, the phenomenon of autoinduction is not restricted to TR upregulated by TH during amphibian metamorphosis but is also seen with the expression of other nuclear receptors, such as those for steroids and retinoids [16, 21, 39]. A model has been proposed, whereby upregulation of a given receptor is a prerequisite for the sequential activation of its target genes that specify the hormone's biological action [11]. It predicts a double threshold of receptor numbers, the first, or lower level, which would be essential and sufficient for the autoinduction of the receptor and the second, higher threshold for the activation of target genes with which the hormone-receptor complex interacts. It also implies that the gene encoding a given receptor is constitutively expressed to produce a very low level of functional receptor in the target tissue at very early stages of development, which indeed is the case for most growth and developmental hormones and growth factors. One way of validating this model would be to measure the relative affinities of interaction between the DNA-binding domain or hormone-responsive elements in the promoters of the receptor and target genes (which specify the phenotype of biological action of the hormone).

6. Single or Multiple Receptors?

For nearly fifty years until the 1980s, most explanations for the mechanism of action of thyroid hormone were based on a direct interaction of the hormone with individual enzymes, membrane, and other cellular preparations with ligand-binding properties, that is, nongenomic mechanisms ([2]; see Table 3). But the mid-1980s saw a sea change in our thinking about the mechanism of action, not only of TH, but also of all steroid hormones, retinoic acid, and many nonsteroidal signalling molecules, with the discovery of nuclear receptors, that is, signals acting via genomic mechanisms [7, 16, 26, 27]. The powerful tools of gene cloning and sequencing and cell transfection have helped place these later genomic studies on a firmer biochemical base. More recently, however, several investigators have reinvoked the possibility that TH as well as steroid hormones and other signalling molecules acting via nuclear receptors also exert nongenomic actions through extranuclear sites. The latter is particularly relevant to those that involve rapid responses to the same signal acting relatively slowly via genomic mechanisms (see, e.g., [40–43]). This, in turn, raises the wider question for all hormonal and nonhormonal signals as to whether they operate through the same receptor, perhaps located in different cellular compartments, or whether the different genomic and nongenomic responses are the outcome of interactions with different receptors, irrespective of their locations. While, unlike nuclear receptors, we still await the isolation and precise information on the structure of "nongenomic" receptors, it is of some interest to consider some of the possible situations arising out of whether the multiplicity of responses to a given hormone arise from its interaction with a unique or multiple receptors, as considered below.

As already shown in Table 1, thyroid hormone exerts a wide range of actions in different tissues and organs (e.g., control of metamorphosis in amphibians and basal metabolic rate in mammals). The same is true of signalling molecules. Furthermore, almost all membrane and nuclear hormone receptors are highly conserved as cellular homologues of the oncogenes c-*erb*B and c-*erb*A, respectively, which explains why several hormones can functionally interact with their receptors in a wide range of phyla. For example, analogous domains of the receptors of insect hormone ecdysone and the vertebrate thyroid hormone can be swapped to activate transcription in insect and mammalian cells in a reciprocal fashion (see [44]). One is thus faced with the question as to how, if both the hormonal signal and its receptor are conserved through evolution, their physiological actions are not. It underlines the importance of an understanding of the postreceptor "Black Box" in order to move further down the pathway in the search for the mechanism of multiple actions of a given hormone.

To further consider the fact that hormone receptors are evolutionarily conserved, but that their downstream responses are not, three hypothetical, oversimplified, models are presented in Figure 3. First, If one accepts that there is a unique receptor for thyroid hormone in a given cell (model 1), is it possible at some point along the chain of postreceptor downstream responses that there is a point where chains of events diverge, such that it could explain the multiplicity of the physiological actions of the hormone, as shown in Table 1? This possibility of divergence has led some investigators to look for drugs to selectively enhance or block some TH actions of, as has been implicit in the blocking of cardiac effects, but not cholesterol-lowering effects of, thyroid hormone derivatives [45]. Another possibility for the multiplicity of responses is that the receptor is the same but that it is present in different cellular locations. For example, it has been suggested by some that the nuclear thyroid and steroid hormone receptors are also located in the plasma membrane and mitochondria which could give rise to different actions with different latency periods [40–42]. Recently, Cheng et al. [46] have claimed that the different responses to TH are initiated not only in the nucleus, but also in the plasma membrane, cytoplasm, and mitochondria (model 2). But firm evidence that these different locations represent distinct functional receptor entities such that each would be responsible for a distinct response to the hormone is however lacking.

The establishment of two distinct receptors for thyroid hormone, TRα and TRβ, has allowed the separation of different actions of TH at the receptor level. It has also meant that a well-characterized human thyroid disorder (thyroid hormone resistance) could be explained on the basis of a mutation in one and not the other TR isoform ([43, 47]; see Refetoff, this issue). For some other hormones or cellular signals, it is also possible to explain different responses to the same hormone on the basis of interactions with distinct receptors. For example, the actions of oestrogen via ERα and ERβ (oestrogen receptors α and β) explain the multiplicity of their biological actions as resulting from an initial interaction with distinct receptors in different cell types. This possibility is confirmed by the identification of oestrogen receptors α and β present in the different cell types but in the same intracellular location, that is, the nucleus [48].

Another possible model suggests that the multiplicity of action is a reflection of separate postreceptor downstream chains of responses (model 3), but, again, no convincing experimental evidence has been provided. One can invoke other models, such as a modification of that shown in model 3, whereby the action of one hormone acting through one receptor is qualitatively or quantitatively modulated by the downstream action via responses to another receptor (known or unknown) located in a different part of the target cell. The downstream modifications of responses could be compared to how Brivanlou and Darnell [49] conceptualize signals generated from the cell membrane can modify the chain of responses to a signal generated from within the cell, such as from the cell nucleus. The second (or third) signal involved in cross-regulation need not be hormonal in all cases and can be a growth factor, vitamin, antibody, and so forth. Scanning the literature on hormone action in general, many examples can be found whereby one or more hormones can modify the action of another, irrespective of whether or not these hormones act through nuclear or membrane receptors. This is depicted in model 3 of Figure 3 in which hormones H_2 and H_3 modify the response to T_3,

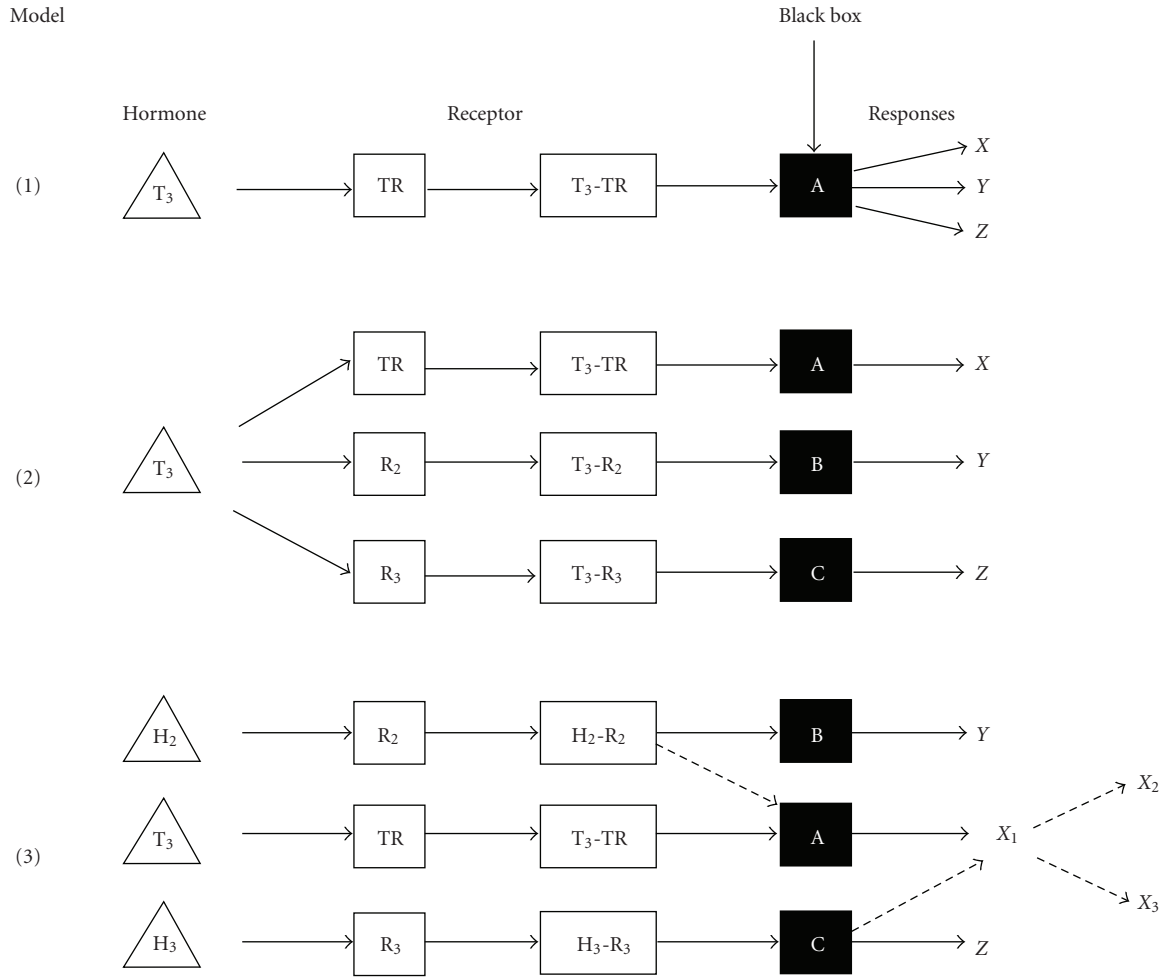

FIGURE 3: Three hypothetical models for thyroid hormone action depicting permutations of multiple hormonal interplay at the postreceptor level, represented by "Black Boxes" A, B, and C. According to model 1, the simplest situation is that T_3 interacting with a single thyroid hormone receptor (TR) modifies the activity of a key postreceptor complex which gives rise to a chain of multiple responses X, Y, Z. The multiplicity of responses to a hormone may also arise from different chemical or structural isoforms of a single receptor, represented in model 2 as TR, R_2 and R_3 together giving rise to multiple actions through functionally different Black Boxes. Alternatively, the same single receptor is present in different cellular locations and behaves effectively as different receptors (TR, R_2, R_3) in the same or different cells. In model 3, the multiplicity is a function of another hormone or signal (H_2, H_3), acting via different receptors (R_2, R_3) to modify the nature, multiplicity, or extent of the action(s) of T_3, either before or after the action at the level of Black Boxes. The multiplicity, can also result from further downstream interactions of the postreceptor responses (e.g., $X_1, X_2,$ and X_3).

each acting through its own receptor. Some examples of hormonal cross-regulation can be found in a most recent review [50].

In all three models depicted in Figure 3, the crucial element is the Black Box. Whereas there has been considerable progress, especially since the molecular cloning of hormonal and nonhormonal receptors, in understanding the fine details of receptor structure, function, and their intermolecular complexes, it has not yet been possible so far to link these data to the final physiological action of a given hormone. It is this gap in our knowledge of receptor function and its relevance to the final physiological action of the hormone (in this case thyroid hormone) that is represented by the Black Boxes in Figure 3. The Black Box could be a one-step or multistep event, the latter most likely if recent ideas of convergence of signals, networking, or proteomics turn out to be the crucial elements for furthering our understanding of mechanisms of hormone action. It represents a major challenge currently to fully understanding the mechanism of hormone action, in contrast to an enormous cataloguing of the effects produced by hormones in order to explain the multiplicity of hormone action. An understanding of the immediate postreceptor response by the target cell to the hormonal signal is essential to enhance our understanding of how hormonal signals accomplish their physiological actions.

7. Are We Anywhere Near Discovering the "Mechanism" of Action of Thyroid Hormone?

A central issue concerning hormone action has been to understand the nature and function of hormone receptors. The application at the end of the 20th century of established and newly emerging technologies of gene cloning, cell transfection, transgenesis and gene knock-out, X-ray crystallography, and NMR analysis of DNA-protein and protein-protein interactions have significantly advanced our understanding of the structure and function of both membrane and nuclear hormone receptors. These advances also point to the important position of cellular homologues of oncogenes in the evolution of cell signalling mechanisms. As regards thyroid hormone, what is most striking from Table 3 is that our views on the mechanism of its action have simply followed the progression of technology needed to understand biological processes in ever more detail. Thus, to answer the main question posed in this review about whether we are any closer to finding the ultimate mechanism of the action of thyroid hormone, the answer has to be a qualified "No." Qualified, because the progression in our search for the mechanism of action of any hormone has been a function of availability of new biochemical, biophysical, genetic, and structural biological technologies. Often, this has simply resulted in the cataloguing of an ever-rapidly accumulation of effects of hormones, but not of their mechanism of action. In other words, the search for the mechanism of hormone action is often largely technology driven and not sufficiently hypothesis based.

So, should one abandon the search for a molecular mechanism of action of thyroid or any other hormone? The answer again is "No." On the contrary, attempts so far to discover the mechanism of hormone action have often led to the discovery of some fundamental principles of biological regulation and signalling. For example, Sutherland and Rall's search for the mechanism underlying how insulin and glucagon regulate metabolic activity in rodent liver [14] led them to discover cyclic AMP which, in turn, unearthed a central pillar of regulatory biology of all living organisms, equally applicable to bacteria, plants, and animals, and not just restricted to hormone action. It was then a relatively short step towards establishing the importance of protein phosphorylation and dephosphorylation in so many vital cellular functions [51], such as energy metabolism, protein and DNA synthesis, and membrane receptor-linked activities, not to mention the subsequent discovery of cyclic GMP and G-proteins, as vital signalling components within cell membranes. Not less important, efforts to unravel the role of thyroid hormone in regulating transcription within the cell nucleus have provided much valuable insight into the multiplicity and functions of RNA polymerases [30] and the importance of structural organisation of chromatin in regulation of gene expression [8, 16].

8. Perspective

In the 1920s, the increasing availability of pure, nondenatured and biologically active proteins, along with the advances in biochemistry of enzymes, soon led to explanations of mechanisms of thyroid hormone action based on hormone-enzyme interaction. Later, the developments of the methods for subcellular fractionation gave rise to hypotheses based on hormones directly interacting with cell membrane enzymes, mitochondria, ribosomes, and the cell nucleus to explain regulation of energy metabolism, protein synthesis, and transcriptional control. Hormone receptors, as cellular homologues of oncogenes, now occupy a central position in our current thinking of signalling mechanisms, whether located in the cell membrane or the nucleus.

It is certain that increasingly rapid emergence of new molecular, cellular, and physical technologies is going to lead us to re-evaluate the validity and importance of our present-day concepts of mechanisms of hormone action. One can safely predict that, just as in the past, every new technical advance is going to demand a rethinking, modification, or even rejection, of our currently accepted models and introduce new concepts of how thyroid hormone elicits its molecular mechanism of action. What are the most likely developments in the future in our search for the mechanism of thyroid hormone action? The discovery of new factors associated with transcription, such as coactivators, corepressors, and integrators, will continue to define in greater detail how thyroid hormone receptors regulate gene expression; many more modulators will be discovered in the future and that these will be found to form even more complex structures with nuclear hormone receptors [16]. Similarly, it is only in the last decade that the true significance of enzymes that add or remove acetyl and methyl groups in histones and other chromosomal proteins has been realized in the context of a network of cellular signals impinging on nuclear and extranuclear sites. As yet we have uncovered the identity of only a fraction of the approximately 300 chromatin-associated proteins. The recent findings on histone modifications emphasize nucleosome and chromatin structure as dynamic entities that make up the transcriptional machinery and which, in turn, is now leading us to a major shift in our thinking on how thyroid (and steroid) hormones control gene expression [8]. Indeed, Flamant and colleagues have more recently put together a number of different ways in which TH and TRs, together and separately, render the mechanisms of signalling by thyroid hormone much more complex than has been thought of earlier [43].

In a wider perspective, there is an increasing realization that the interaction between different signalling mechanisms operating through membrane, nuclear, and cytoplasmic sites has to be considered as converging mechanisms, rather than as individual or isolated pathways [49]. For example, recently, Kress et al. [9]; (see Plateroti, this issue) have emphasized the importance of considering crosstalk between TH-controlled and other signalling mechanisms. The same group have also highlighted another facet of TH action, namely that the same signal can elicit dual and divergent actions, in this case of cell proliferation and differentiation and further propose that multiple gene networks will also have to be taken into account [10]. They compare the multiplicity of responses to the hormone with what has been

well known during amphibian metamorphosis where TH can promote cell growth, differentiation, or cell death in different tissues of the larva [12]. More indirectly, factors governing the availability of thyroid hormone to the receptor, or other sites in the pathway of its action, have also been invoked. For example, the generation or inactivation of T_3, the active thyroid hormone, by iodothyronine deiodinases has also to be considered as regulators of thyroid hormone action in controlling metabolism and development [4, 52]. Clearly, much has yet to be learned about how thyroid hormone signalling regulates specific gene expression and diverse cellular functions from early development to cell death. Finally, all future advances will have to be considered in the context of evolutionary aspects of hormones and their actions, while not losing sight of the history of earlier attempts to explain the mechanism of hormone action.

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Review Article

Thyroid Hormone and the Neuroglia: Both Source and Target

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Thyroid hormone plays a crucial role in the development and function of the nervous system. In order to bind to its nuclear receptor and regulate gene transcription thyroxine needs to be activated in the brain. This activation occurs via conversion of thyroxine to T3, which is catalyzed by the type 2 iodothyronine deiodinase (D2) in glial cells, in astrocytes, and tanycytes in the mediobasal hypothalamus. We discuss how thyroid hormone affects glial cell function followed by an overview on the fine-tuned regulation of T3 generation by D2 in different glial subtypes. Recent evidence on the direct paracrine impact of glial D2 on neuronal gene expression underlines the importance of glial-neuronal interaction in thyroid hormone regulation as a major regulatory pathway in the brain in health and disease.

1. Introduction

Thyroid hormone is a fundamental regulator of biological processes, including cell proliferation, differentiation, and metabolic balance [1]. Thyroid hormone plays a crucial role in brain development, which is illustrated by the dramatic neurologic impairment observed in untreated neonatal hypothyroidism, a condition leading to cretinism [2–4]. The adult brain is also sensitive to thyroid hormone in view of mood disorders, depression, memory, cognitive and motoric impairments frequently observed in hypothyroid patients [5].

The major secretory product of the human thyroid gland is thyroxine (T4), a prohormone that does not efficiently bind thyroid hormone receptor (TR). T4 has to be converted to 3,5,3′-triiodothyronine (T3) in order to bind to TR and initiate thyroid hormone-mediated changes in gene expression profiles. Notably, a significant amount of brain T3 is derived from the local activation of prohormone T4 to T3 (80% in the cortex), suggesting that most T3 acting in the brain is generated *in situ* from T4 deiodination [6]. Plasma T3 was also shown to enter the brain [7], and studies on the monocarboxylate transporter 8 (MCT8) knock-out mice indicate that MCT8 plays an important role in this process, but other transporters might also be involved [8, 9]. However, only supraphysiological doses of T3 were sufficient to suppress pro-TRH mRNA in the hypothalamic paraventricular nucleus of hypothyroid rats [10] indicating that T4 uptake into the brain is important for normal function of T3-mediated processes in this tissue. Further studies are required to better understand the transport of different thyroid hormone derivatives across the blood-brain and CSF-brain barrier, the consequences of this mechanism, and the factors affecting this process (see also Section 3.3).

Local T3 generation in the brain is catalyzed by the type 2 deiodinase (D2), a tightly controlled selenoenzyme [11–14]. D2 is the only known protein capable of producing T3 in the human brain [15]. Beyond D2-mediated T3 generation, type 3 deiodinase (D3) is also similarly important for thyroid hormone economy in the brain [16, 17]. D3 inactivates T3 and converts T4 to reverse-T3 that cannot bind to TR. Thus, in contrast to D2, D3 catalyzes the inactivation pathway of thyroid hormone metabolism.

D2 is expressed in glial cells, including astrocytes in different brain regions and tanycytes, the specialized glial cells in the walls and floor of the third ventricle of the mediobasal hypothalamus [18–20]. In contrast to the glial D2, D3 expression in the brain is restricted to neurons [21].

While historically glial elements of the brain were viewed as a type of connective tissue of the CNS without any real function, this view was overturned by the abundant data on the complex role of glial cells in brain metabolism [22]. According to this more recent hypothesis, glial D2 provides T3 for neighboring neurons that express TR but lack T3 generating capacity [4, 19, 20, 23–25].

Thyroid hormone exerts its biological effects predominantly via binding to its nuclear TR, but specific nongenomic effects have also been suggested [26–29]. Two TR isoforms, α and β, act as ligand-regulated transcription factor and have a central role in transducing the hormonal signal into a cellular response in the brain (reviewed in [30, 31]). Despite accumulating evidence demonstrating that thyroid hormone alters astrocytes function (see Section 2), the presence of TR in astrocytes remains controversial.

The presence of TR in astrocytes has been suggested by *in vitro* studies [32–34], but lower receptor concentrations have been detected compared to oligodendrocytes or neurons [34]. The presence of TR could also be detected in purified glial nuclei from postnatal rat brain [35]. In contrast, *in vivo* data suggested that thyroid hormone would mediate astrocyte function indirectly, based on the lack of immunofluorescence staining of TR receptor isoforms α1, β1, and β2 in GFAP positive astrocytes of the adult rat brain [36]. Interestingly the same group used immunofluorescent to locate TR in cultured astrocytes [37], in line with other *in vitro* data. Astrocytes from distinct developing brain regions are differently responsive to thyroid hormone, with the highest sensitivity in the hemispheres [38]. Thyroid hormone has been shown to be essential for maturation of rat cerebellar astrocytes [39], and TRα1 knock-out mice display astrocyte maturation defects suggesting the role of this TR isoform to mediate a direct effect of thyroid hormone action in astrocytes [40]. Presently, most studies agree with the presence of TRα1 isoform in astrocytes; discrepancies remain in the case of TRβ receptor subtypes. Expression of different TR isoforms has been reported in human astrocytomas [41]. Thyroid hormone action occurs within limited time windows, a spatially and timely controlled phenomenon. Cultured cells and most astrocytomas are devoid of the same control conditions, which normally act on cells in the brain, and this could be a background of the different experimental results obtained between *in vitro* and *in vivo* data. In addition, cultured astrocytes are most likely reactive astrocytes, and the heterogeneity of the *in vitro* experimental results regarding TR expression in these cells may reflect the different experimental conditions, such as the brain region and the age of the animals used for cultivation or the different culture conditions.

Active transport of thyroid hormone into brain cells adds to the complexity of thyroid hormone economy in the central nervous system [42, 43]. The MCT8 (SLC16A2) and organic anion transporter 1C1 (OATP1C1) are the best studied thyroid hormone transporters [44, 45]. MCT8 seems to be the predominant neuronal T3 transporter, and its mutations are associated with the Allan-Herndon-Dudley syndrome characterized by congenital hypotonia that progresses to spasticity with severe psychomotor delays [44, 46, 47]. OATP1C1 has high affinity to T4 and is expressed in brain endothelial cells and also in vascular end-feet of astrocytes [48]. Interestingly, tanycytes seem to coexpress MCT8 and OATP1C1 [48]. Other thyroid hormone transporters have also been identified including MCT10, which seems to transport T3 more effectively than MCT8 [49] and the L-type amino acid transporters (LATs) [50]. MCT10 expression was demonstrated in microglia while LAT1 and LAT2 expression was found both in astrocytes and neurons; LAT2 was also present in microglia [51]. Studies on the MCT8 deficient mouse revealed that in the absence of functional MCT8, alternative thyroid hormone transporters play an important complementary role in neuronal T3 transport. In contrast, the lack of alternative pathways, for example, LAT2 in developing human neurons, might be involved in the devastating neurodevelopmental phenotype seen in MCT8-deficient patients with Allan-Herndon-Dudley syndrome [8, 52].

Studies on transgenic mice with targeted inactivation of different members of the deiodinase enzyme family, MCT8, or their combined deletion provided important information on the complex nature of functional interactions between factors regulating thyroid hormone metabolism and transport in the brain and other tissues [8, 9, 53–58]. Despite the relatively mild brain phenotype of D2KO or MCT8KO mice, their combined inactivation led to aggravated manifestation of thyroid hormone deprivation and resulted in similar effects as observed in hypothyroidism [9, 59]. These data confirmed the crucial role of D2 in local T3 generation in the brain and suggested that changes in D2 expression could compensate for defects in MCT8 function in the rodent brain.

Numerous aspects of deiodinase-mediated changes of thyroid hormone metabolism have been carefully reviewed and provide a comprehensive view on the molecular and biochemical properties, structure, regulation, and biological functions of these enzymes in the brain and different tissues [24, 60–69]. In the present paper we will focus on the role and regulation of thyroid hormone in neuroglia, representing an exciting aspect of emerging significance for thyroid hormone economy. We provide a concise overview on the most important effects of thyroid hormone on glial cells, followed by the discussion of novel data on D2-mediated glial T3 generation and its role under specific physiological and pathophysiological conditions.

2. Thyroid Hormone-Mediated Changes in Neuroglial Cells

Brain development provides the best studied model of thyroid hormone action in the brain. It has been known for decades that hypothyroidism can result in numerous brain defects, including decreased dendritic arborization of Purkinje cells, diminished axonal outgrow and myelinization, and insufficient cortical layer organization [70]. Although thyroid hormone also impacts the adult brain, the underlying cellular and molecular events are less understood [71, 72]. Various aspects of thyroid hormone-mediated brain function were extensively reviewed (see Section 1).

Available data on thyroid-hormone-regulated gene networks are yet limited, but accumulating evidence indicates that various sets of genes are regulated along this pathway. In a recent study, thyroid hormone action on adult rat striatum was monitored using gene expression profiling [73]. The numerous up or down regulated sets of genes involved various pathways affecting for example, circadian regulation, oxidative stress response, phenylethylamine degradation, MAPK pathway, phosphate metabolism, signal transduction, and cell structure. These findings revealed novel aspects of brain related thyroid hormone action that need to be studied in details. Numerous examples of thyroid hormone-dependent gene expression in the brain are related to neurons, which could be a result of direct neuronal effect or an indirect glia-mediated signal [74]. Neuroglial cells are heavily involved in the regulation of neuronal metabolism and activity, glucose supply, cerebral blood flow, and neurotransmitter levels in mature brain [22]. The detailed description of mechanisms how glial cells are involved in this process is an ongoing effort and requires further studies. We will briefly summarize below how thyroid hormone targets glial cells and mediates their function that has also consequence on glia-mediated neuronal activities.

2.1. Differentiation, Maturation. Thyroid hormone affects the differentiation and maturation of different glial subtypes including astrocytes, oligodendrocytes, and microglia [75–77]. Although many aspects of glial linages are yet controversial, evidence has been obtained *in vitro* that oligodendrocytes and astrocytes are derived from a common precursor, the glial restricted precursor cells (GRP) [78]. GRPs are tripotential cells, owning the ability to divide into myelin producing oligodendrocyte or two types of astrocytes, depending on the factors contained in growth medium. *In vitro* studies demonstrated that mature oligodendrocytes were developed from precursor cells in the presence of thyroid hormone and platelet-derived growth factor (PDGF) [79]. The concept that these two glial cell types originate from a common lineage was also supported by findings that show reciprocal changes in oligodendrocyte/astrocyte cell density in the rat white matter upon changes in serum T4 level [80]. Furthermore, the number of matured oligodendrocytes and astrocytes was reduced in the brain of hypothyroid animals within white matter tracts [81, 82]. Morphological differentiation of astrocytes from progenitors to mature cells has been explained by thyroid hormone-mediated actions affecting cytoskeletal proteins (F-actin, GFAP) [40, 83]. In hypothyroid neonatal rats, there was reduced GFAP content in hippocampus and basal forebrain [84]. In cell cultures, T3 upregulates GFAP production and reorganizes GFAP filaments and transforms the flat polygonal astrocytes into process-bearing cells [85, 86]. Not only T3, but T3-mediated growth factor secretion can enhance GFAP expression, thanks to several growth factor binding domains in its promoter region [87, 88].

Microglial cell development is also affected by thyroid hormone. In the cortical forebrain of hypothyroid neonatal rat there is diminished amount of cell bodies and less abundant microglial process density. T3 favored survival of microglias *in vitro* and have triggered their process extension [77, 89].

The mechanisms by which thyroid hormone promotes differentiation were not yet fully revealed, but there are several candidates for this process, for example, cell-cycle modulators like E2F-1, cyclin D1, and p27 [90]. E2F1 is a key transcription factor, that controls G1 to S phase transition and has an impact on cyclin D1 [91]. E2F-1 and cyclin D1 expression is down regulated via TR-mediated transcriptional repression [92, 93]. Another candidate of this pathway, p27 cyclin-dependent kinase inhibitor was upregulated in response to T3 [94, 95]. Decreased amount of E2F-1 and cyclin D1 protein and increased levels of the p27 cell-cycle inhibitor may shift cell fate towards differentiation.

2.2. Myelination. Myelination represents the best characterized T3-dependent glial action in the brain [96–98]. Thyroid hormone regulates oligodendrocyte differentiation and myelin production via TR-mediated transcriptional effects [34, 99]. Thyroid hormone depletion resulted in delayed expression of oligodendrocyte-specific markers [100] and decreased the number of oligodendrocyte cell bodies in the main white matter tracts [81]. Hypothyroidism delayed the expression of genes encoding structural proteins of myelin, for example, myelin basic protein (MBP), proteolipid protein (PLP), and myelin-associated glycoprotein (MAG) [101] and resulted in reduced numbers of myelinated axons and lower myelin content [102]. Sensitivity period of these genes for thyroid hormone extends from the end of the first postnatal week up to the end of the first month in rat [76, 103].

2.3. Extracellular Matrix Formation and Cytoskeleton Organization. Thyroid hormone action on astrocytes during brain development is illustrated by enhanced secretion of extracellular matrix proteins and growth factors. Astrocytes were previously shown to produce laminin and fibronectin [104, 105]. Subsequent studies demonstrated T3-induced expression of laminin and fibronectin in cultured cerebellar astrocytes and revealed that both laminin and fibronectin were organized in fibrillar pattern on the cell surface, while hypothyroid conditions changed this distribution for a disorganized extracellular matrix of punctuate pattern [106]. As an underlying mechanism it was suggested that astrocytes modulate extracellular matrix composition via T3-mediated growth factor secretion [104, 107].

Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) are secreted by cerebellar astrocytes in response to T3 and seem to promote extracellular matrix protein secretion and organization in an autocrine manner [106]. EGF was suggested to exert its effect on extracellular matrix protein secretion through MAPK/phosphatidylinositol 3-kinase pathway [108]. Astrocytes also secrete nerve growth factor (NGF) in a T3-dependent manner, which allows potent control of neurite growth and survival [109–111].

Beyond T3, the effect of T4 on astrocytes was also demonstrated suggesting that thyroid hormone could also

impact astrocytes via a nongenomic pathway. T4 exerts its effect on the microfilament network of astrocytes by dynamically organizing F-actin filaments, facilitating integrin clustering, and focal contact formation [112, 113]. Polymerized actin filament network was observed in cultured astrocytes after treatment with T4 and reverse T3 while T3 did not affect the polimerization rate [114, 115].

Adhesive interactions among the extracellular matrix protein laminin, integrins, and the microfilament network play a fundamental role in the regulation of neural cell migration during brain development. *In vitro* studies on neurite development demonstrated that neurons, cocultured with astrocytes under thyroid hormone-depleted conditions, showed reduced total neurite length and decreased neurite numbers [108]. As a consequence, these data suggest that thyroid hormone-mediated actions on astrocytes are important events in neuron migration and axon formation.

3. Thyroid Hormone Activation in Neuroglia

3.1. Regulation of Type 2 Deiodinase in Glial Cells. While thyroid hormone impacts glial function in various manner (see above), neuroglia is not only target but also the predominant source of T3 in the brain. As mentioned above, astrocytes and tanycytes express type 2 deiodinase (D2), the enzyme catalyzing thyroid hormone activation. Below, we will discuss factors and conditions affecting D2 regulation in glial cells, since they can contribute to the better understanding of thyroid hormone signaling in the brain.

3.1.1. Thyroid Hormone. D2 is negatively regulated by thyroid hormone, through a mechanism that involves product (T3-) mediated transcriptional downregulation of the *Dio*2 gene and substrate (T4-) induced posttranslational decrease of D2 protein levels (reviewed in [66], see Section 3.2). The negative regulation of D2 activity suggests a homeostatic regulation of T3 generation [116–118]. However, region-specific differences within the brain regarding the response to hyper- or hypothyroidism are reflected by changes in D2 regulation. D2 is reciprocally regulated by thyroid hormone in various brain regions but shows only modest response in the hypothalamus [19, 119–121]. The fact that D2 activity in the hypothalamus is concentrated in tanycytes [19] suggests marked differences between astrocytes and tanycytes regarding thyroid hormone response and balance. While in astrocytes T3 production seems to serve homeostatic purposes, the relative insensitivity of D2 to T3 in tanycytes would indicate that other signals act more importantly on D2 expression, thus controlling local T3 production [25]. The mechanisms responsible for this difference between astrocytes and tanycytes remain to be determined. However, a link has been suggested between the developmental state of astrocytes and their responsiveness to thyroid hormone [38]. Although tanycytes are still considered as terminally differentiated cells, data have been accumulating that at least a subpopulation of this inhomogeneous cell layer might behave as progenitor cells. This is supported by observations that the tanycyte layer in the wall of the third ventricle regenerates in two weeks following alloxan-induced destruction [122], and tanycytes could be considered a neurogenic niche in response to IGF-I [123]. This is presently unclear whether differences in differentiation stages or a more specific factor is responsible for the different responsiveness to thyroid hormone of the two cell types.

3.1.2. Infection, Nonthyroidal Illness. It has been suggested that D2-generated T3 in tanycytes of the mediobasal hypothalamus could play a role in the pathogenesis of nonthyroidal illness during infection [63, 66, 67, 124]. Nonthyroidal illness syndrome (euthyroid sick syndrome or low T3 syndrome) is accompanied by low T3 and sometimes low T4 serum levels and associated with nonelevated or inappropriately elevated TSH levels during infection, sepsis, starvation, malignancy, life-threatening trauma, and other critical illness [125–128]. Although the syndrome has been known for decades, it is still a matter of debate whether the changes of thyroid hormone profile provide physiologic compensation for illness or it represents pathological conditions [129–131]. Systemic administration of bacterial lipopolysaccharide (LPS) increased D2 mRNA expression in tanycytes and D2 activity in the rat mediobasal hypothalamus (Figure 1) accompanied by falling serum thyroid hormone and TSH levels [132]. This phenomenon was also observed in mice, immediately followed by decreased expression of thyroid receptor $\beta 2$, TSHβ in the pituitary and decreased type 1 deiodinase mRNA in the pituitary and liver [133].

LPS induced suppression of TRH expression in the hypothalamic paraventricular nucleus of wild type but the effect was abolished in the D2 knock-out mice (Figure 2) (see Section 3.3) [23]. Although this model is not suitable to dissect the role of specific glial subtypes in this mechanism, it clearly demonstrated the fundamental role of D2 activity in TRH suppression during infection and supported the hypothesis of a close interaction between neurons and glial cells and their role in regulating brain functions via T3 availability. Importantly, while the continuous increase in D2 activity of cortical astrocytes seemed to be the consequence of falling T4 levels, D2 activation in tanycytes followed kinetics that was independent of thyroid hormone levels [132, 134]. It was also demonstrated that LPS-induced D2 expression on the mediobasal hypothalamus was not dependent on circulating corticosterone, either [135]. Unexpectedly, cultured astrocytes of the rat cerebral hemispheres increased their D2 activity in response to LPS, and glucocorticoids enhanced this effect [136]. It is presently not clear why this effect is not reflected *in vivo* by the kinetics of cortical D2 induction. Importantly NF-κB, a potent effector of LPS-induced signaling, transcriptionally activated the D2 encoding *Dio*2 gene, and a functional NF-κB binding site was identified and characterized in the human *Dio*2 5′ flanking region [132, 137]. NF-κB was also involved in the LPS-induced increase in D2 activity in cultured astrocytes [136].

Further studies on the kinetics of LPS-induced activation of the NF-κB pathway in the rat mediobasal hypothalamus indicated that NF-κB activation contributes to sustaining the LPS-induced D2 response in a subset of α tanycytes [138]. However, this is not the initiating mechanism of LPS-induced D2 response in tanycytes. The same study suggested

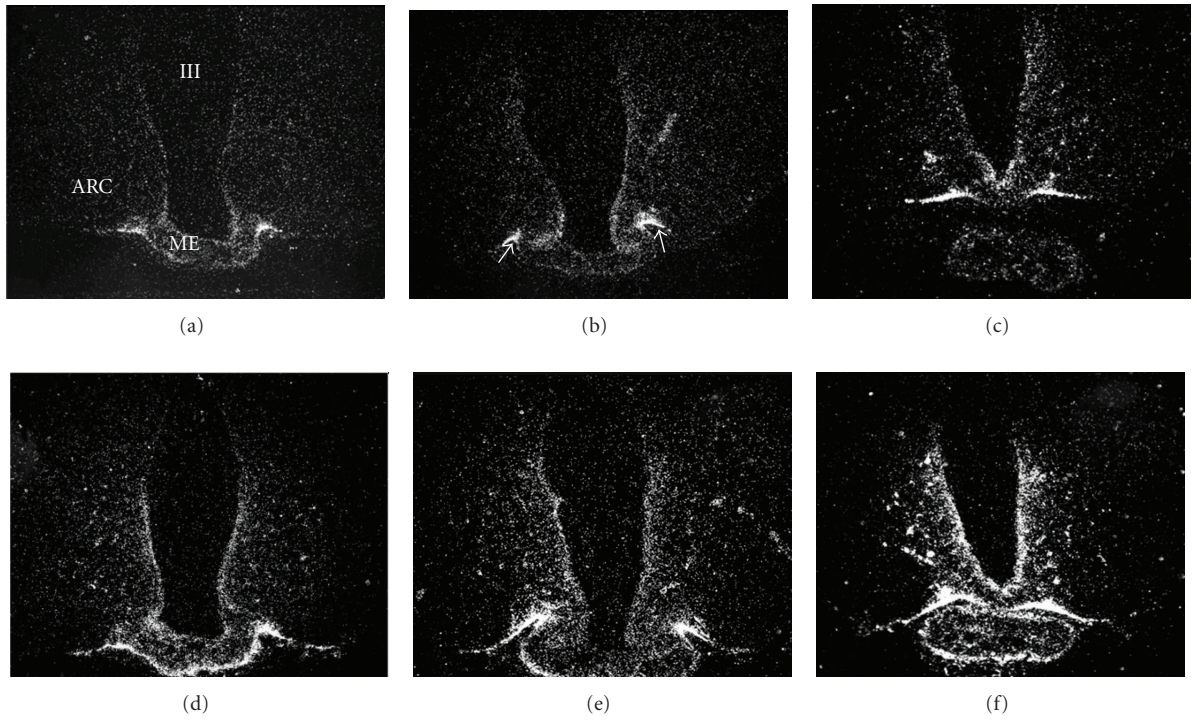

Figure 1: Infection upregulates D2 expression in tanycytes of the rat mediobasal hypothalamus. Dark-field micrographs from three different rostrocaudal levels of the median eminence (ME) showing the effect of *i.p.* LPS treatment on D2 mRNA expression. (a)–(c), Controls; (d)-(f), LPS-treatment. Note: D2 *in situ* hybridization signal is increased in the tanycytes lining the wall of the third ventricle (III), and in the tanycyte processes in the tuberoinfundibular sulci (arrows), in the external zone of the ME. ARC, arcuate nucleus. Reprinted with permission from Fekete et al. [132], The Endocrine Society.

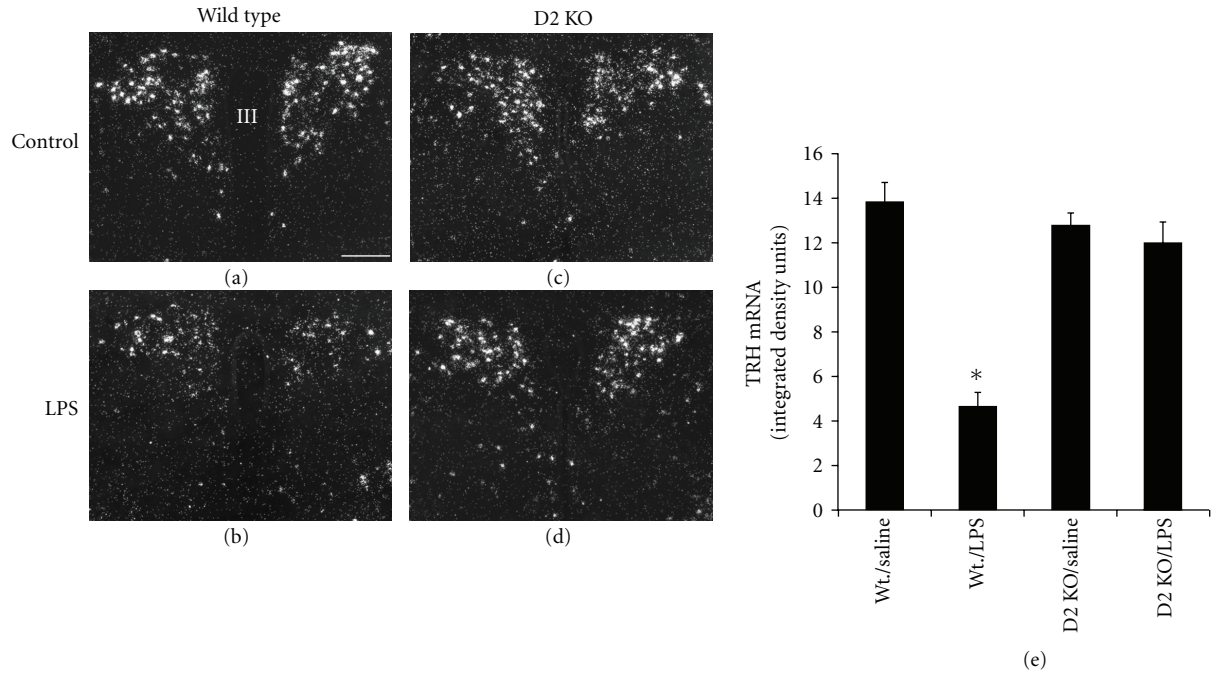

Figure 2: LPS-induced infection downregulates TRH mRNA expression in the hypothalamic paraventricular nucleus of wildtype but in D2 knock-out mice. (a,b), wild-type; (c,d), D2 KO mice; (a,c), control; (b,d), *i.p.* LPS-treated animals; (e), quantification of the TRH mRNA signal by densitometry. Printed from Freitas et al. American Society for Clinical Investigation [23].

that TSH of the part tuberalis could also play a role in this process [138]. The factor(s) that initiate tanycytal D2 induction in the starting phase of LPS-evoked infection are presently not known. However, taking into account the highly active nature of D2-catalyzed T3 generation even a subpopulation of tanycytes could provide a significant amount of T3 for the modulation of TRH expression. To asses this appropriately, it would be important to understand in details the pathways that allow tanycyte-generated T3 to reach hypophysiotropic TRH neurons in the paraventricular nucleus.

3.1.3. Iodine. Iodine availability is critically important to maintain proper thyroid hormone levels. During moderate iodine deficiency most thyroid hormone target tissues are only mildly affected, due to rapid physiological adaptations of the hypothalamo-hypophyseal-thyroid axis, which maintains plasma T3 at the normal range [139, 140]. Via glial D2 and neuronal D3, the brain is capable of adapting to iodine deficiency in a complex manner. A moderately severe iodine deficiency resulted in increased D2 mRNA and activity in different brain regions [140]. D2 sensitivity to iodine deficiency was region specific, the hippocampus and cerebral cortex represented the most responsive regions. D2 induction in this regions indicated that astrocytes increase their T3-generating activity under iodine deficiency. Tanycytes of the mediobasal hypothalamus also increased their D2 expression and activity although their response was lower compared to astrocytes in the cortex and hippocampus. Since increase of D2 activity was higher than that of mRNA expression, it could be speculated that not only pretranslational events are involved here in D2 regulation but also prolonged D2 half-life due to decreased D2 ubiquitination (see Section 3.2) could contribute to this effect. Increased glial D2 in iodine deficiency was paralleled with reduced neuronal D3 [140]. Thus mitigating the effects of iodine deficiency by both increased T3 generation and reduced T3 degradation reflected the particular importance of adaptation of the brain to iodine deficiency. Various aspects of iodine deficiency modulated alterations of thyroid hormone deiodination were extensively reviewed elsewhere [141].

3.1.4. Fasting. D2 expression in tanycytes is modulated by food restriction. Fasting resulted in twofold increase in D2 mRNA expression and activity in rat mediobasal hypothalamus, and it was straightforward to suggest that this could suppress TRH expression in the hypothalamic paraventricular nucleus and downregulate this way the hypothalamo-hypophyseal-thyroid axis. [121]. However, fasting-mediated decrease of TRH expression in the paraventricular nucleus of the TRβ2-null transgenic mice remained unaffected although TRβ2 represents the key TR isoform involved in T3-mediated negative regulation of TRH expression in transgenic mice [142]. This finding demonstrated that tanycyte-generated T3 during fasting should not have major direct effects on TRH expression in the paraventricular nucleus. As an alternative pathway, the hypothalamic ventromedial nucleus was also suggested as a target translating changing hypothalamic T3 levels into the modulation of food intake [143]. The role of glial D2-mediated hypothalamic T3 in fasting is not yet resolved, and related data are reviewed elsewhere [25, 61, 63, 66].

3.1.5. Light. D2 expression in the mediobasal hypothalamus is controlled by light, and this has consequences on reproductive function. Light exposure-induced D2 expression in the mediobasal hypothalamus of the Japanese quail (*Coturnix japonica*) represents a crucial event in the signal transduction pathway ensuring photoperiodic response of gonads. Intracerebroventricular administration of T3 mimicked the photoperiodic response, whereas the D2 inhibitor iopanoic acid prevented gonadal growth [144]. Interestingly, beyond median eminence and infundibular nucleus D2 induction was also observed in the dorsal and lateral hypothalamus. Based on this finding it cannot be excluded that not only tanycytes but other cell types, for example, hypothalamic astrocytes could be also involved in this mechanism, but this aspect was not studied in details. A mechanism for light-induced D2 expression in the mediobasal hypothalamus was also revealed in quail showing a preceding peak of TSHβ-subunit expression in the pars tuberalis via a cAMP-dependent mechanism. It was demonstrated that intracerebroventricular administration of TSH to short-day quail stimulated gonadal growth and D2 expression and proved that TSH in the pars tuberalis therefore seems to trigger long-day photoinduced seasonal breeding [145].

A homology between avian and mammalian photoperiodic regulation of reproduction has been observed since D2 expression was also increased in Djungarian (Siberian) hamsters (*Phodopus sungorus*) under long days; the signal was weaker under short days while melatonin injection decreased D2 expression under long days [146]. These results indicate that D2 expression in tanycytes may be involved in the regulation of seasonal reproduction both in mammals and birds. Regulation of seasonal reproduction by photoperiodic regulation of hypothalamic thyroid hormone levels also involves reciprocal changes of D2 and D3 expression that is reviewed elsewhere along with data on other models of seasonal reproduction [66, 147, 148].

3.1.6. Trauma. After traumatic brain injury D2 mRNA was upregulated in reactive astrocytes in rat. In the cerebral cortex near the contusion D2 mRNA was upregulated on the first day after injury; in the following days the signal was shown to have expanded to the hippocampus, where the astrocytic localization of upregulated D2 mRNA was obvious and bordered the neuronal granule cell layer [149]. Furthermore, different stressors including relatively mild ones (e.g., handling) increased D2 activity in a stressor- and brain-region dependent manner [150]. The frontal cortex showed the highest D2 response, and motor stress was the most dominant stressor in this region while no effect was seen in the cerebellum. A stressor-dependent decrease of T4 tissue concentration was also observed but stressor-dependent deviations were also found since, for example, gently handling resulted in elevated T4 in the frontal cortex.

A strict correlation between D2 activity and tissue T4 levels could not be found suggesting the role of specific factors and not simply the falling T4 level in stress-related D2 increase [150].

3.1.7. Development. Deiodinases are tightly regulated during various developmental processes (reviewed in [4, 151]). It was shown that the human fetal brain is already sensitive to thyroid hormones before the onset of the fetal thyroid [152, 153]. The presence of high-affinity T3 binding sites with a specificity that resembles that of the nuclear T3 receptors was also demonstrated in the human fetal brain and its concentration increased by tenfold from ten to sixteen weeks [154]. D2 expression and activity was detected in the human fetal cortex already from seven to eight weeks of gestation [155]. It has been also demonstrated that during the second trimester T3 increases in the cortex due to D2 activity, while it remains very low in cerebellum because of D3-mediated thyroid hormone inactivation [156]. Although deiodinase activities were also studied in the developing rat brain, [157], data are limited on ontogenic aspects of D2 expression in different glial subtypes. Increasing D2 expression was detected in the developing chicken brain in perivascular localizations probably localized to glial cells [158]. It was also demonstrated that D2 was expressed in chicken tanycytes before the onset of thyroid hormone-dependent negative feedback. Furthermore, D2 and Nkx2.1 were coexpressed at E13 and P2 in tanycytes but not in the perivascular glia indicating a glial-subtype-specific regulation of D2 expression [159].

3.1.8. Other Factors. It has been demonstrated that D2 expression in glial cells is under the control of multiple factors. These include the increase of D2 activity upon cAMP induction [160, 161] that is in line with the finding of an evolutionary conserved CRE site in the *Dio*2 promoter [61, 162–164]. Selenium dependence [165], phorbol esters and glucocorticoids [166], acidic fibroblast growth factor [167] also impact D2 activity.

3.2. Posttranslational Regulation of Glial D2 Activity. It has been demonstrated that D2 activity in the brain undergoes rapid and substrate-induce changes [116, 117]. The underlying mechanism was later identified demonstrating that D2 undergoes substrate-induced ubiquitination followed by its degradation in the proteasome [168–170]. This was a unique example of substrate-induced selective proteolysis that involves ubiquitination of an endoplasmic reticulum resident enzyme and represented the first demonstration that such a regulatory pathway controls activation of a hormone [170]. The pathway works also in primary cultures of astrocytes, since MG132 a proteasome uptake inhibitor could block substrate-induced D2 inactivation [171]. Interestingly, D2 inactivation via ubiquitination does not necessarily involve proteasomal proteolysis. It has been revealed that D2 forms homodimers that undergo ubiquitination-mediated transient and reversible conformation changes. Since dimerization of D2 monomers is crucial to maintain the proper conformation of the active center of the enzyme, ubiquitination-mediated changes result in the rapid loss of D2 activity [172].

Since D2 ubiquitination represents a rapid way for the regulation of T3 generation its mechanism was studied in detail in the past several years. UBC6 and 7 were identified as the ubiquitin conjugases (E2) involved in the ubiquitination of D2 [173, 174], while USP-33 and USP-20 (VDU1 and 2) deubiquitinate D2 and prolong its half-life [175]. A novel type of ubiquitination motif containing a 18-aa-loop structure of the D2 protein was identified and characterized [176, 177]. WSB1 (Swip1) was recognized as a sonic hedgehog-induced SOCS-box containing protein of unknown function [178, 179]. Importantly, it could be shown that WSB1 serves as the ubiquitin ligase (E3) that links D2 to the Elongin BC-Cul5-Rbx1 ubiquitinating catalytic core complex [176]. Later, Teb4 has been also identified as a D2 E3 ligase [180].

Data are accumulating on how crucial elements of the D2 ubiquitination machinery are expressed in D2-expressing glial subtypes. The available data revealed cell-type-specific differences in the expression of crucial elements of the D2 ubiquitinating/deubiquitinating machinery in the rodent brain. WSB1, the D2 E3 ligase, is expressed both in GFAP-expressing astrocytes in different brain regions and in tanycytes in the mediobasal hypothalamus (Figure 3) [181]. This suggested that the WSB1-D2 interaction, a process required for D2 ubiquitination, could be functional in these cells. In contrast to WSB1, the TEB4 E3 ligase could not be detected in GFAP-expressing astrocytes, only in the cerebellum, but it was expressed in tanycytes [180]. Furthermore, the USP33 (VDUI) D2 deubiquitinase is co-expressed with D2 only in tanycytes but not in astrocytes (Figure 3) [181]. WSB1 and USP33 expression in the brain was not affected by thyroid hormone status indicating that these genes are not involved in the homeostatic response to hypo- or hyperthyroidism [181].

The available data suggested that kinetics of D2 ubiquitination and consequent selective proteolysis in the proteasome could be different among different subtypes of glial cells. Among D2-expressing cell types of the brain, tanycytes express the most comprehensive set of genes involved in ubiquitination-mediated D2 regulation, that ensures both WSB1- and TEB4-mediated ubiquitination ligation to D2- and also USP33 deubiquitinase-mediated D2 reactivation. In astrocytes D2 deubiquitinaton is either not possible, or it works via USP20 or other unidentified D2 deubiquitinases.

3.3. Neuroglial Thyroid Hormone Metabolism Affects Neuronal Gene Expression. Astrocytes and tanycytes of the neuroglial compartment are the predominant source of T3 present in the brain while TR in neurons represents a major target of thyroid hormone. As discussed above, neurons cannot generate T3 but express type 3 deiodinase (D3), the T3 degrading enzyme. While numerous observations suggested that glial thyroid hormone metabolism could affect neuronal function (see Section 3.1), until recently no direct evidence could be obtained to prove the existence of deiodinase-mediated transcriptional T3 footprints in neurons. Recently a two-dimensional coculture was used based on the D2-expressing

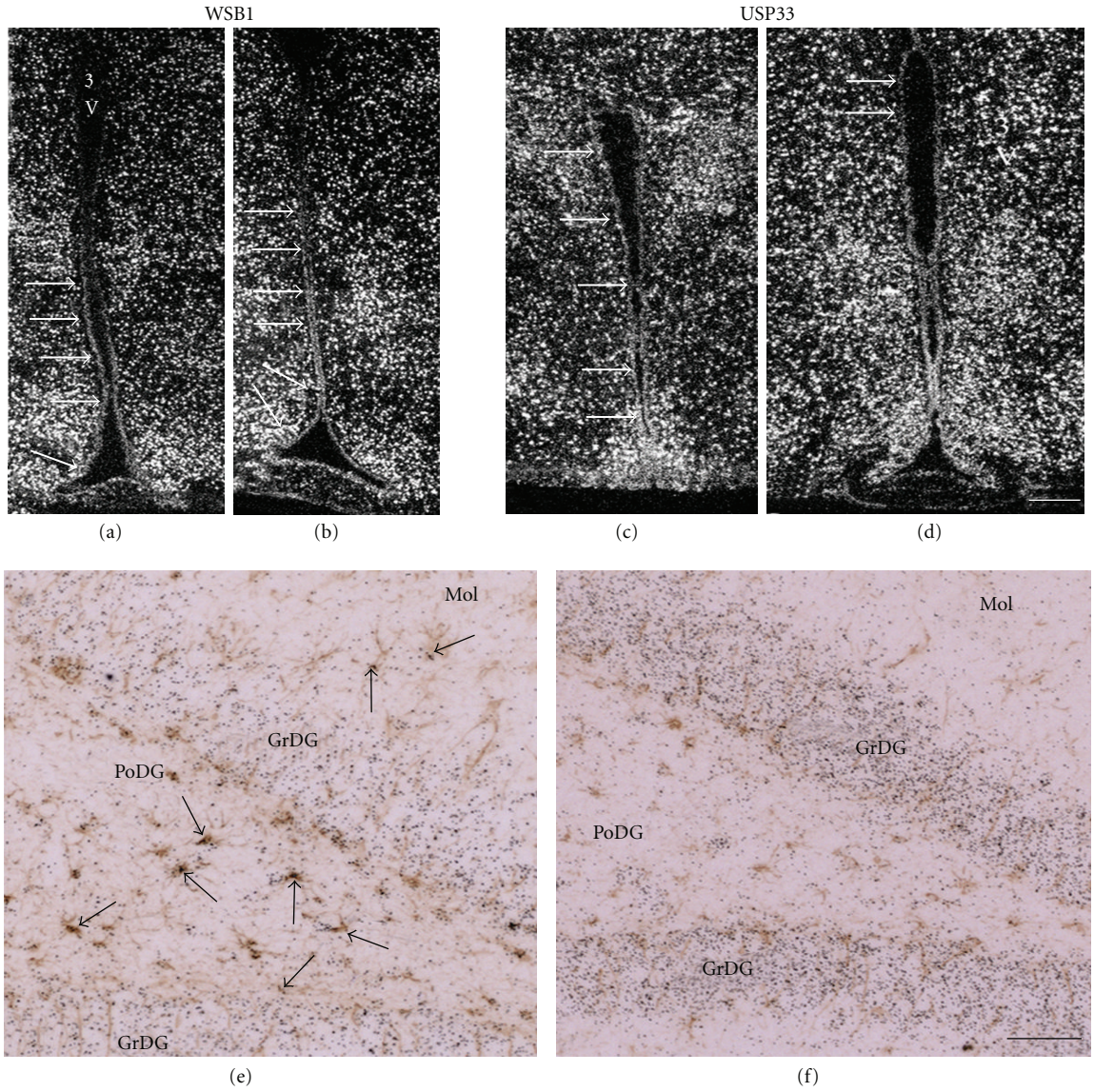

Figure 3: Expression of crucial elements of the D2 ubiquitination machinery in glial cells in the rat brain. (a,b) mRNA of WSB1, the D2 ubiquitin ligase is expressed in tanycytes lining the wall of the third ventricle (3V). WSB1 expression *(arrows)* extended from the anterior lower part (not shown) to the lower two thirds of the wall of the third ventricle in more caudal regions. Neuronal cells also express WSB1. (c,d) Hybridization signal of the USP33 D2 deubiquitinase was also detected over tanycytes and ependymal cells lining all regions of the wall of the third ventricle *(arrows)*. Neuronal cells also express USP33. (e) WSB1 *in situ* hybridization signal (arrows) is observed over the majority of GFAP-expressing astrocytes (brown immunoreactivity) demonstrated here in the hippocampal dentate gyrus. (f) The mRNA of USP33 was absent from GFAP-expressing astrocytes (brown) in the hippocampus, but it was expressed in granular neurons. The sense probes for WSB1 or USP33 did not produce any signal (not shown). Mol, molecular layers of the dentate gyrus; GrDG, granular layer of the dentate gyrus; PoDG, polymorph layer of the dentate gyrus. Reprinted with permission from Fekete et al. [181] The Endocrine Society.

H4 glioma cells and the D3-expressing SK-N-AS neuronal cell line. It has been shown that T4 could activate the endogenously expressed T3-sensitive ENPP2 gene of the neuronal compartment only if the glial compartment was present. This model led to the demonstration that D2-mediated glial T3 generation from physiological amount of T4 can directly affect thyroid hormone-dependent gene expression in a paracrine fashion [23]. A different approach using expression profiling-based assessment of thyroid-hormone-regulated gene expression in the cerebral cortex of the MCT8, D2, and MCT8/D2 knock-out mice suggested that negative regulation required D2-generated T3, while peripheral T3 entering the brain should be sufficient to maintain normal expression of positively regulated genes [59].

Specific signals as hedgehog proteins [176, 182], bacterial lipopolysaccharide (LPS) [132, 133, 137, 183], and hypoxia [184] have been established as regulators of deiodinase activities. It was also studied how these specific signals impact neuroglial thyroid hormone metabolism in the coculture system. The sonic hedgehog morphogene decreases glial thyroid

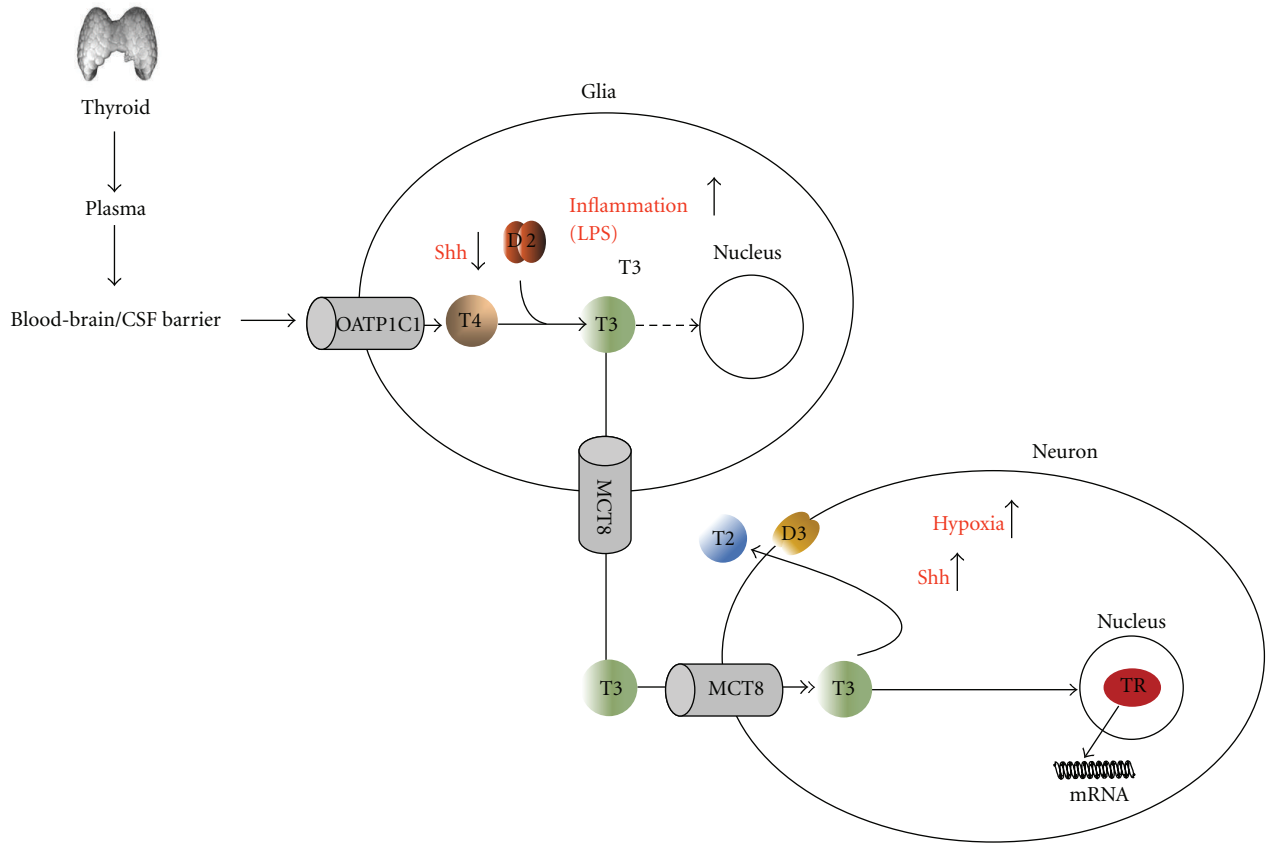

Figure 4: Proposed model of neuroglia-neuron interaction of thyroid hormone signaling in the brain. D2 activates the prohormone T4 in glial cells (astrocytes and tanycytes); the generated T3 exits the glial compartment and enters adjacent neurons, where it establishes a transcriptional footprint via liganding TR. Only the two best-characterized thyroid hormone transporters, OATP1C1 and MCT8, are indicated, but data are also accumulating on the role of LAT1 and LAT2 in the thyroid hormone transport both in neurons and astrocytes (discussed in Section 1). In the glial compartment LPS activates D2 transcription while sonic hedgehog (Shh) promotes D2 inactivation via WSB1—mediated ubiquitination; both hypoxia and Shh activate D3 gene transcription in neurons. Figure modified from Freitas et al. American Society for Clinical Investigation [23].

hormone activation via WSB1-mediated posttranslational downregulation of D2 (see Section 3.2) and increases neuronal D3 expression [23]. This demonstrates the existence of a mechanism ensuring a fine-tuned balance between sonic hedgehog-mediated proliferation and T3-evoked differentiation. This is interesting since astrocytes are targets of sonic hedgehog signaling [185]. It has been also demonstrated that in the brain T3 upregulates crucial elements of the sonic hedgehog signaling pathway that could represent a compensatory feedback loop for sonic hedgehog-mediated T3 regulation [186].

The effect of LPS on D2 expression and its relation to nonthyroidal illness were discussed in Section 3.1. In contrast to sonic hedgehog, LPS-induced glial D2 activity and decreased neuronal D3 in the H4-SK-N-AS system and as a consequence resulted in a decreased T3-mediated gene expression in the neuronal compartment [23]. These data were complemented with *in vivo* observation on the LPS evoked model of nonthyroidal illness. LPS could not induce TRH suppression on the paraventricular nucleus of the D2 knock-out mice only in wild types (Figure 2) (see also Section 3.1). This indicated that glial (highly probably tanycytal) D2-generated T3 in the hypothalamus could play an important role in T3-mediated suppression of the hypophysiotropic TRH neurons and consequently in the decreased activity of the hypothalamo-hypophyseal-thyroid axis during the infection-evoked subtype of nonthyroidal illness [23].

In contrast, hypoxia affected predominantly neuronal D3 activity in the H4- SK-N-AS system. This effect could be also demonstrated in a rat *in vivo* hypoxia/ischemia model showing D3 induction in cortical neurons and in the hippocampal pyramidal and granular cell layers [23]. This suggested that lowered local T3 levels improve neuronal survival under hypoxic challenge. The glial aspect of this phenomenon requires further studies since an independent study on primary cultures of astrocytes demonstrated hypoxia-induced increase of D2 activity [171]. These data established deiodinase enzymes as glial and neuronal control points for the regulation of thyroid hormone action in the brain during health and disease (Figure 4) [23].

Abbreviations

D2: type 2 deiodinase
D3: type 3 deiodinase
GFAP: glial fibrillary acidic protein
T4: thyroxine
T3: 3,5,3′-triiodothyronine
TR: thyroid hormone receptor
LPS: bacterial lipopolysaccharide
TSH: thyrotropin
TRH: thyrotropin-releasing hormone.

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Review Article

Thyroid Disorders and Diabetes Mellitus

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Studies have found that diabetes and thyroid disorders tend to coexist in patients. Both conditions involve a dysfunction of the endocrine system. Thyroid disorders can have a major impact on glucose control, and untreated thyroid disorders affect the management of diabetes in patients. Consequently, a systematic approach to thyroid testing in patients with diabetes is recommended.

1. Introduction

Thyroid diseases and diabetes mellitus are the two most common endocrine disorders encountered in clinical practice. Diabetes and thyroid disorders have been shown to mutually influence each other and associations between both conditions have long been reported [1, 2]. On one hand, thyroid hormones contribute to the regulation of carbohydrate metabolism and pancreatic function, and on the other hand, diabetes affects thyroid function tests to variable extents. This paper demonstrates the importance of recognition of this interdependent relationship between thyroid disease and diabetes which in turn will help guide clinicians on the optimal screening and management of these conditions.

2. Frequency of Thyroid Disorders in the General Population and in Patients with Diabetes

Thyroid disorders are widely common with variable prevalence among the different populations. Data from the Whickham survey conducted in the late 1970s in the north of England revealed a prevalence of 6.6% of thyroid dysfunction in the adult general population [3]. In the Colorado Thyroid Disease Prevalence study involving 25,862 participants attending a state health fair, 9.5% of the studied population were found to have an elevated TSH, while 2.2% had a low TSH [4]. In the NHANES III study, a survey of 17,353 subjects representing the US population, hypothyroidism was found in 4.6% and hyperthyroidism in 1.3% of subjects [5]. The latter further observed an increased frequency of thyroid dysfunction with advancing age and a higher prevalence of thyroid disease in women compared to men and in diabetic subjects compared to nondiabetic.

Several reports documented a higher than normal prevalence of thyroid dysfunction in the diabetic population. Particularly, Perros et al. demonstrated an overall prevalence of 13.4% of thyroid diseases in diabetics with the highest prevalence in type 1 female diabetics (31.4%) and lowest prevalence in type 2 male diabetics (6.9%) [6]. Recently, a prevalence of 12.3% was reported among Greek diabetic patients [7] and 16% of Saudi patients with type 2 diabetes were found to have thyroid dysfunction [8]. In Jordan, a study reported that thyroid dysfunction was present in 12.5% of type 2 diabetic patients [9]. However, thyroid disorders were found to be more common in subjects with type 1 diabetes compared to those with type 2 diabetes. Additionally, a 3.5-fold increased risk of autoimmune thyroiditis was noticed in GADA positive patients [10]. Thyroid disorders remain the most frequent autoimmune disorders associated with type 1 diabetes. This was shown in a cross-sectional study involving 1419 children with type 1 diabetes mellitus, where 3.5% had Hashimoto's thyroiditis [11]. In addition, positive TPO antibodies have been reported in as high as 38% of diabetic individuals and have been

shown to be predictive for the development of clinical and subclinical hypothyroidism [12–14]. Very recently, Ghawil et al. documented that 23.4% of type 1 diabetic Libyan subjects had positive TPO antibodies and 7% had positive TG antibodies [15]. The association between AITD and T1DM has been recognized as a variant of APS3 referred to as APS3 variant [16]. Common susceptibility genes have been acknowledged to confer a risk for development of both AITD and type 1 diabetes mellitus. Currently, at least four shared genes have been identified including HLA [17–22], CTLA-4 [23], PTPN22 [24, 25], and FOXP3 genes [26].

3. Effects of Thyroid Hormones on Glucose Homeostasis

Thyroid hormones affect glucose metabolism via several mechanisms. Hyperthyroidism has long been recognized to promote hyperglycemia [27]. During hyperthyroidism, the half-life of insulin is reduced most likely secondary to an increased rate of degradation and an enhanced release of biologically inactive insulin precursors [28, 29].

In untreated Graves' disease, increased proinsulin levels in response to a meal were observed in a study by Bech et al. [30]. In addition, untreated hyperthyroidism was associated with a reduced C-peptide to proinsulin ratio suggesting an underlying defect in proinsulin processing [31]. Another mechanism explaining the relationship between hyperthyroidism and hyperglycemia is the increase in glucose gut absorption mediated by the excess thyroid hormones [32, 33].

Endogenous production of glucose is also enhanced in hyperthyroidism via several mechanisms. Thyroid hormones produce an increase in the hepatocyte plasma membrane concentrations of GLUT2 which is the main glucose transporter in the liver, and consequently, the increased levels of GLUT-2 contribute to the increased hepatic glucose output and abnormal glucose metabolism [34, 35]. Additionally, increased lipolysis is observed in hyperthyroidism resulting in an increase in FFA that stimulates hepatic gluconeogenesis. The increased release of FFA could partially be explained by an enhanced catecholamine-stimulated lipolysis induced by the excess thyroid hormones [36]. Moreover, the nonoxidative glucose disposal in hyperthyroidism is enhanced resulting in an overproduction of lactate that enters the Cori cycle and promotes further hepatic gluconeogenesis. The increase in GH, glucagon and catecholamine levels associated with hyperthyroidism further contributes to the impaired glucose tolerance [37–39].

It is well known that diabetic patients with hyperthyroidism experience worsening of their glycemic control and thyrotoxicosis has been shown to precipitate diabetic ketoacidosis in subjects with diabetes [40, 41].

As for hypothyroidism, glucose metabolism is affected as well via several mechanisms. A reduced rate of liver glucose production is observed in hypothyroidism [42] and accounts for the decrease in insulin requirement in hypothyroid diabetic patients. Recurrent hypoglycemic episodes are the presenting signs for the development of hypothyroidism in patients with type 1 diabetes and replacement with thyroid hormones reduced the fluctuations in blood glucose levels as demonstrated by Leong et al. [43]. In a case control study involving type 1 diabetic patients, those with subclinical hypothyroidism experienced more frequent episodes of hypoglycemia during the 12 months prior to the diagnosis of hypothyroidism compared to euthyroid diabetics. On the other hand, both clinical and subclinical hypothyroidisms have been recognized as insulin resistant states [44–46]. In vivo and in vitro studies have shown that this is due to impaired insulin stimulated glucose utilization in peripheral tissues [44, 45, 47, 48]. A recent study involving subjects from a Chinese population found a higher TSH level in patients with metabolic syndrome compared to that in the nonmetabolic syndrome group suggesting that subclinical hypothyroidism may be a risk factor for metabolic syndrome [49]. More recently, Erdogan et al. found an increased frequency of metabolic syndrome in subclinical and overt hypothyroidism compared to healthy controls [50]. Therefore, it seems prudent to consider hypothyroidism in newly diagnosed metabolic syndrome patients. This raises the issue whether routine screening for thyroid disease in all patients newly diagnosed with metabolic syndrome will be cost effective. Furthermore, an increased risk of nephropathy was shown in type 2 diabetic patients with subclinical hypothyroidism [51] which could be explained by the decrease in cardiac output and increase in peripheral vascular resistance seen with hypothyroidism and the resulting decrease in renal flow and glomerular filtration rate [52]. In 2005, Den Hollander et al. reported that treating hypothyroidism improved renal function in diabetic patients [53]. As for retinopathy, Yang et al. demonstrated recently that diabetic patients with subclinical hypothyroidism have more severe retinopathy than euthyroid patients with diabetes [54].

The increased risk of retinopathy and nephropathy observed in diabetic patients with subclinical hypothyroidism provides evidence in favor of screening patients with type 2 diabetes for thyroid dysfunction and treating when present.

4. Leptin, Adiponectin, Ghrelin, and Thyroid Hormones

Thyroid hormones may influence carbohydrate mechanisms via its interaction with adipocytokines and gut hormones. Among these adipocytokines, adiponectin is the most abundant adipokine secreted by the adipose tissue and has important insulin-sensitizing properties. Low levels of adiponectin have been shown to confer a higher risk for development of type 2 diabetes. Adiponectin and thyroid hormones share some biological properties including reduction in body fat by increasing thermogenesis and lipid oxidation [55]. It has been suggested that adiponectin might influence thyroid hormone production through its interaction with gC1q receptor found in thyroid mitochondria [56]. On the other hand, it was recently shown that T3 exhibited an inhibitory effect in rat models on adiponectin mRNA expression particularly on white adipose tissue [57]. The relationship between thyroid hormones and adiponectin remains to be

clarified and limited studies addressing this issue have shown inconsistent results. Some studies found that adiponectin are increased in hyperthyroidism [58–60], whereas other studies report unchanged levels in states of excess thyroid hormones [61, 62]. In hypothyroidism, reduced levels of adiponectin have been shown by Dimitriadis et al. [44], and comparable levels of adiponectin were observed in hypothyroid patients and controls in a study by Nagasaki et al. [63]. Therefore, no definite conclusion can yet be drawn, and further studies are needed to clarify the above controversies.

Leptin is another hormone produced by adipocytes that regulates energy expenditure and body weight. A correlation between leptin and thyroid hormones has been demonstrated in several studies. However, results have also been discordant. Some studies showed a decrease in leptin levels in hyperthyroidism [61, 64], whereas others observed unchanged levels [65–67]. Similarly, increased [64, 67], unchanged [66], and even decreased [65] values of leptin have been reported in hypothyroid patients. An increase in serum leptin and insulin have been described in hypothyroid dogs [68]. On the other hand, leptin, by enhancing the activity of type I iodothyronine 5′-deiodinase enzyme, could result in an increase in circulating T3 level [69]. The changes in fat mass accompanying thyroid diseases complicates the interpretation of the results of studies on leptin and thyroid dysfunction. However, the complex interplay between thyroid hormones and leptin and its possible influence on carbohydrate metabolism remains to be elucidated.

Ghrelin is an orexigen secreted from the fundus of the stomach. It has been shown to exert several diabetogenic effects including decreasing secretion of the insulin sensitizing hormone adiponectin [70]. In addition, ghrelin circulates in two different forms acylated and desacylated ghrelin with the latter constituting the major circulating form. Ghrelin levels are lower in obese subjects and those with type 2 diabetes, states associated with hyperinsulinemia [71]. Reduced ghrelin levels were observed in hyperthyroid patients [72, 73], and these levels rose to normal values after pharmacological treatment of hyperthyroidism [74–76]. Hyperthyroidism, being a state of negative energy balance should result in an increase in ghrelin levels. Interestingly, ghrelin levels in thyroid dysfunction states seem to correlate with insulin resistance rather than food intake and energy balance [77]. Hyperthyroidism is associated with insulin resistance [77] and hyperinsulinemia suppresses ghrelin levels [78]. Increased levels of ghrelin has been observed in hypothyroid patients, and these levels normalized with L-thyroxine treatment [74, 79].

In hypothyroid rat models, increased circulating ghrelin and gastric ghrelin mRNA levels were demonstrated by Caminos et al. [80]. However, in other studies, hypothyroid patients were reported to have comparable ghrelin levels to that of healthy subjects and those levels were not significantly altered after thyroid hormone replacement [77, 81, 82]. Therefore, the limited number of studies assessing the link between thyroid dysfunction, on one hand, and ghrelin and adipokines, on the other hand, have yielded conflicting results. These discrepancies could be potentially explained by differences in individuals' characteristics, changes in fat mass and energy expenditure accompanying hyper- or hypothyroidism, duration and degree of thyroid dysfunction, and variability in the assays used for hormonal measurements particularly for ghrelin. As previously mentioned, ghrelin circulates in two major forms, acyl ghrelin which exerts a stimulatory effect on food intake and desacyl ghrelin which reduces food intake inducing a state of negative energy balance. Measuring either form or measuring total ghrelin will lead to confounding results.

5. Thyroid Function and Energy Expenditure

Besides all of the above described mechanisms, thyroid hormones can indirectly affect glucose metabolism through modulation of energy homeostasis. Although the underlying mechanisms have not yet been clearly defined, thyroid hormones have been shown to alter the expression of uncoupling proteins in brown adipose tissue involved in effective thermoregulation [83].

More recently, a role for thyroid hormones and TRH in the central regulatory pathways for thermogenesis has been identified. TRH neurons in the hypothalamus express both thyroid hormone nuclear receptors (TRs) and type 4 melanocortin receptor (MC4R), a key receptor involved in central energy regulation [84]. Activation of MC4R reduces food intake and increases energy expenditure and inactivating mutations in MC4R are associated with obesity [85]. The repressive effect of T3 on the expression of MC4R helps in conserving energy in hyperthyroid states [86]. Furthermore, both the POMC (pro) and AgRP (Agouti-related protein) neurons of the arcuate nucleus act at the MC4R. Thus, T3, by reducing the expression of MC4R, has been shown to decrease the hypothalamic sensitivity of the POMC and AgRP signaling [86].

AMP-activated protein kinase (AMPK), a cellular energy sensor, mediates the effects of various nutritional and hormonal signals in the hypothalamus.

Mice lacking AMPKα2 in POMC neurons developed obesity due to a reduced resting metabolite rate and a defective nutrient handling. On the other hand, AMPKα2 knockout mice in AgRP neurons remained lean with an enhanced sensitivity to melanocortin agonists [87]. On the other hand, injecting an adenovirus expressing the dominant-negative form of AMPK (Ad-DN AMPK) into the hypothalamus of male rats resulted in significant decrements in glucose production. Recently, López et al. showed that hyperthyroidism or central administration of T3 reduced the activity of hypothalamic AMPK [88]. Consequently, thyroid hormones could indirectly alter glucose metabolism via their interaction with various hypothalamic signals. However, the exact mechanisms behind this complex interaction remains to be clarified.

6. Effects of Diabetes Mellitus on Thyroid Hormones and Thyroid Diseases

Altered thyroid hormones have been described in patients with diabetes especially those with poor glycemic control. In diabetic patients, the nocturnal TSH peak is blunted

or abolished, and the TSH response to TRH is impaired [89]. Reduced T3 levels have been observed in uncontrolled diabetic patients. This "low T3 state" could be explained by an impairment in peripheral conversion of T4 to T3 that normalizes with improvement in glycemic control. However, in a study by Coiro et al. involving type 1 diabetes patients with absent residual pancreatic beta cell function, an amelioration in glycemic control did not restore the normal nocturnal TSH peak suggesting a diabetes-dependent alteration in the central control of TSH [90]. Higher levels of circulating insulin associated with insulin resistance have shown a proliferative effect on thyroid tissue resulting in larger thyroid size with increased formation of nodules [91, 92]. A higher prevalence of type 1 diabetes is observed in patients with Grave's orbitopathy than in the normal population. Furthermore, the vasculopathic changes associated with diabetes renders the optic nerve more susceptible to the pressure exerted by the enlarged extraocular muscles. Consequently, a higher incidence of dysthyroid optic neuropathy is observed in diabetic subjects with Graves ophthalmopathy compared to nondiabetic [93].

7. Conclusion

The relationship between thyroid disorders and diabetes mellitus is characterized by a complex interdependent interaction. Insulin resistance states may increase thyroid gland nodularity and coexisting diabetes may increase risk of visual loss in patients with Graves' disease. Hyperthyroidism impairs glycemic control in diabetic subjects, while hypothyroidism may increase susceptibility to hypoglycemias thus complicating diabetes management. Additionally, thyroid hormones may further alter carbohydrate metabolism via its interaction with leptin, adiponectin, and gut hormones, namely, ghrelin. However, this association and the resulting alteration in metabolic effects need further research. It has been shown that thyroid dysfunctions are more prevalent in people with diabetes and particularly type 1 diabetes. Furthermore, it seems that unidentified thyroid dysfunction could negatively impact diabetes and its complications. A higher frequency of retinopathy and nephropathy was observed in diabetic patients with subclinical hypothyroidism, and more severe retinopathy was noted as well. Therefore, management of subclinical hypothyroidism in patients with diabetes may prove beneficial. We conclude that a systematic approach to thyroid testing in diabetic subjects is favorable; however, no definitive guidelines exist regarding screening for thyroid dysfunction in diabetic patients. Finally, whether all patients with diabetes should be screened for thyroid function or whether patients with subclinical thyroid disease should be treated merits reconsideration.

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Review Article

Pleiotropic Effects of Thyroid Hormones: Learning from Hypothyroidism

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Hypothyroidism induces several metabolic changes that allow understanding some physiopathological mechanisms. Under experimental hypothyroid conditions in rats, heart and kidney are protected against oxidative damage induced by ischemia reperfusion. An increased resistance to opening of the permeability transition pore seems to be at the basis of such protection. Moreover, glomerular filtration rate of hypothyroid kidney is low as a result of adenosine receptors-induced renal vasoconstriction. The vascular tone of aorta is also regulated by adenosine in hypothyroid conditions. In other context, thyroid hormones regulate lipoprotein metabolism. High plasma level of LDL cholesterol is a common feature in hypothyroidism, due to a low expression of the hepatic LDL receptor. In contrast, HDL-cholesterol plasma levels are variable in hypothyroidism; several proteins involved in HDL metabolism and structure are expressed at lower levels in experimental hypothyroidism. Based on the positive influence of thyroid hormones on lipoprotein metabolism, thyromimetic drugs are promising for the treatment of dyslipidemias. In summary, hypothyroid status has been useful to understand molecular mechanisms involved in ischemia reperfusion, regulation of vascular function and intravascular metabolism of lipoproteins.

1. Introduction

The influence of thyroid hormones on metabolic processes has been widely recognized, particularly the profound changes in ATP metabolism. ATP production is primarily driven by mitochondrion, along with other relevant processes, including intracellular Ca^{2+} regulation, redox signaling, and apoptosis triggering [1]. In this respect, it is known that hypothyroidism diminishes oxygen consumption and promotes low metabolism that lead to disturbances in hemodynamic, cardiac, and renal functions, as well as in lipid metabolism.

Adenosine is a vasoactive compound that may induce dysfunction of several physiological mechanisms. In this regard, little attention has been given to the relationship between the effects of the nucleoside adenosine, a metabolite of ATP, and the thyroid status, specifically in hypothyroidism. Lipoproteins profile is another aspect affected in hypothyroidism; increased low-density lipoproteins- (LDL-) cholesterol plasma levels is the most common feature. The metabolism of high-density lipoproteins (HDLs) is also deeply impaired; several genes coding for proteins involved in the intravascular metabolism of HDL are regulated by thyroid hormones. However, the altered HDL metabolism has not been considered in its real dimension because HDL-cholesterol plasma levels vary widely among different studies in patients or animal models of hypothyroidism.

In this paper, we will focus on hypothyroidism as model to understand new physiological mechanisms that influence the mitochondrion, the cardiovascular system, the regulation of vascular tone, the kidney, and lipid metabolism.

2. Hypothyroidism and Mitochondria

Thyroid hormones regulate the expression of several membrane-associated respiratory enzymes and metabolite carriers in mitochondria; whether the effect of such regulation is deleterious or beneficial remains controversial [1–5].

In hypothyroidism, it is known that the electron transport chain complexes and the F_1-F_o ATPase decrease their activity in many tissues [6, 7]. Also cardiolipin, a dimeric phospholipid synthesized by the mitochondrial enzyme cardiolipin synthase, is activated by the thyroid hormones [8]. As a consequence, lower mitochondrial content of this phospholipid has been reported in hypothyroidism, contributing to the constellation of intracellular abnormalities in this physiopathological situation [9, 10]. The function of several mitochondrial proteins seems to be related to cardiolipin content. Paradies et al. [11] showed that the activity of cytochrome c oxidase was diminished in mitochondria isolated from hypothyroid rats, and that its normal activity was recovered after the addition of cardiolipin. Other mitochondrial protein affected by the hypothyroid status is the adenine nucleotide translocase (ANT). Normal ADP/ATP exchange activity of ANT depends on cardiolipin [12]; Paradies et al. [11] demonstrated that cardiolipin content in liver mitochondria from hypothyroid rats was 24 nmol/mg versus 40 nmol/mg in euthyroid rats. These values correlated with a diminution in ANT levels, from 58 nmol/mg in control rats to 40 nmol/mg in hypothyroid rats. In addition to its normal function, ANT forms the inner membrane channel of the mitochondrial permeability transition pore (MPTP) [13–15] which allows the movement of matrix ions and metabolites through the mitochondrial membrane (Figure 1). Binding of cyclophilin-D (CyP-D) to ANT matrix surface facilitates a calcium-triggered conformational change converting it from a specific transporter to a nonspecific pore. Both proteins along with the voltage-dependent anion channel (VDAC) and cardiolipin probably represent the minimum MPTP configuration [13–15]. Once integrated, MPTP opening is favored by Ca^{2+} accumulation in the matrix or by inhibiting the ADP/ATP translocase activity with the specific inhibitor carboxyatractyloside (CAT) [16]. As mentioned above, the translocase activity in the membrane depends on the lipid constitution of the bilayer, in other words, on the cardiolipin content [17, 18]. Since mitochondria from hypothyroid rats are characterized by low cardiolipin content and by low expression of ANT [11], MPTP formation in these mitochondria is impaired [17]. Experiments carried out with mitochondria from thyroidectomized rats, whose blood T_4 levels diminished from 6.42 ± 0.024 to 1.49 ± 0.12 μg/dL after surgery, showed that the transition pore remained closed, regardless of the Ca^{2+} overload [17].

A controversial issue in hypothyroidism is the apparent protection against tissue injury derived from ischemia reperfusion [18–20]. Tissue injury by reperfusion is an important complication involved in organ transplantation; in kidney transplantation, a delay in graft function has been attributed to the deleterious effect of excessive release of reactive oxygen species (ROS) [21]. The increased production of ROS induces a leakage of mitochondria matrix components, likely as a consequence of membrane protein and lipid peroxidation [22]. Mitochondrial membrane increased permeability leads to massive Ca^{2+} loss; in mitochondria isolated from euthyroid-rat kidneys subjected to ischemia/reperfusion, the initial Ca^{2+} accumulation inside mitochondria was followed almost immediately by a slow release of the cation. In contrast, mitochondria isolated from ischemic/reperfused kidney of hypothyroid rats were capable of maintaining the accumulated Ca^{2+}.

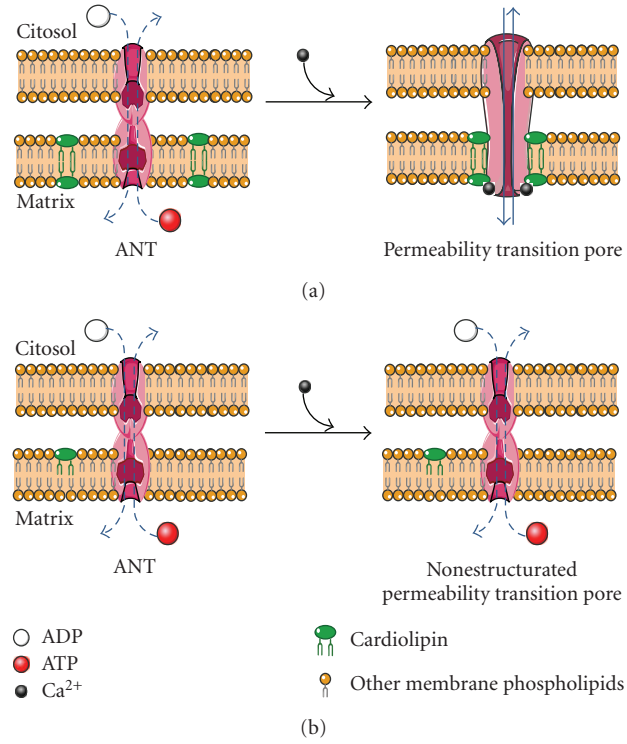

Figure 1: Conversion of the adenine nucleotide translocase (ANT) to permeability transition pore. (a) Under euthyroid conditions, ANT allows the selective exchange (dotted lines) of ADP and ATP between the citosol and the mitochondrion matrix. Ca^{2+} triggers the assembling of ANT with cardiolipin and other proteins (not shown in the figure) to integrate the permeability transition pore. (b) In hypothyroidism, the low content of cardiolipin in the mitochondrion membrane limits the pore formation, thus conserving the ANT function and matrix content unaltered. See the text for details.

Oxidative stress induces opening of the pore, which allows depletion of several compounds from the mitochondrial matrix including NAD^+ and cytochrome c [19, 23]. In this context, NAD^+ and cytochrome c content in kidney mitochondria also depend on thyroid status. Matrix NAD^+ fell down to about 10% of control levels in mitochondria isolated from euthyroid rats after ischemia/reperfusion. In contrast, matrix NAD^+ increased around 40% in mitochondria isolated from hypothyroid rats subjected to ischemia/reperfusion with respect to control mitochondria. As previously reported, the MPTP lies under the progression of the apoptotic mitochondrial pathway, providing mitochondrial cytochrome c release [19, 23]. Mitochondria isolated from ischemic/reperfused euthyroid kidney showed lower levels of cytochrome c, as compared with mitochondria isolated from reperfused kidney from hypothyroid rats (40% versus 10%) [19].

Hypothyroidism protection, against nonspecific membrane leakage, has also been shown by using inhibitors of the electron transport and oxidative phosphorylation uncouplers [19, 20].

Based on the aforementioned evidence, we can suggest that the thyroid status influences mitochondrial membrane permeability, that is, hypothyroidism renders mitochondria membrane less prone to leak calcium. In support of the evidence mentioned above, it is important to mention that mitochondria isolated from hyperthyroid rats have an increased nonspecific permeability, since they undergo permeability transition in the absence of inducing agents [24]. The resistance to increased permeability in mitochondria from hypothyroid rats should be due to the inability of ANT to convert itself into a nonspecific pore. Such a failure must be attributed to the low content of cardiolipin, which is part of the annulus of the carrier [12, 15] and is indispensable for its adequate function. Furthermore, it has been proposed that cardiolipin would be the target site for Ca^{2+} to induce pore opening (Figure 1). Therefore, in mitochondria with low content of cardiolipin, Ca^{2+} fails to induce membrane leakage. It should be noted that cardiolipin content in kidney mitochondria from hypothyroid rats was about 60% of those isolated from control rats [25].

Thyroid hormones affect other mitochondrial functions that are mediated by mechanisms different to the cardiolipin membrane content; hypothyroidism affects the expression of mitochondrial proteins from the respiratory chain. In this regard, it has been shown that Coenzyme Q10 levels decreased, as well as the antioxidant capacity of mitochondria [26, 27]. Of particular interest, T3 regulates the energy dissipation related to proton leakage. Basal mitochondrial proton leakage accounts for about 25% of whole-body resting energy expenditure; therefore, the modulation of proton leakage by T3 may result in significant control of total body energy expenditure. In this context, the authors in [28] have shown that mitochondrial PTP gating could not be ascribed to T3-induced changes in the mitochondrial content of integral PTP components, that is, ANT, cyclophilin D, or VDAC, despite T3-induced increases in the expression of ANT and cyclophylin D. *In vivo* T3 treatment resulted in pronounced increase in liver mitochondrial Bax together with pronounced decrease in mitochondrial Bcl2, while hypothyroidism resulted in opposite effects that may be reversed by T3 [29]; mitochondrial free Bax induced by T3 results in a low-conductance PTP gating [28], the consequent proton leakage, and finally energy expenditure. The comprehension of the T3 transduction pathway that is involved in regulating mitochondrial energetics may offer novel targets for thyromimetics designed to modulate energy expenditure.

3. Hypothyroidism and Heart Reperfusion Damage

Heart reperfusion achieved through the implant of intracoronary stents or coronary by-passes in patients with ischemic heart disease is an obligatory therapy to maintain the myocardium viable after ischemia [30]. In this regard, sudden reoxygenation occurring when the vessels are opened causes considerable cell injury produced by the action of oxygen-derived reactive species, generated mainly by mitochondria [31, 32], associated to the Ca^{2+} overload which contributes to the myocardial insult. Although the controversial evidence of beneficial or deleterious effects of hypothyroidism in heart reperfusion has not been completed elucidated, several studies have shown an increased recovery of cardiac function compared to the observed in normal animals, as well as the protection against arrhythmias [33].

Increasing evidence suggests that heart injury by reperfusion results from mitochondrial Ca^{2+} overload, oxidative stress, adenine nucleotide depletion, elevated phosphate concentration, and depolarization which in turn induces the increase of nonspecific permeability [13]. As mentioned above, mitochondria from hypothyroid rats are resistant to permeability transition induced by Ca^{2+}. In this sense, myocardial tissue from hypothyroid rats is also resistant to the damage exerted by reperfusion after an ischemic period [34]. As shown in Figure 2(a) during the ischemic period, hearts from control rats maintained sinusal rhythm; however, after removing the occlusion, reperfusion arrhythmias were evident and continued during 10 min, up to the end of the experiment. Figure 2(b) shows ECG tracings from hypothyroid rats, in which the cardiac frequency remained unchanged during the ischemic period, as observed in the control rats. Remarkably, when blood flow was reestablished, no ventricular tachycardia was detected, and the hearts remained in sinus rhythm along the experiment.

Figure 2 shows the electrocardiogram (ECG) tracing from control and hypothyroid rats. Panel (a) illustrates the electric profile of a control rat, and Panel (b) of a hypothyroid rat.

The mechanisms involved in the protection remain under investigation, since they cannot been explained only by the metabolic changes induced by hypothyroidism, that is, low consumption of oxygen, higher myocardial glycogen levels, and slow decline of ATP levels during ischemia. Modification of signal transduction molecules seems to play an important role in the response to reperfusion of hypothyroid hearts. PKCε is overexpressed, while phospho-p46 and p54 JNK does not increase after I/R, suggesting that they play an important role in the hypothyroidism-induced cardioprotection [20]. In addition, a shift from α to β myosin isoform expression and a lower expression of SERCA (sarco/endoplasmic reticulum Ca^{2+} ATPase) occurred in hypothyroid hearts. Further experiments are needed to completely understand the pathways involved in the protective effect of hypothyroidism in reperfusion.

4. Innovative Mechanism to Explain Altered Kidney Function in Hypothyroidism

The renal alterations observed in hypothyroidism are well known, among them: inability to produce maximally concentrated or diluted urine, reduced corticopapillary tissue concentration gradients for urea, impaired urinary acidification, and decreased glomerular filtration rate [35–37]. The abnormalities in glomerular hemodynamics at the single nephron level are characterized by hypoperfusion and decreased permeability of the glomerular capillaries, resulting in a reduction of whole glomerular filtration rate [38]; these hemodynamic changes suggest the presence

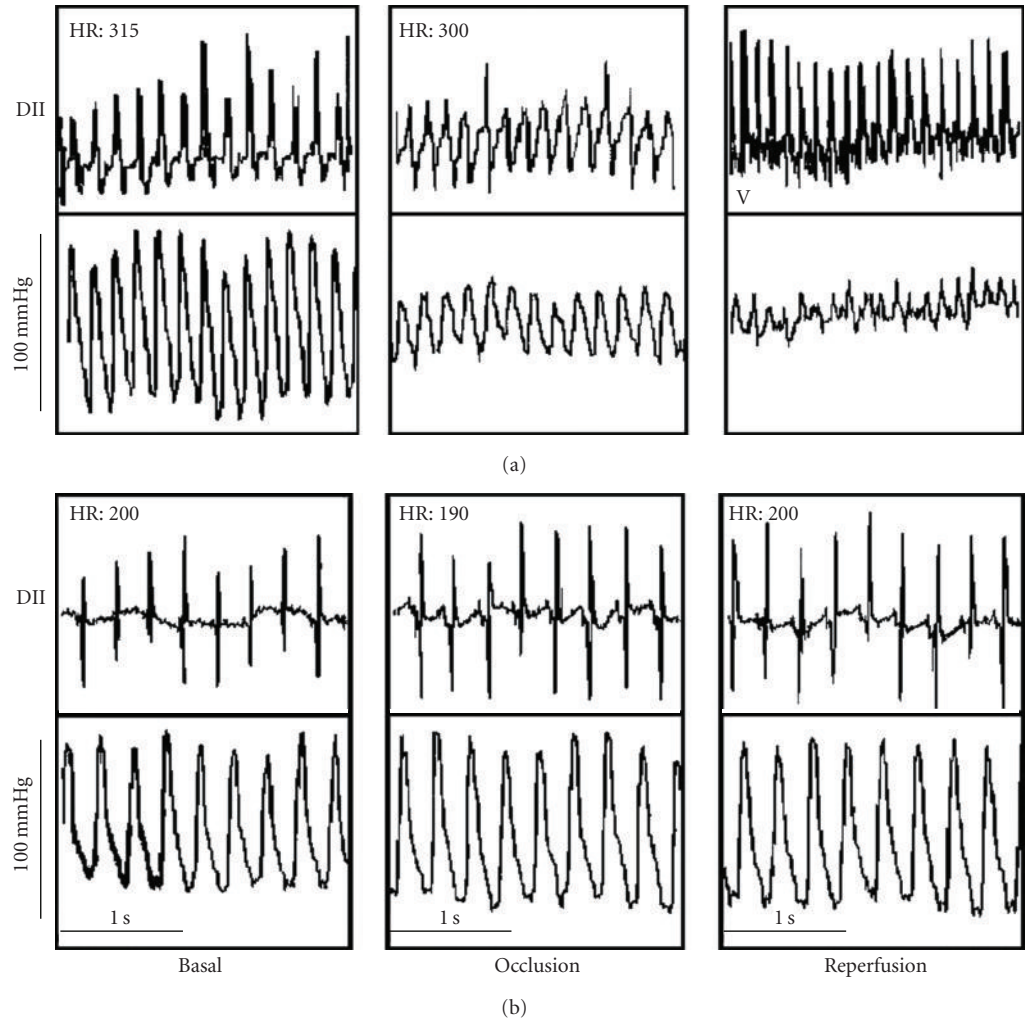

Figure 2: Electrocardiogram of control (a) and hypothyroid (b) rats (modified from [34]). See text for explanation.

of vasoconstrictor compounds influencing renal function, among them, participation of angiotensin II has been suggested [39]. However, the alterations of ATP metabolism play a greater role in the physiopathological mechanisms involved in the abnormalities of renal function in hypothyroidism. In the presence of an abnormal nucleoside metabolism characterized by a low production of ATP such as in hypothyroidism, ATP degradation leads to a decreased adenosine (ADO) production. To this regard, ADO has been implicated in the regulation of glomerular filtration rate because of its unique vasoactive properties [40–42]; the nucleoside induces systemic vasodilation and renal vasoconstriction. We demonstrated that administration of an ADO receptors antagonist (PSPX) or administration of exogenous ADO reverts the renal vasoconstriction of rats with acute hypothyroidism (Figures 3 and 4) induced by surgical thyroidectomy with parathyroid reimplant [38].

The observation that in hypothyroid rats the response in renal hemodynamics to ADO administration is opposite to the changes observed in sham-operated rats is remarkable; these results suggest that ADO production is low and stimulates preferentially ADO A1 receptors, which mediate vasoconstriction.

Indeed, when a dose-response curve of single nephron glomerular filtration rate was obtained with intra-arterial infusion of adenosine, the difference in the response between sham-operated (Sham) and thyroidectomized (HTX) rats was clearly demonstrated; single nephron glomerular rate (SNGFR) decreased progressively with the various doses of adenosine, as is well recognized [35–39]; in contrast, in the HTX rats, there was an initial decrease of SNGFR, followed by no changes and a marked increase with the highest dose of adenosine, supporting the notion that ADO was low in the kidney of HTX, and that increased concentrations of adenosine were able to activate ADO A2 receptors that induce vasodilation.

When ADO tissue content was measured in the kidneys of Sham and HTX rats, the later had a very low content of the nucleoside (Figure 5). These responses in glomerular hemodynamics are in accordance with the fact that high affinity ADO A1 receptors are preferentially activated by low concentrations of ADO, and the low affinity ADO2 receptors

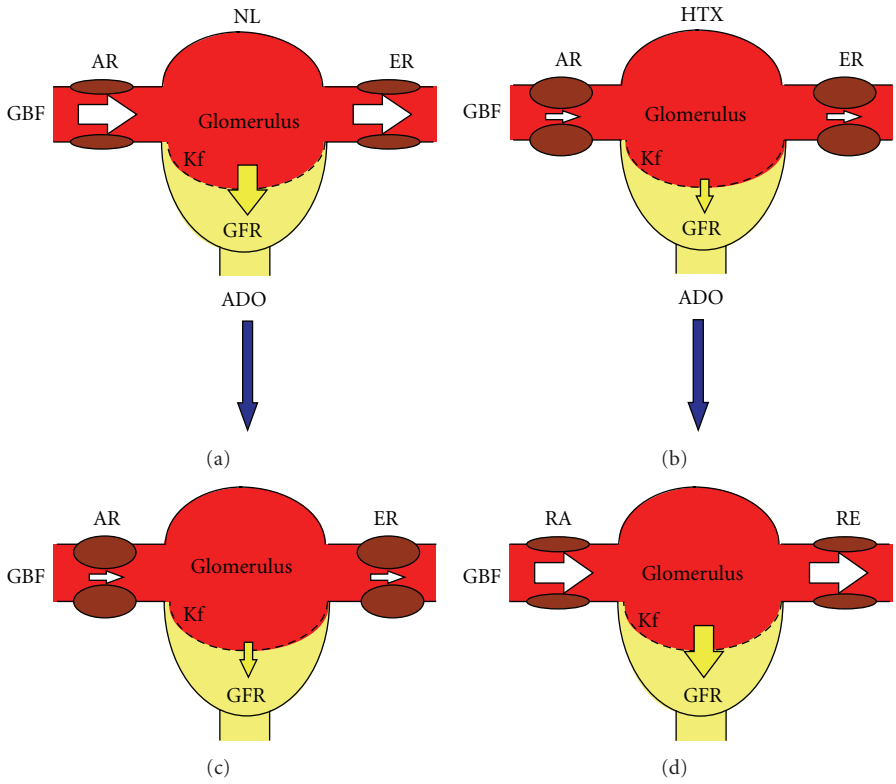

Figure 3: Effect of adenosine (ADO) on glomerular hemodynamics in normal and (NL) and hypothyroid (HTX) rats. (a) The balance of glomerular blood flow (GBF), the afferent and efferent resistances (AR, ER, resp.), and the permeability of the glomerular capillary membrane (Kf) maintain a normal glomerular filtration rate (GFR). (c) Administration of exogenous adenosine to normal rats produces an increase in afferent and efferent resistances, which in turn result in decreased glomerular blood flow and glomerular permeability; as a final consequence, the glomerular filtration rate diminishes. (b) Glomerular hemodynamics of the hypothyroid kidney. There is a glomerular vasoconstriction due to an increase in afferent and efferent resistances, a decrease in glomerular blood flow, and permeability of the glomerular capillary membrane, which results in a decreased glomerular filtration rate. (d) When exogenous adenosine is administrated to HTX rats, glomerular hemodynamics and GFR reach values near to normal. White arrows are indicative of magnitude of AR or ER, whereas yellow arrows represent the magnitude of the glomerular filtration rate.

are activated by high concentrations of the nucleoside (Figure 5).

Hypothyroidism induces important changes in the enzymes involved in the metabolism of adenosine, and these changes seem to be organ specific; thus, an increase in 5′ nucleotidase activity and a decrease in adenosine kinase have been reported in brain tissue of hypothyroid rats [43]. Moreover, 5′-nucleotidase and adenosine deaminase activities in brown and white adipocytes have been shown to decrease [44] associated with a lower adenosine content [45]; these cells develop an enhanced sensitivity to the nucleoside. These findings are in agreement with the results obtained on renal hemodynamics in the kidney of hypothyroid rats in our study.

5. Participation of Adenosine Receptors in the Regulation of Renal Function in Hypothyroidism

To further investigate the mechanisms of renal vasoconstriction observed in hypothyroid rats, we performed binding studies for A1 and A2 adenosine receptors in the kidneys from hypothyroid rats [46]. We should point out that the response of receptors to an agonist or antagonist depends on both the number and affinity of the receptors. We were able to demonstrate two binding sites, a high (K_H) and a low (K_L) affinity for adenosine A1 and A2 receptors with the radioligands (3H)CPA (ADO A1 agonist) and (3H) CGS21680 (ADO A2 agonist) in the renal cortex and medulla of Sham and hypothyroid rats. In the renal cortex from hypothyroid rats, no differences seem to exist in the high-specificity state of the A1 receptor; however, significant lower values were observed in the low-specificity state for affinity and number of A1 receptors [46]. In contrast, at the A2 receptors, an increase in affinity and number of the K_H receptors was observed, with a decrease in affinity and number of the K_L A2 receptors. These results are in agreement with the cortical renal vasoconstriction observed in hypothyroid rats, as well as the vasodilator response to adenosine infusion [37].

The collecting ducts and loop of Henle of the juxtamedullary nephrons are located mainly in the outer strip of the medulla. The fine regulation of blood flow and

solute reabsorption occur in these structures [47], and the medullary segments regulate the concentration of urine. A1 adenosine receptors are mainly in the low-affinity state of the outer and inner medulla in the hypothyroid kidney, but both low and high affinity A2 receptors increase and predominate in the outer and inner medulla. In hypothyroidism, an inability to achieve either maximal concentration or dilution of urine is recognized [48–51]. A2 adenosine receptors have been proposed to have a role in the regulation of the vasa recta and transport mechanism in the medullary segments of the nephron [52]. Thus, the changes in A1 and A2 adenosine receptors in the medullary segments may increase blood flow in the vessels, which results in a washout of interstitial solutes, decreasing medullary tonicity, thereby producing an impairment in the concentration of tubular fluid and a decrease in sodium and water reabsorption, [52–54]. Thus, the changes in adenosine receptors in the renal medullary segments from hypothyroid rats may contribute to induce an inability to concentrate urine and a decrease in tubular reabsorption, as observed in hypothyroidism.

6. Regulation of Renal Nitric Oxide Production by Adenosine in Hypothyroidism

It has been suggested that, in smooth muscle, the vasodilation produced by adenosine may implicate nitric oxide production (NO) [55]. In this regard, the vasodilator action of ADO A2 analogs in coronary vasculature is mediated by NO production, since A2 receptors are expressed in the endothelial cells [56]. In isolated renal arteries, N6-cyclopentyl adenosine (ADO A1 agonist) and 5′-N-ethylcarboxamido adenosine (ADO A2 agonist) induce vasodilation by activation of ADO receptors located in the endothelium and in smooth muscle, the latter involving NO release [56]. Furthermore, ADO stimulates NO production in endothelial cell culture [57]. However, controversial evidence has been obtained regarding the effects of ADO on intact aortas and aortic endothelium-denuded preparations contracted with phenylephrine [58].

In the kidney, the release of NO increases, opposing the vasoconstrictor effects of ADO; the same mechanism occurs with various vasoconstrictor substances such as angiotensin II and norepinephrine [59]; however, interaction between ADO and NO production has not been completely elucidated. As mentioned above, in hypothyroidism, exogenous adenosine vasodilates the kidney instead of exerting the known vasoconstrictive effect [38]. Thus, it is possible that nitric oxide might be involved in this effect. We have demonstrated that whole glomerular filtration rate (GFR) decreased in response to exogenous ADO in Sham rats; in contrast, in hypothyroid rats, the baseline of whole GFR was significantly lower than in the Sham group and increased during administration of exogenous ADO. However, ADO administration stimulated nitrite-nitrate (NO_2^-/UNO_3^-) production in both normal (NL, or Sham) and HTX groups, but this effect was significantly higher in the HTX rats (3.6 times increase in HTX versus 2.2 times increase in Sham). To further study the effects of ADO in the HTX kidney, ADO A1 receptors were blocked with a specific receptor blocker (DPSPX), which increased GFR in both groups; urinary excretion of NO_2^-/UNO_3^- (UNO_2^-/UNO_3^-) increased in NL and HTX groups, but in the HTX rats, it reached the same values as in Sham rats. These results suggest a role of ADO A2 receptors in the increased GFR and UNO_2^-/UNO_3^- in NL and HTX rats. Nevertheless, the dependency of GFR on NO production seems to be very important in hypothyroidism. To support the evidence that the increased UNO_2^-/UNO_3^- induced by ADO and the ADO A1 blocker was actually due to changes in NO production, NO synthase was inhibited with L-NAME. L-NAME + ADO in NL rats decreased GFR and prevented the increase in UNO_2^-/UNO_3^-; in HTX rats, GFR as well as UNO_2^-/UNO_3^- remained unchanged, since L-NAME completely prevented the renal vasodilator effects of ADO (Figure 6) [60].

As it can be observed, adenosine markedly stimulates the production of NO in the hypothyroid rats, which is associated with an increase in glomerular filtration rate; this effect is completely blocked when L-NAME is coinfused with adenosine. These results are in agreement with the results obtained by Quesada et al. [61] who show that nitric oxide synthase activity remains within the normal range in the cortex and medulla from hypothyroid rats, which explains the increased NO results obtained with ADO in the hypothyroid rats and supports the notion that in hypothyroidism the production of NO is regulated by adenosine. In contrast, Moreno et al. demonstrated, in the isolated kidney of hypothyroid rats, that the vascular reactivity of the kidney is reduced to NO donors, and it is insensitive to NO blockade; however, renal function was not evaluated in this study [62].

7. Participation of Adenosine in the Regulation of Vascular Relaxation in Hypothyroidism

A limited number of studies have investigated systemic vascular relaxation under hypothyroid conditions; the evidence available indicates no alterations in response to acetylcholine (Ach) or $CaCl_2$ in aortic vessels [36, 63]; however, the vasodilation mediated by ADO has not been completely elucidated. In this regard, it has been suggested that the aorta has heterogeneous populations of A1 and A2 ADO receptors [64], which inhibit or stimulate adenylate cyclase, respectively. A3 receptors [65] activate guanylate cyclase and mediate cellular responses by inducing nitric oxide production [66]. In this regard, it has been clearly demonstrated that HTX aortas show a reduced contractile response to noradrenaline (NA) or phenylephrine (PE) when compared to normal arteries (NL) [67–69], indicating a lower response to receptor-mediated vasoconstrictors and nonreceptor-mediated smooth muscle (KCl) in HTX vessels.

Adenosine has been used as a physiological agonist to investigate the activation of various receptors, and CPA (N^6-cyclopentyladenosine) and NECA (5′-ethylcarboxamidoadenosine), the former a specific agonist for A1, and for A2a, A2B, and A3 receptors the latter have been used to investigate the participation of those receptors in vascular regulation; more specific A2 (CGS21680) or A3 (IB-MECA) agonists have produced uncertain results in the NL aorta, suggesting activation of an undefined receptor

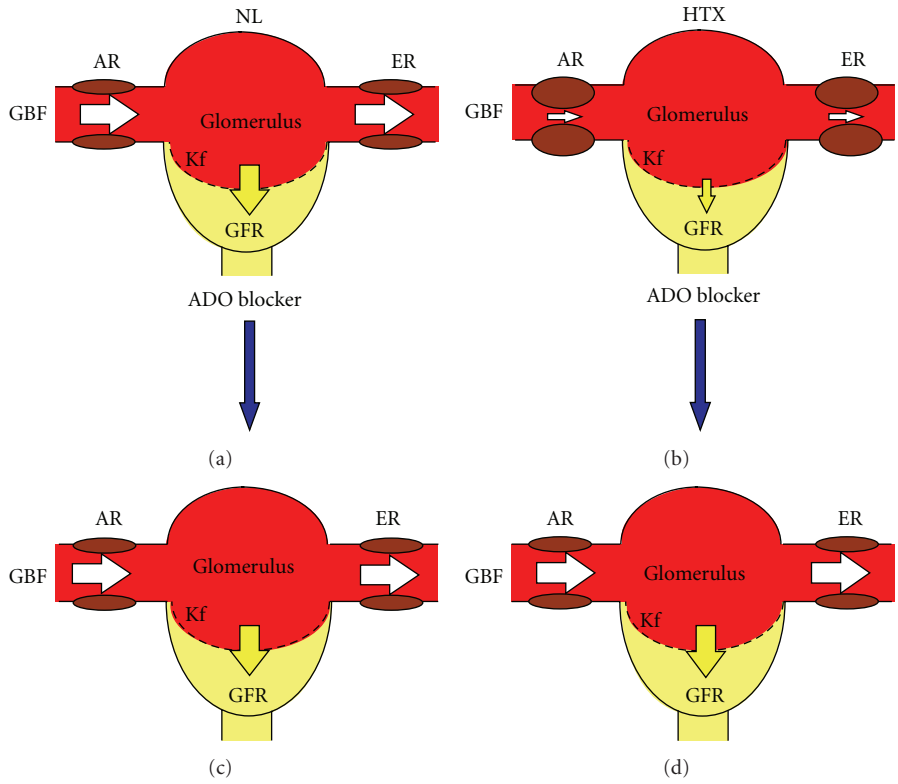

Figure 4: Response to an adenosine (ADO) blocker (PSPX) in glomerular hemodynamics from normal (NL) and hypothyroid (HTX) rats. (a) The balance of glomerular blood flow (GBF), the afferent and efferent resistances (AR, ER, resp.), and the permeability of the glomerular capillary membrane (Kf) maintain a normal glomerular filtration rate (GFR). (c) Since normal glomerular hemodynamics is not importantly regulated by adenosine, the administration of an adenosine blocker does not modify the GFR. (b) In contrast, renal vasoconstrictions are observed under hypothyroid conditions. (d) The administration of an adenosine blocker completely reverses the alterations of glomerular hemodynamics in the hypothyroid kidney. White arrows are indicative of magnitude of AR or ER, whereas yellow arrows represent the magnitude of the glomerular filtration rate.

[64]. We studied the effect of ADO and ADO agonists, CPA and NECA, in aortic rings from HTX rats, and they clearly produced an increase in the maximal relaxation in NA-PE-, and KCl-contracted rings from the HTX group as compared with normal vessels (Figure 7).

The A2 ADO receptors are the most abundant in the normal rat thoracic aorta [70], and they are located in both endothelium and smooth muscle cells. However, the predominant action of ADO seems to be mediated by direct smooth muscle receptor activation [67]; the potency of responses, NECA>CPA>ADO in NL and HTX aortas, found in our study was consistent with this report; although the vasodilator response to ADO has not been explored in hypothyroidism, our results suggest a higher sensitivity of the receptors or an increase in number by an upregulation mechanism [71].

It is generally accepted that vasodilation is endothelium dependent and is mediated by NO in normal vessels [71]. Some studies have demonstrated that the vasodilator effects of ADO are mediated by NO production [72]. Certainly, this is the main mechanism mediating vasodilator responses in some vessels, such as the guinea pig coronary vasculature [73], rat mesenteric arteries [74], and renal arteries [75]. In general, the vasodilator effect of ADO has been considered partially endothelium dependent [58], but controversial evidence has been found for the ADO stimulatory effects on NO production of endothelial cell cultures [73, 76]. As we observed in our experiments, L-NAME did not significantly block the effects of ADO and its analogs in NA and PE-contracted NL or HTX aortic rings; in contrast, when the effect of A1 and A2 ADO receptor antagonist (DPSPX) was evaluated, blocking of ADO receptors with high concentrations of DPSPX significantly reduced the effects of ADO on HTX vessels but had a small contribution in decreasing the vasodilation in NL vessels. The vasodilator response in the HTX rings was not completely inhibited by DPSPX, suggesting the presence of A3 ADO receptors, since these receptors are resistant to blockade with several xanthine derivatives, among them DPSPX [77, 78]. The inhibition of relaxation with DPSPX in HTX aortas greatly supports the hypothesis that ADO receptors mediate the relaxation response under these conditions. It has been demonstrated that A2 ADO receptors located in the endothelial cells induce an age-dependent vasodilatation response through generation of NO, which stimulates cyclic GMP in aortic rings [79]. HTX rats may express ADO

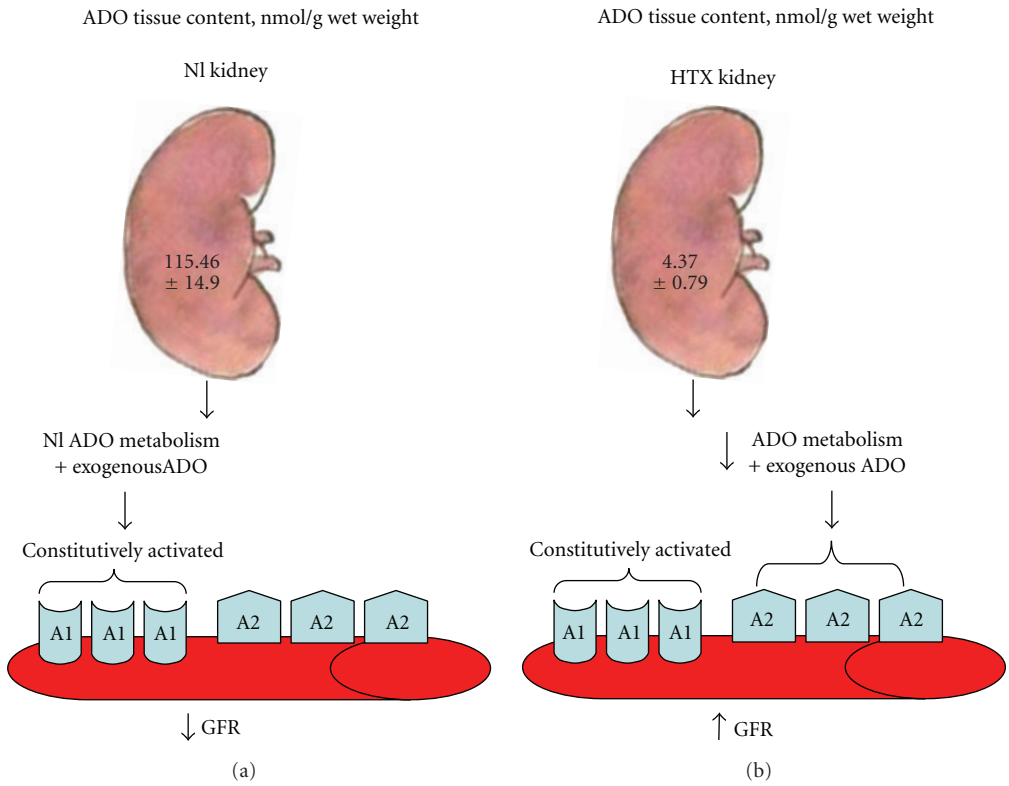

Figure 5: The adenosine tissue content is markedly affected by hypothyroidism; under normal conditions, the metabolism of adenosine is very fast, thus the administration of exogenous adenosine within the μmolar range activates preferentially the high-affinity A1 receptors and induces vasoconstriction (a). In contrast, under hypothyroid conditions, since the metabolism of adenosine is abnormally low, exogenous adenosine reaches concentrations high enough to activate adenosine A2 receptors and induce vasodilation, (b).

receptors in a similar manner to that of aged rats. Thus, it is quite possible that ADO receptors located in the smooth muscle mediate relaxation in the HTX model. The ADO-mediated vasodilation increase in aortic rings of HTX rats suggests a novel mechanism in which ADO receptors may be involved in regulating the vascular tone in hypothyroidism [67].

In summary, hypothyroidism is characterized by changes in adenosine receptor number and sensitivity in the kidney, which results in renal vasoconstriction and induces an impairment in the ability to concentrate or dilute urine. Nitric oxide production in the kidney of hypothyroid rats is mediated by ADO receptors and seems to play an important role in the regulation of glomerular filtration rate and renal hemodynamics. In addition, adenosine receptors play an important role in the regulation of vascular tone in the hypothyroid rat; however, at least in the aorta, the effect is directly mediated by ADO receptors with a minor contribution of NO.

8. Hypothyroidism and Intravascular Metabolism of Lipoproteins

Atherosclerosis is a common feature in hypothyroidism. The early stages of the atherogenic process are represented in Figure 8; high-density lipoproteins (HDLs) play a protective role by promoting cholesterol efflux and improving endothelial function [80]. Moreover, HDLs have antioxidant [81–83], antithrombotic [84], and anti-inflammatory properties [85, 86]. HDLs stimulate the synthesis of nitric oxide by the endothelium [84] and contribute to the endothelial function through the enzyme paraoxonase-1 (PON1) [81–83]. This enzyme catalyzes the conversion of the proatherogenic LDL lipoperoxides to the corresponding lipohydroxides; the former generated by the reactive oxygen species produce severe damage to the endothelium, and the latter are biologically innocuous [72].

In this context, thyroid hormones have been extensively associated with atherosclerosis; overt hypothyroidism induces high plasma levels of low-density lipoprotein (LDL) cholesterol, diastolic hypertension, increased coagulability, and vascular smooth muscle impaired function [87]. There is substantial epidemiological support for the direct relationship between hypothyroidism and atherosclerosis. In a cross-sectional analysis of the Rotterdam study [80], women with subclinical hypothyroidism had a significantly higher prevalence of aortic atherosclerosis and a higher prevalence of myocardial infarction than euthyroid women, after adjustment for age, body mass index, high-density lipoprotein (HDL), blood pressure, and smoking status. Women with antibodies to thyroid peroxidase in the absence of thyroid function test abnormalities had a similar prevalence of

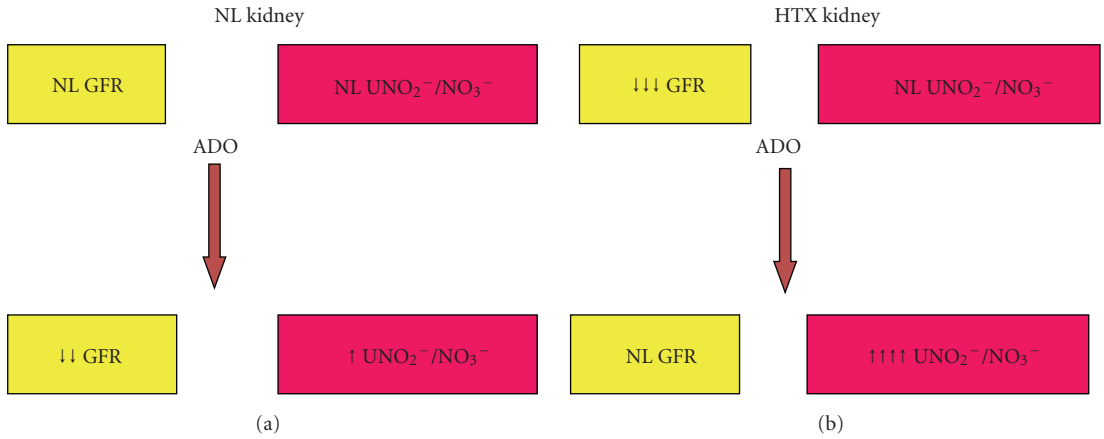

Figure 6: The effect of adenosine on urinary excretion of NO_2^-/NO_3^- (NO_2^-/NO_3^-). Exogenous adenosine induces a decrease of the glomerular filtration rate (GFR) in normal (NL) kidneys and slightly increases the urinary excretions of nitrites. In contrast, in the hypothyroid (HTX) kidneys, despite the vasoconstriction, the urinary excretion of nitrates is within the normal range. When exogenous adenosine is administrated to HTX kidney, the glomerular filtration rate returns to normal values but is associated with a marked increase in the UNO_2^-/NO_3^-, suggesting that this effect is mediated by nitric oxide.

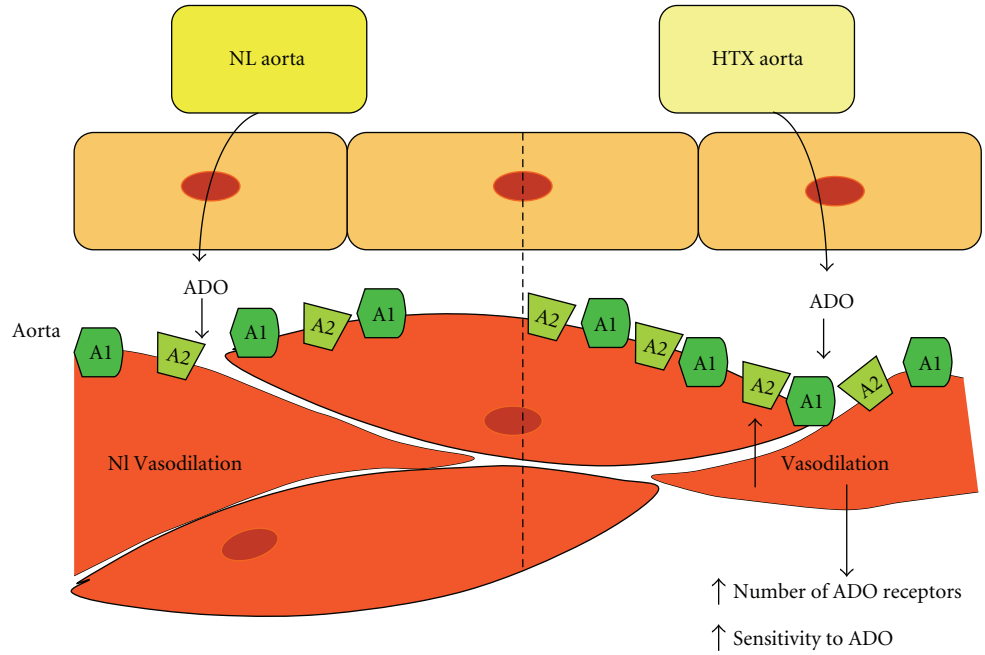

Figure 7: In spite of a similar norepinephrine-induced vasoconstriction of the aortic rings from normal (NL) and hypothyroid (HTX) rats, adenosine- (ADO-) induced vasodilation is markedly higher in the HTX aortic rings. Increased number of adenosine receptors or a higher sensitivity to the receptors to exogenous adenosine may explain the higher sensitivity of HTX aortic rings to ADO.

aortic atherosclerosis and myocardial infarction as euthyroid women without antibodies, suggesting that the increased atherosclerosis was mediated by relative T4 deficiency rather than immune dysfunction.

In agreement with this notion, the Whickham Survey revealed an association between incident ischemic heart disease (IHD) events and IHD-related mortality with subclinical hypothyroidism over a 20-year followup [88, 89]. However, subsequent treatment of subclinical hypothyroidism with levothyroxine attenuated IHD-related morbidity and mortality, supporting the concept that T4 plays a direct role on the protection against coronary events. Besides, in a recent meta-analysis, it was suggested that subclinical hypothyroidism is associated with an increased risk of congestive heart failure among older adults with a TSH level of 7.0 mIU/L or greater, but not with coronary heart disease, stroke, peripheral arterial disease, or cardiovascular-related mortality [90].

The molecular basis of the protective role of thyroid hormones against atherosclerosis is mainly related with

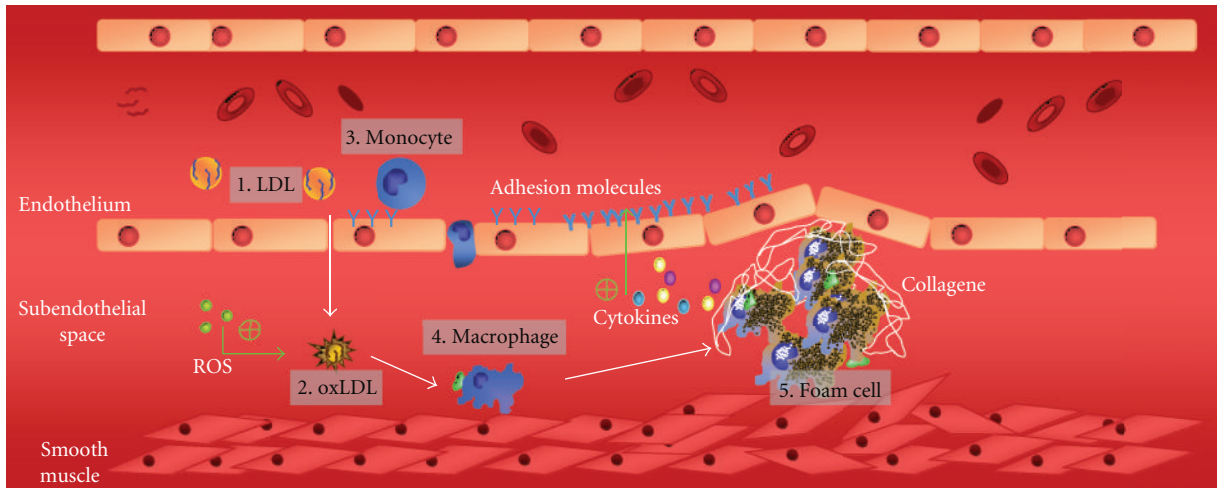

Figure 8: Early stages of the atherogenic process. (1) Low-density lipoproteins (LDLs) reach the subendothelial space. (2) LDLs become oxidized by reactive oxygen species (ROS). (3) Oxidized LDLs act as chemotactic molecules for monocytes. (4) Monocytes differentiate in macrophages within the subendothelial space (5) Macrophages further phagocyte oxidized LDL with the consequent lipid accumulation in their cytoplasm, originating a foam cell that favours an inflammatory response by stimulating the endothelial expression of adhesion molecules.

the regulation of the intravascular metabolism of lipoproteins. Elevated levels of total cholesterol, LDL-cholesterol, and apo B are well-documented features of hypothyroidism [91]. The metabolic origin of such abnormalities has been described in previous studies; propylthiouracil-induced hypothyroidism in rats resulted in nearly 50% decrease of LDL receptor mRNA levels in the liver. In agreement with these observations, LDL receptor mRNA levels increase more than 50% in euthyroid rats supplemented with T4 [92]. Therefore, decreased LDL receptor expression explains the low LDL clearance observed in cultured human skin fibroblasts [93]. Low LDL clearance was also observed in early studies using isotopically labeled lipoproteins in humans [94] and reviewed elsewhere [87]. These findings were supported by an "*in vivo*" study in a hypothyroid woman whose receptor-mediated LDL catabolism was reduced, compared with euthyroid controls, with significant improvement after T4 replacement therapy [95].

Further studies have established the molecular mechanisms that explain the regulation of LDL receptors by thyroid hormones; the promoter region of the gene coding for LDL receptor contains functional thyroid response elements. When the LDL receptor promoter was linked to a reporter gene and cotransfected with the β1 isoform of the thyroid hormone receptor into a hepatic cell line, activity of the chimeric gene was observed when it was stimulated with T3 [96]. The specificity of different thyroid hormone receptors has been recently considered for drugs design, and they are discussed below.

Thyroid hormones also contribute to regulate the intravascular metabolism of high-density lipoproteins (HDLs) (Figure 9). HDLs, as other lipoproteins, are complex macromolecules composed ofphospholipids and free cholesterol on the surface and cholesteryl esters and triglycerides nonpolar lipids in the core. The physicochemical stability of HDL is guaranteed by several apolipoproteins; apo AI represents up to 70% of the protein mass whereas apo AII is the second more abundant protein of HDL. HDLs are secreted as discoid particles containing lipid-poor apo AI, and then they rapidly acquire phospholipids and unesterified cholesterol from tissues via the ABC-A1 transporter to become spherical HDL particles (Figure 9). HDLs facilitate a process known as reverse cholesterol transport (RCT) in which cholesterol in peripheral tissues is delivered to these lipoproteins and ultimately returned to the liver for excretion in bile and feces.

The important role of HDL in the antiatherosclerotic process [81–86, 97] highlights the function of thyroid hormones on the intravascular metabolism of these lipoproteins. Clinical studies have reported conflicting results about HDL-cholesterol plasma levels in hypothyroidism; significantly lower HDL-cholesterol plasma concentrations were described when 52 patients with subclinical hypothyroidism and 18 with overt hypothyroidism were compared with 46 euthyroid controls [98]. Caron et al. [99] also reported that the HDL cholesterol level was significantly decreased among 29 women who had subclinical hypothyroidism, compared with 41 euthyroid women matched for age and metabolic parameters. Furthermore, a significant increase in HDL cholesterol plasma levels was observed after T4 therapy, which normalized the serum TSH concentration [99]. In contrast, other reports conclude that HDL cholesterol plasma levels are normal or even increased in human hypothyroidism [100–102] with a significant decrease after T4 treatment [103]. The effect of T4 on HDL-C in this study was independent from LDL receptor and CETP polymorphisms [103]. In addition, a controlled trial in which 66 women with

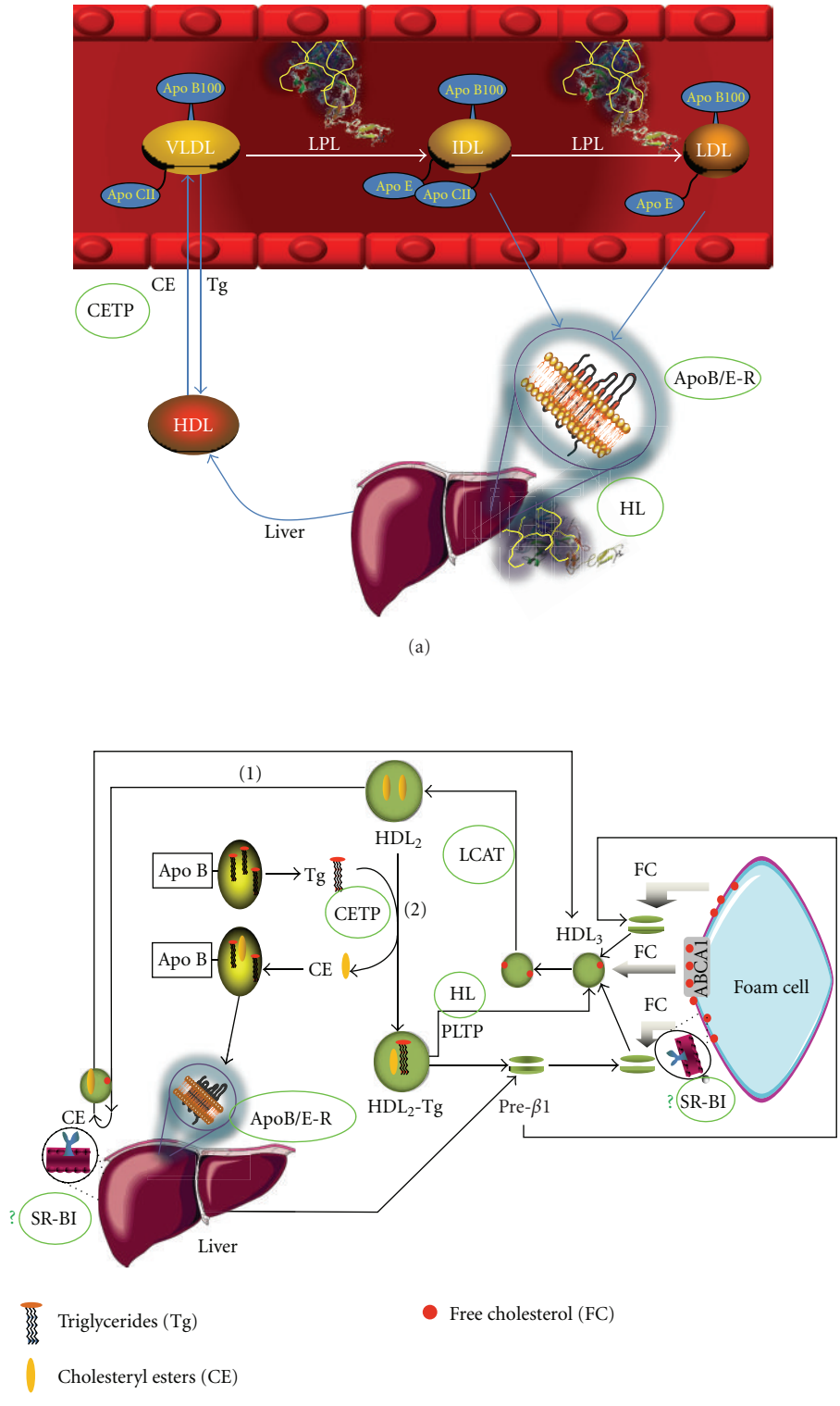

Figure 9: Intravascular metabolism of lipoproteins. (a) Very low-density lipoproteins (VLDLs) synthesized by the liver follow a lipolstic cascade up to the formation of low-density lipoproteins (LDLs), which are cleared from plasma by the apo B/apo E hepatic receptor (apoB/E-R). The cholesteryl esters transfer protein (CETP) facilitates the exchange of triglycerides from apo B containing lipoproteins to HDL and cholesteryl esters in the opposite sense. (b) High-density lipoproteins (HDL) pick up cholesterol from peripheral tissues, including the foam cells. HDL cholesterol becomes esterified by the activity of the lecithin:cholesterol acyl transferase (LCAT). Cholesteryl esters are cleared from plasma by the hepatic scavenger receptor class B, type I (SR-BI) or via CETP. Proteins involved in lipid metabolism that are regulated by thyroid hormones have been highlighted with green circles; low expression levels of SR-BI have not been reported (indicated by a green question mark).

subclinical hypothyroidism were randomly assigned to T4 or placebo treatment found no significant change in either HDL cholesterol or apolipoprotein AI [104].

Hence, there is not a constant pattern concerning HDL-cholesterol in hypothyroidism. Interestingly, several proteins related with HDL metabolism are affected by thyroid hormones (Figure 9(b)). Cholesteryl ester transfer protein (CETP) activity has been reported in human hypothyroidism [92, 93]; low CETP activity induces cholesterol accumulation in HDL particles that result in high HDL-C plasma levels [97]. In contrast, thyroidectomized rats, an animal species that lacks CETP activity, were characterized by a significant decrease of HDL-cholesterol and apo AI of about 26% and 23%, respectively, as compared to controls. These paradoxical results suggest that the variability of HDL cholesterol among hypothyroid patients is not related to CETP activity.

Another key enzyme in intravascular HDL metabolism is LCAT (Figure 9(b)); the activity of this enzyme was found to be lower in hypothyroid rats than in controls [105], probably due to a decreased secretion of the protein by the hepatocyte [106]. It could be suggested that low LCAT activities are consistent with increased levels of HDL cholesterol, since free cholesterol is unable to continue its clearance from plasma. However, thyroidectomized rats exhibit low HDL cholesterol plasma levels as mentioned above, associated to low LCAT activities, suggesting a minimal contribution of this enzyme to abnormal HDL concentrations [106].

Low hepatic lipase (HL) seems to be another feature of hypothyroidism [105, 107]. HL catalyzes the hydrolysis of HDL triglycerides with the consequent remodeling of these lipoproteins towards smaller particles (Figure 9(b)). In agreement with this idea, HDL isolated from hypothyroid rats was significantly larger than those from euthyroid rats [108]. Since large HDLs are the best ligands of hepatic scavenger receptor B, class I (SR-BI), cholesteryl esters are cleared faster from plasma; the consequence of such enhanced interaction would be decreased HDL cholesterol plasma levels, as has been observed in hypothyroid rats [105, 108].

Another protein that regulates the metabolism of HDL and whose expression is likely controlled by thyroid hormones is the hepatic SR-BI [109]. SR-BI promotes the selective uptake of cholesteryl esters contained in HDL, particularly from the largest subclasses of these lipoproteins [110]. Studies with mice treated with GC-1, an analog of thyroid hormones with high affinity for the thyroid receptor beta induced an overexpression of SR-BI [109] which suggests that in hypothyroidism the synthesis of this protein is decreased. To our knowledge, low levels of SR-BI hepatic expression have not been described yet. However, Huesca-Gómez et al. described large HDL isolated from plasma of thyroidectomized rats [108]. This feature is consistent with a low expression of hepatic SR-BI since HDL become smaller after interacting with the receptor [110].

Reduced paraoxonase-1 activity paraoxonase-1 (PON1) activities is another important consequence probably related to the abnormal HDL structure in hypothyroidism [108]. PON1 is an enzyme that plays an important role against the toxicity of organophosphosphate pesticides [111], but it also catalyzes the conversion of lipoperoxides characteristic of the first stages of atherome formation to the corresponding lipohydroxides that are biologically innocuous [112]. In this context, PON1 has been considered as an antioxidant and antiatherogenic enzyme. PON1 is physically associated to HDL by hydrophobic interactions, the smallest HDL particles being the best carriers [83, 113]. Since HDL size is increased during the hypothyroid status, low plasma levels of PON1 could be expected. Indeed, low PON1 plasma activities have been consistently reported in hypothyroid patients [114, 115] with significant increases after thyroxine replacement [115].

Low levels of thyroid hormones are also associated with an important decrease of apo AI catabolic rates of about 40%, as demonstrated in thyroidectomized rats [108]. Such a low apo AI clearance was associated to very low synthesis rates of the protein, almost 60% lower than those of controls. The final balance of this metabolic behavior was a low plasma level of apo AI in hypothyroid rats [108]. This study clearly showed that hypothyroidism affects both synthesis and catabolism of the major component of HDL. Genetic and environmental factors may influence the final balance between the reduced synthesis and catabolism of HDL-apo AI in hypothyroid subjects; consequently, HDL plasma levels may be low, unchanged, and even increased, in function of the environmental and genetic background of each individual.

Aggressive reduction of LDL cholesterol is the basis of preventive cardiovascular care [116], but additional therapeutic approaches to reduce atherogenesis are still needed. Since thyroid hormones have a vast constellation of beneficial effects on lipoprotein metabolism, T4 analogs were considered to treat hypercholesterolemia. The apparent deleterious effects on heart function as well as the introduction of statins led to the discontinuation of clinical studies with T4 analogs in the 1970s. However, thyromimetic drugs are being explored to treat dyslipidemias (for a review, see [117]). Of particular interest have been the thyromimetics with high affinity for the thyroid receptor (TR) beta-1 which is the predominant isoform of the hepatocyte that is almost absent in the heart [117, 118]. In consequence, TR-beta-1 agonists would improve lipoprotein metabolism without the negative impact on the heart [119]. The preliminary results with some thyromimetics are promising, and the final outcomes will define whether or not these drugs will become an alternative for statins.

In summary, high plasma levels of LDL cholesterol are a common feature of hypothyroidism. The metabolic basis of this abnormality is a low expression of the hepatic LDL receptor, since the gene coding for this protein is positively regulated by T4. Concerning the HDL, the profile is uncertain in hypothyroid subjects; proteins related to HDL metabolism such as HL, LCAT, possibly SR-BI, and the main constituent of HDL, apo AI, are expressed at lower levels under hypothyroid conditions. Consequently, the HDL metabolism is greatly affected in hypothyroidism, but the impact of such metabolic impairment on HDL cholesterol plasma levels is variable. On the basis of the positive

influence of thyroid hormones on lipoprotein metabolism, thyromimetic drugs with specificity for TR-beta-1 are very promising for the treatment of dyslipidemia.

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Review Article

Thyroid Hormone and Cardiac Disease: From Basic Concepts to Clinical Application

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Nature's models of regeneration provide substantial evidence that a natural healing process may exist in the heart. Analogies existing between the damaged myocardium and the developing heart strongly indicate that regulatory factors which drive embryonic heart development may also control aspects of heart regeneration. In this context, thyroid hormone (TH) which is critical in heart maturation during development appears to have a reparative role in adult life. Thus, changes in TH -thyroid hormone receptor (TR) homeostasis are shown to govern the return of the damaged myocardium to the fetal phenotype. Accordingly, thyroid hormone treatment preferentially rebuilds the injured myocardium by reactivating developmental gene programming. Clinical data provide further support to this experimental evidence and changes in TH levels and in particular a reduction of biologically active triiodothyronine (T3) in plasma after myocardial infarction or during evolution of heart failure, are strongly correlated with patients morbidity and mortality. The potential of TH to regenerate a diseased heart has now been testing in patients with acute myocardial infarction in a phase II, randomized, double blind, placebo-controlled study (the THiRST study).

1. Introduction

The recognition of analogies between developmental processes and changes occurring in the damaged myocardium has refocused research on the pathophysiology of cardiac disease. In fact, divergent environmental stressful stimuli as pressure overload, hypoxia, ischaemia, metabolic disturbances, and so forth, seem to induce a common response of the heart which is characterized by the suppression of the postnatal gene program, resulting in the predominance of the fetal gene programming. The developmental switch of the diseased myocardium is not fully understood and much controversy surrounds this issue [1]. However, recent research provides evidence that reactivation of the fetal phenotype which leads to cell dedifferentiation may constitute a permissive state for regeneration. In this context, developmental regulators of cell differentiation/cell dedifferentiation process, such as thyroid hormone (TH), may be implicated in this response with important therapeutic potentials [2]. In fact, there is accumulating evidence that TH while having a critical role during development may serve a regenerative/reparative role during adult life [2, 3]. With this evidence in mind, this paper will highlight the basic concepts on the role of TH in the pathophysiology of heart disease with emphasis in the ischaemic heart disease and the potential clinical applications.

2. Cardiac Remodeling: Reactivation of the Fetal Phenotype

Cardiac remodeling is a stress response process to an index event such as ischaemia, mechanical loading, and metabolic alterations. Early in this process, a variety of compensatory mechanisms are in operation, such as activation of the inflammatory and neuro-hormonal systems. In the short term, this response seems to restore cardiovascular function to a normal homeostatic range but with time, sustained activation of these systems can lead to end-organ damage. One of the main characteristics of this response is the

reactivation of the fetal gene programming that drives cells to de-differentiate. Thus, features of fetal heart metabolism re-emerge and include the preference of glucose metabolism over fatty acids as substrates for energy provision while early response genes such as c-myc and c-fos are highly expressed with isoform switches of many other proteins, including metabolic enzymes and sarcomeric proteins (decrease in α-MHC expression and increase in β-MHC expression) [1, 4].

3. Why Fetal Phenotype Is Reactivated ?

The physiological relevance of the return of the heart to fetal gene programming remains still a debatable issue. There is a long held controversy and has been the source of many false starts and therapeutic strategies which have been abandoned over years (e.g., treatment with beta blockers once contraindicated and currently is cornerstone therapy). Heart failure physiologists view this as a maladaptive response which leads to the progressive decline in cardiac function. However, fetal reprogramming may also result in a "low-energy state" which adapts and protects the damaged myocardium upon stress [5]. In fact, the remodeled myocardium appears to be resistant to hypoxia or ischaemia-reperfusion injury [6, 7].

4. Return to Fetal Phenotype: An Opportunity for Regeneration: A Strong Hypothesis

In our laboratory, studies on cardiomyocyte cell dedifferentiation/redifferentiation processes, has led us to the hypothesis that return to fetal phenotype and cell dedifferentiation may be a prerequisite permissive state for regeneration after stress. In fact, de-differentiated cells seem to have the ability to proliferate and/or grow and then to re-differentiate to specialized cells that comprise the regenerated structure or organ [8]. This mechanism appeared early in evolution and allowed living organisms to adapt to environmental stresses. Thus, it has long been recognized that salamanders have the ability to replace whole body parts or anurans can recover body parts at the embryonic stages through induction of cell dedifferentiation/redifferentiation processes [9]. Mammals retain the ability to regenerate but in a more restrictive form. Thus, neonatal mouse heart displays a regenerative potential early after birth in which differentiation is not complete [10]. This regenerative potential may be regained in adult life after stress-induced dedifferentiation and return to fetal phenotype. However, the ability of the cells to re-differentiate may be diminished upon intense and sustained stressful stimuli. This redifferentiation "deficit" may result in heart failure, cancer, and so forth [11], while interventions which potentially could enhance endogenous redifferentiation may restore tissue integrity and function (Figure 1).

5. TH: a Regulator of Cell Dedifferentiation/Redifferentiation

The molecular pathways which control cell dedifferentiation/redifferentiation process in response to stress are not fully understood. However, recent research points out an important physiological role of TH signaling in this process (Figure 1).

5.1. Evidence from Evolution. It is now recognized that important mechanisms that allowed living organisms to adapt to the environment and evolve, may be conserved in mammals and be of physiological and therapeutic relevance. Amphibian metamorphosis is one the natures' paradigms of tissue remodeling and regeneration and this process seems to be thyroid hormone dependent. Thus, almost 100 years ago, JF Gudernatsch made the remarkable discovery that equine thyroid extracts could accelerate the metamorphosis of tadpole into juvenile frogs [12]. Since then, several studies, if not all, have shown that the morphological and functional changes of metamorphosis are the result of alterations in the transcription of specific sets of genes induced by TH. Thyroid hormone system seems to be an ancestral hormone system and interestingly, thyroid hormone receptor (TR) gene is present at the base of biletarians before TH production, indicating an important physiological role of this receptor. Living organisms acquired the ability of producing TH with increasing evolutionary complexity [13].

The role of TH as developmental signal which can trigger the onset of amphibian metamorphosis has been documented in several studies with Xenopus laevis to be an ideal system for ascertaining the developmental roles of TH and its receptor [14]. Interestingly, this system reveals that regulation of TH/TR axis allows the same simple molecule TH to induce completely opposite morphological responses in distinct tissues. In this context, the TH receptor alpha 1 (TRα1) seems to play an important physiological role. This receptor appears to have a dual action which is dependent on its liganded or unliganded (with repressive action) state [15]. Thus, at the early developmental stages in which TH is low, TRα1 receptor is highly expressed and at its unliganded state acts as a repressor of TH-positive-regulated genes and prevents precocious metamorphosis [16]. At later stages, the rise in TH levels results in the conversion of the unliganded TRα1 to the liganded state and triggers cell differentiation and completes metamorphosis [17].

The developmental changes of TH signaling seem to be of important physiological relevance for the response to stress. Thus, anurans can regenerate body parts after injury and this regenerative ability is higher when trauma occurs at early embryonic stages in which cell dedifferentiation prevails due the repressive actions of the unliganded TRα1. The regenerative potential is lost at later stages in which differentiation is progressed by the rise of TH. This response clearly shows that dedifferentiation may be a prerequisite for regeneration [14] (Figure 1).

5.2. Evidence from Cell Based and Animal Models. Based on the evidence obtained from the amphibian models of tissue remodeling, we investigated the potential role of TH signaling in a model of mammalian cardiac cell dedifferentiation/redifferentiation induced by exposure of cardiomyocytes to stressful stimuli. Neonatal cardiomyocytes cultures were

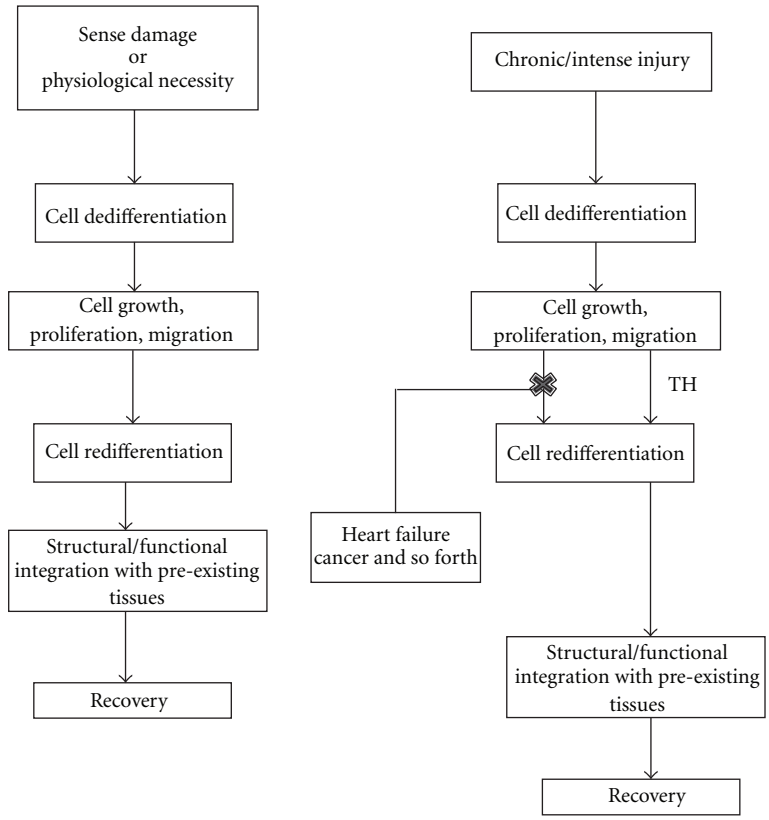

Figure 1: Schematic of the sequence of biological events occurring in response to environmental stimuli and lead to tissue restoration. In the case of intense and sustained stressful stimuli redifferentiation "deficit" occurs and results in a disease state (e.g., heart failure, cancer, etc.). Exogenous TH can enhance redifferentiation and restore damage.

exposed to phenylephrine (PE, an α1-adrenergic agonist) which is a progrowth stimulus and its effects are mediated by activation of intracellular growth kinases such as ERK and mTOR [18]. Thus, PE administration (in the absence of TH in the medium) resulted in marked cell dedifferentiation: PE treated cardiomyocytes, unlike the nontreated cardiomyocytes (which were almost of circular shape with poorly organized cytoskeleton), appeared to be large with disoriented, dense myofibrils, and undefined shape with filopodia-like structures [19]. Myosin isoform expression switched to a fetal pattern with a marked increase in β-MHC expression. This response was associated with a redistribution of TRα1 with increased content of TRα1 in the nucleus and decreased in the cytosol and was abolished by inhibition of activation of ERK signaling with PD98059 [19]. Furthermore, an intact mTOR signaling pathway was found to be required for this response. In fact, inhibition of mTOR signaling with rapamycin not only abolished PE-induced nuclear TRα1 overexpression but resulted in marked TRα1 decrease with cell atrophy [20] (Figure 2). These data clearly show that stress-induced TRα1 overexpression may be a potential mechanism of cardiomyocyte dedifferentiation. Transfection and pharmacological cell-based studies and studies in animals provide further support to this notion. Thus, overexpression of unliganded TRα1 in neonatal cardiomyocytes resulted in cell growth with fetal pattern of myosin isoform expression [21], and cardiomyocytes from mice with dominant negative TRα1 displayed impaired calcium handling and contraction [22]. Similarly, pharmacological inhibition of T3 binding to TRα1 prevented cardiac embryonic cells differentiation [19]. In accordance to this evidence, TRα1 overexpression was shown to occur during the development of pathological hypertrophy after myocardial infarction in rats and decline with the progression to heart failure [20]. Further support to the role of TRα1 as a critical regulator of cell dedifferentiation/redifferentiation in response to stress was provided by the effects of addition of TH in the medium of cells stressed by PE. Thus, as in anurans, we raised the levels of T3 in the medium to convert the unliganded TRα1 to a liganded receptor. Interestingly, this resulted in cell redifferentiation as this was evident by the switch of fetal-like pattern of MHC expression to the adult pattern and changes in cell morphology: cells displayed an elongated shape, with a filamentous actin pattern organized into orderly arrays [19] (Figure 2).

6. TH Promotes Endogenous Regeneration of the Damaged Heart

On the basis of the evidence obtained from cell and animal models it seems likely that treatments targeting the TH

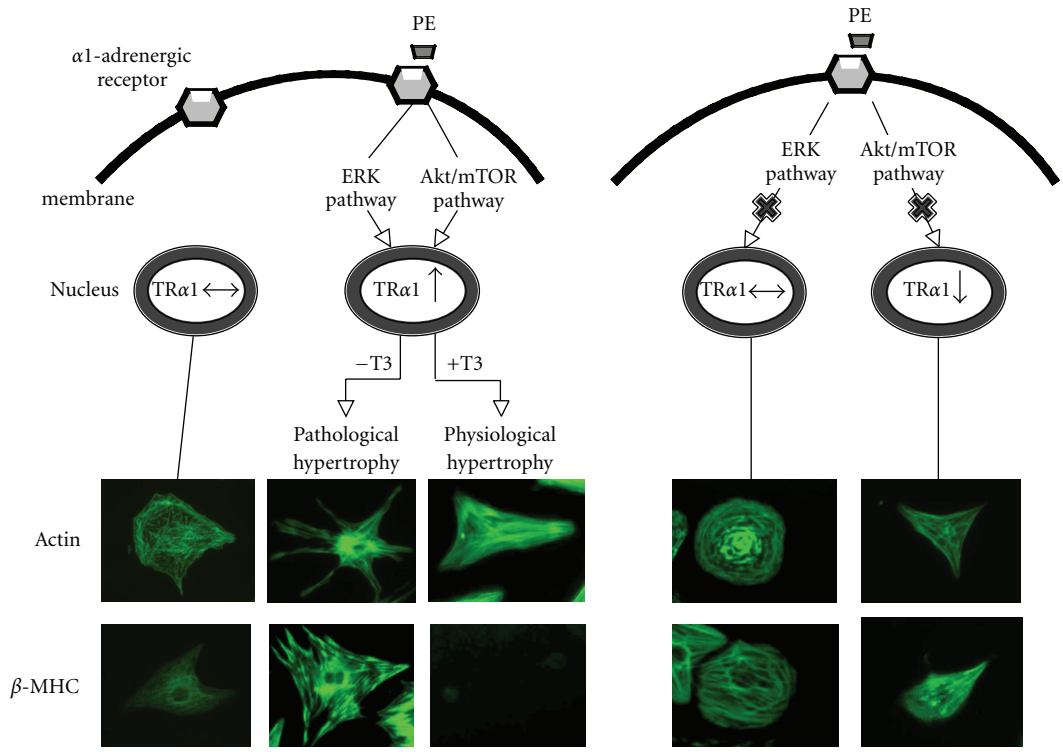

Figure 2: Cardiac cells are de-differentiated upon exposure to progrowth stimuli such as phenylephrine (PE). This response is mediated via ERK/TRα1 and requires an intact mTOR signaling. Inhibition of mTOR signaling with rapamycin not only abolishes PE-induced nuclear TRα1 overexpression but results in marked TRα1 decrease with cell atrophy. De-differentiated cells retain the ability to re-differentiate when T3 is added to the medium. TRα1 by its dual action (liganded versus unliganded) seems to act as a regulator of the cell dedifferentiation/redifferentiation process.

signaling may promote endogenous regeneration of the damaged myocardium [23]. Several lines of experimental evidence support this notion [24–29] (Figure 3). Thus, TH treatment early after infarction resulted in marked improvement of contractile indices independent of loading conditions, such as +dp/dt and −dp/dt, due to favorable cell remodeling as indicated by the switch of MHC isoform expression from the fetal to adult pattern [26]. Furthermore, TH increased cardiac mass and normalized wall stress while it preserved left ventricular geometry to the ellipsoidal shape. These important structural and functional changes were translated to enhanced myocardial performance as indicated by significant improvement of left ventricular ejection fraction [27]. Similar effects were evident even after the development of pathological hypertrophy in old myocardial infarction. In fact, TH was shown to have the ability to convert pathological to physiological hypertrophy [30] (Figure 3).

7. TH and Cell Differentiation: Potential Underlying Mechanisms

Cell differentiation seems to be a redox regulated process. This mechanism has been demonstrated in cultures of glial oligodendrocyte/astrocyte progenitor cells in which the more oxidizing cytoplasmic environment induced by growth factors such as thyroid hormone and bone morphogenic protein 4 and the chemical oxidant tert-butyl hydroperoxide favored cell oligodendrocyte formation. By contrast, a more reducing cytoplasmic environment induced by growth in the presence of a basic fibroblast growth factor, platelet-derived growth factor, or their combination favored the maintenance of the progenitor cells [31]. Similarly, the induction of cardiac hypertrophy by TH is shown to involve redox regulated signaling pathways [32]. Furthermore, TH can alter cardiac cell shape and growth via activation of distinct growth kinase signaling pathways. Thus, TH involves ERK signaling to change cell shape and geometry whereas cell growth is an mTOR-dependent process [33].

8. The TH "Paradox"

It is now recognized that cell differentiation and cell survival may share common signaling pathways. Thus, the TH-induced activation of redox-regulated signaling pathways which mediate cell differentiation, results also in the up-regulation of redox-regulated cardioprotective molecules, such as heat shock proteins which can increase tolerance of the cell against ischaemia [34, 35]. Furthermore, TRα1 receptor which is critical in cell differentiation appears also to mediate TH-induced cardioprotection (unpublished data). These data provide an explanation regarding the paradox

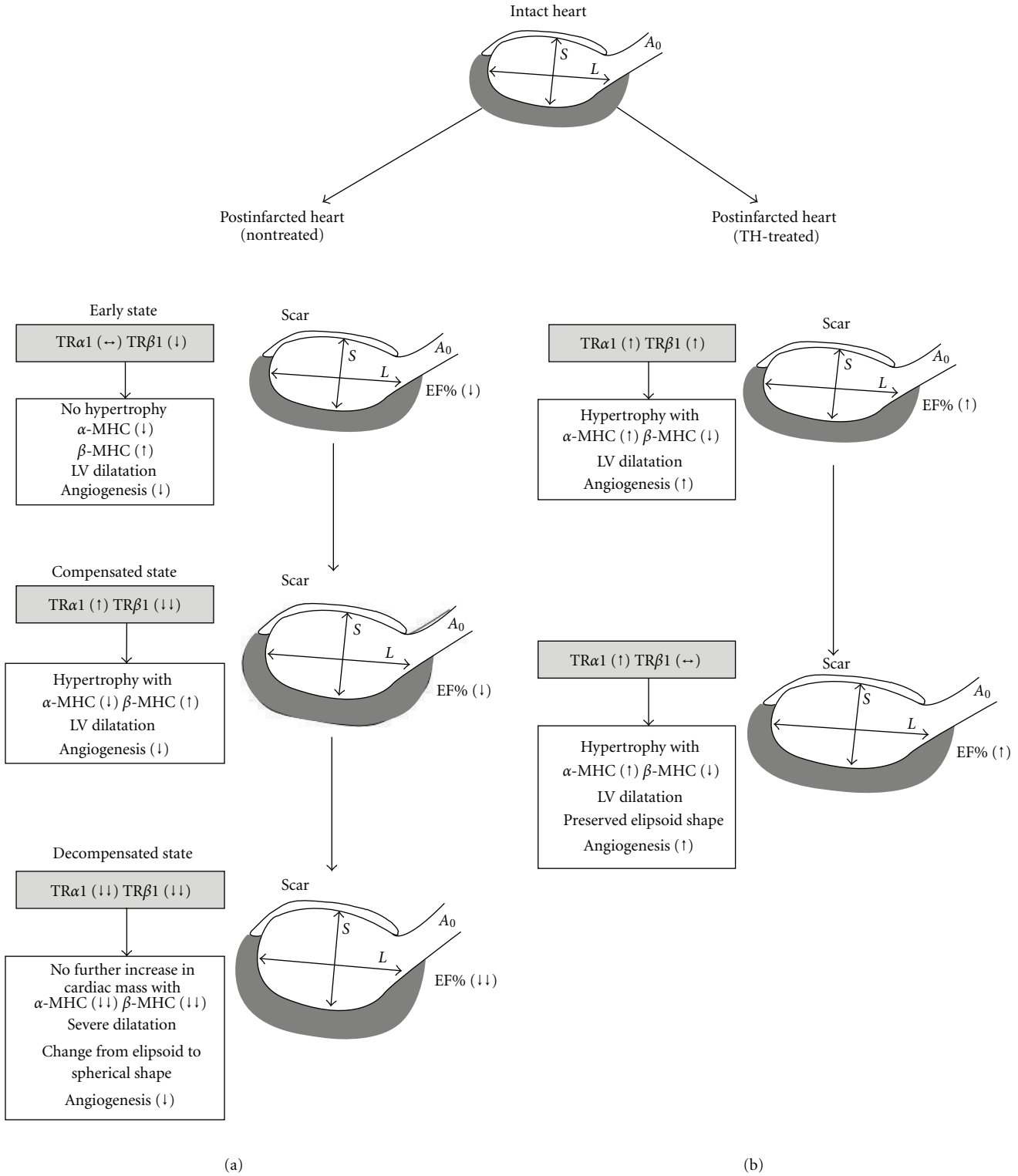

Figure 3: Schematic of molecular, structural, and functional changes during post-ischaemic cardiac remodeling in untreated (a) and TH-treated hearts (b). TH shortly after myocardial infarction induces favorable changes in left ventricular chamber remodeling in a time-dependent manner; TH treatment accelerates the development of cardiac hypertrophy which normalizes wall tension and reshapes left ventricular chamber towards a more ellipsoidal shape at later stages [20].

of TH being rather protective than detrimental for the ischaemic heart although it increases oxygen consumption (by accelerating heart rhythm and increasing cardiac contractility) and depletes the heart from glycogen [36]. Here, it should be noted that the protective effect of TH has also been observed in models of neuronal injury [37].

9. Does TH Availability for a Diseased Human Myocardium Represent a Critical Point?

As clearly documented by several experimental animal data, most of the effects of TH on cardiac function are mediated by binding of biologically active T3 to nuclear TR. Thus, T3 tissue concentration represents the switching factor in TH signalling. Not surprisingly, in patients with primary thyroid mild dysfunction, cardiovascular adverse manifestations have widely been described [38, 39]. An altered TH bioavailability has also been documented in cardiac patients without primary thyroid disorders. The most frequent change in TH metabolism in patients with severe cardiac disease including heart failure, myocardial infarction, and coronary artery bypass is a significant fall in circulating biologically active T3 and a corresponding increase in reverse T3 (rT3), the inactive T3 metabolite[40, 41]. The low-T3 syndrome occurs in approximately 20–30% of patients with heart failure [42–44] with significantly higher incidence in patients with NYHA functional class III-IV than in those with NYHA I-II [43, 45]. The occurrence of a low T3 syndrome, however, does not represent a peculiar pattern of cardiac disease because it has been documented in several noncardiac illnesses. The underlying pathophysiological mechanisms are the reduced enzyme activity of 5′ monodeiodinase responsible for converting T4 into T3 and the increased enzyme activity of 5 monodeiodinase responsible for converting T4 into the inactive r-T3 in peripheral tissues [46].

Regardless of the cause, type (i.e., acute or chronic), severity, and time course of the disease, the first and dominant pathophysiological hypothesis on the meaning of low-T3 syndrome was that decreased T3 concentrations are merely the result of an adaptive process finalized to reduce energy expenditure and thus, having beneficial effects through the reduction in metabolic demand [47]. However, experimental and clinical evidence challenged this interpretation. In an ex vivo study on human myocardial biopsies, we demonstrated that long-term T3 deprivation negatively affected both overall tissue architecture of cultured myocardial slices and calcium handling of the outgrowing cardiomyocytes [48] (Figure 4), indicating that a low-T3 state, as observed during evolution of cardiac disease, can be considered a true hypothyroid-like cardiac condition that worsens per se cardiomyocyte remodeling and dysfunction. In line with the above interpretation, clinical observational studies have shown the important negative prognostic impact of low-T3 syndrome in patients after acute myocardial infarction and cardiopulmonary bypass and during evolution of heart failure [49, 50]. A negative correlation was also found between plasma FT3 concentrations and the extent of myocardial damage after AMI [51].

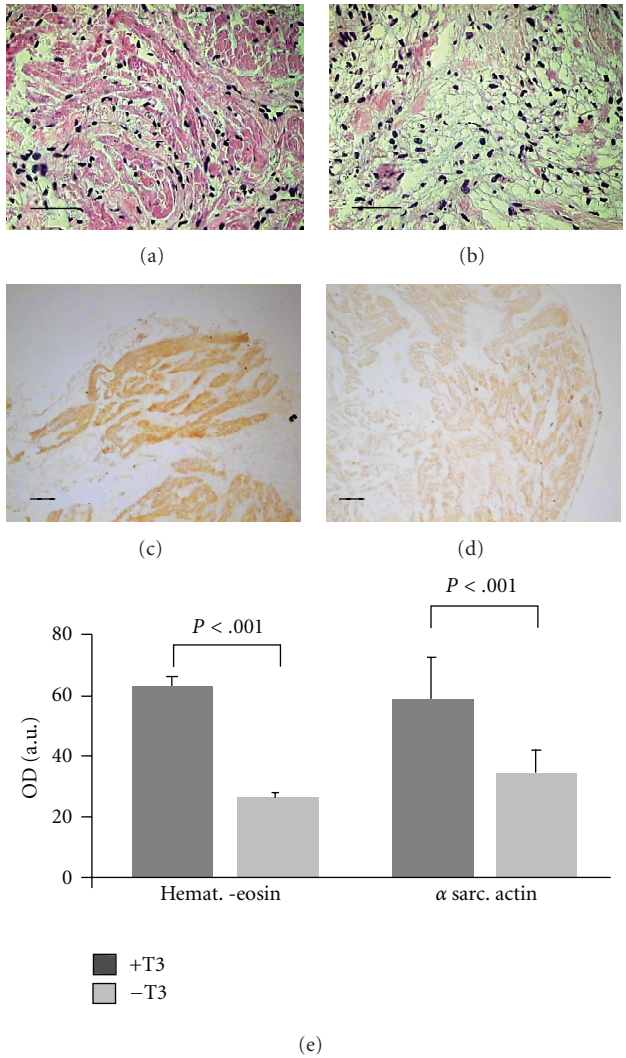

Figure 4: Structural and phenotypic effects of T3 supplementation. After 10 days of culture, T3-supplemented myocardial fragments presented better preserved cardiomyocytes both at histological (a) and immunohistochemical examination (c). Weaker staining and progressive atrophy was observed in the corresponding untreated samples ((b) and (d), resp.). Myocardial sections were stained hematoxylin eosin (a), (b) for total protein content evaluation or immunostained for α-sarcomeric actinin (c), (d) for sarcomeric specif protein content. Images were acquired with a CCD camera and optical density was measured (scale bar = 50 μm). (e) Quantification of the histological results obtained from hematoxylin-eosin and α-sarcomeric-actinin staining of 10-day-old human myocardium in culture. P was determined using the two-tailed unpaired Student's t-test; data are expressed as mean ± SEM (experimental data derived from [28]).

Independently of the parameter used (i.e., a reduction in circulating levels of T3, an increase in inactive rT3, a low free T3 index/rT3 ratio, and an free T3/free T4 ratio), previous works showed that an altered peripheral TH pathway was in any case associated with high incidence of fatal events consisting of cardiac or cumulative death, or heart transplantation [42, 43, 52]. In a study from our laboratory

the probability of death, either cardiac or cumulative, was significantly higher in patients with than without low-T3 syndrome [53]. In another study that enrolled 281 in-patients with ischemic and nonischemic heart [45], we found that total T3 and left ventricular ejection fraction were the only independent predictive variables at multivariate analysis of both cardiac and cumulative death. More recently, these results have been confirmed in a larger cohort of patients with low-T3 syndrome being the cardiac and overall deaths, respectively, 3.4% and 7.3% in euthyroidism, and 6.5% and 13.1% in low-T3 syndrome patients [54].

10. TH Therapy in Patients with Heart Disease

10.1. General Considerations. Besides experimental findings on animal studies, several lines of evidence suggest that some components of the TH signaling could be impaired also in dysfunctioning human heart.

Important acquisitions of knowledge have been derived from a pioneering experiment in a patient with severe hypothyroidism and heart failure in whom several myocardial biopsies were taken; during hypothyroidism MHC alpha mRNA was markedly lower with the opposite for MHC beta. Also phospholamban was 10-fold higher in the hypothyroid heart when compared with euthyroid heart. With restoration of euthyroidism all changes reverted to normal and a normal cardiac function was restored [55]. These direct in vivo human observations are the basis of the afterwards so-called activation of a fetal gene program otherwise indicated as "recapitulation of the fetal phenotype" [56, 57] secondary to the hypothyroid state, all contributing to the decreased contractile function typical of adult HF and a reversal of genotype/phenotype to a more fetal-like cardiac state.

Notably, in a therapeutic perspective, the activation of the fetal gene program observed during evolution of heart failure in adult life appears to be potentially reversible when the TH profile is normalized. The restoration of a physiological TH/TR interaction might counteract progression of heart disease by different mechanisms including: (1) a positive remodelling through the modulation of myocardial gene expression; (2) an improvement in cardiac systolic and diastolic function with a consequent improvement in haemodynamics; (3) an improvement in myocardial perfusion. Last but not least, a positive effect on quality of life by combining multisystems actions of the TH. Actually the rationale for systemic administration of TH is based on the additional systemic actions of THs, in particular on skeletal muscle, kidney, and brain that could be important components of the progression from organ-limited to whole-body disease.

10.2. TH-Based Therapy in Cardiac Patients: From Fear to Hope. Treatment with TH represents a challenging field in cardiac patients with an altered TH metabolism. The potential of novel TH therapies that address the molecular biology of thyroid dysfunction and heart disease represents thus an attractive area of multidisciplinary interest. Based on the available experimental evidence the final goal of TH-based treatment should be to restore and maintain over time a physiological thyroid state when an altered TH metabolism and action does occur as in postischemic remodeling. On the other hand, in normal, nonischaemic heart, excessive TH may produce several cardiovascular deleterious manifestations including increased heart rate, susceptibility to atrial and ventricular arrhythmias, increased oxygen demand, and cardiac hypertrophy both in humans and animals [58, 59]. Subjects with long-term TH excess may also develop cardiac hypertrophy caused by both direct effects of TH on functional contractile and structural protein synthesis as well as indirectly by increasing cardiac load [60]. In addition, long-term L-T4 treatment has been shown to produce significant left ventricular hypertrophy and increase in left ventricular mass index [61]. TH excess has been also implicated as primary cause of impaired cardiopulmonary exercise tolerance probably due to a reduction in cardiac functional reserve [60]. The mechanisms of transition from cardiac hypertrophy to heart failure, however, still remain largely unclear [59]. An increase in LV mass has also been reported in long-term thyroid hyperactivity and could counteract the beneficial effect of TH on diastolic performance by worsening diastolic ventricular filling and systolic performance on effort [62]. Moreover, long-term TH overload may enhance the sensitivity of myocardium to catecholamines and increase the activity of renin-angiotensin system in myocardium [63]. Consequently, chronically elevated catecholamines and/or angiotensin II in myocardium may reduce cardiac contractility and promote apoptosis of cardiomyocytes in humans and animals [64]. By these premises, it is not surprising that some patients with endogenous or exogenous subclinical hyperthyroidism may show significant changes in echocardiographic parameters associated with systolic and diastolic dysfunction, both potentially contributing to increased cardiovascular risk [65, 66]. The small number of patients and the lack of control [65] of the above studies may limit, however, the clinical relevance of data. Observational, large-scale studies on relationships between cardiovascular morbidity and mortality in exogenous subclinical hyperthyroidism induced by long-term LT4 treatment have yielded, however, conflicting results [67] and only in patients with endogenous subclinical hyperthyroidism an increased risk of nonfatal morbidity and dysrhythmia has been reported [68]. An increased cardiovascular and cerebrovascular mortality has also been described in a community-based review of subjects with subclinical hyperthyroidism followed over a 10-year period [69]; the role of dysrhythmias may be critical in accounting for some of the excess cardiovascular and cerebrovascular mortality observed in those patients [70].

The emerging clinical and experimental knowledge, taken as a whole, suggests that TH treatment may provide more beneficial than harmful effects including reversal and/or limitation of remodeling, and progression to cardiac dysfunction. However, despite indications on positive actions of restoring a physiologic TH signalling in a damaged heart, interventional trials by the use of THs or THs analogs have been largely discouraged in cardiac patients. Common belief of physicians is that exogenous TH (or TH analogue)

therapy represents in any case an injudicious and dangerous approach with risk of arrhythmia, myocardial ischemia/infarction, and worsening of congestive heart failure. The origin of this belief is, however, largely debatable and mostly based on results from the Coronary Drug Project (CDP) study [71].

CDP demonstrated adverse outcomes, particularly in regards to the proarrhythmic effects of Dextro (D)-T4 [71].The D-T4 preparation (Choloxin, Flint Laboratories) used in the CDP was contaminated with a high level of active L-T4 [72]. Therefore, the cumulative dose of administered L-T4 was toxic, being equivalent to several times the L-T4 dose that would be given to a patient to correct hypothyroidism. For this reason, the CDP did not provide helpful information regarding the therapeutic use of physiological TH in the treatment of cardiac patients. To our knowledge, all pilot interventional studies with the use of synthetic TH conducted in cardiac disease have suggested, on the contrary, its safety [50, 73] and provided some evidence for beneficial effects, mainly in patients undergoing cardiac surgery as well as in those with heart failure with or without cardiogenic shock [50, 73–77]. Type, dosage, and timing of TH-based treatment still remain, however, important open issues which are awaited to be addressed in clinical trials.

10.3. TH-Based Therapeutic Strategies in Cardiac Patients. From a theoretical point of view, therapeutic strategies that can be employed to optimize TH signalling in the presence of HF can be summed up in the following five points: (1) administration of synthetic L-T4; using this approach the main physiological pathway of secretion and peripheral metabolism of TH system is preserved; (2) administration of synthetic L-T3: by this way the physiological pattern of peripheral conversion from T4 into T3, which is impaired in presence of a low-T3 syndrome, is bypassed through direct application of the biologically active TH; (3) administration of a TH analogue, that is of a synthetic and, as much possible, cardiotropic compound showing similar positive effects than the natural TH but lower adverse effects; (4) genetic manipulation of the expression of cardiovascular deiodinases: in this case the main goal is to increase local production and bioavailability of T3; (5) genetic manipulation of the cardiovascular TR pattern: in this case the goal is to increase hormonal signalling at the receptor level while preserving T3 availability. At present, the first three approaches have been applied in a few human studies, and with a low number of patients [78–80] whereas the remaining therapeutic options are still in the experimental animal stage [81, 82].

10.4. L-T4 Administration. Moruzzi et al. published two studies in which synthetic L-T4 was given orally at the "physiological" dose of 0.1 mg per day and cardiovascular effects were assessed after short-term (one-week) treatment and after continuous three-month treatment [79, 83]. In particular, the latter is the only published study that tried to assess the long-term effects of TH in a model of randomised placebo versus L-T4 therapy study. In both studies L-T4 was well tolerated and induced significant improvement in cardiac pump function, consisting of enhanced resting LVEF, resting cardiac output, and functional capacity during exercise.

10.5. L-T3 Administration. In the pioneer study by Hamilton et al. a protocol based on administration of intravenous bolus dose followed or not by a few hours of L-T3 infusion in patients with advanced HF and low T3 levels and/or elevated rT3 concentrations was adopted [78] However, the study's main weakness was the wide variability in scheduled time and dose of the administered L-T3 (cumulative dose from 0.15 to 2.7 μg/kg with infusion time ranging from a minimum of 6 to a maximum of 12 h) in a small, nonrandomized patient population. Although circulating T3 obtained after L-T3 administration was widely variable and in all cases clearly above the upper limit of normal range, no side effects (i.e., no myocardial ischemia or complex arrhythmias) have been documented. In particular, in patients who received the largest doses of L-T3, cardiac output improved significantly starting from 2 h after administration with a parallel decrease in systemic vascular resistance. Importantly, heart rate did not change nor did metabolic demand as assessed by indirect calorimetry. Nevertheless, the administration of L-T3 at high doses, mimicking a hyperthyroid status, may be detrimental when used for long term. In fact, experimental data clearly documented that high doses of L-T3 were able to induce myocardial hypertrophy, and that they were only initially associated with an improved cardiac performance, but followed by a decline after 1 month of treatment [84]. This finding was also associated with increased expression of uncoupling proteins (UCP2 and UCP3) which may be responsible for the decrease in mitochondrial efficiency during thyroid hyperfunction [85].

In a pilot study from our laboratory L-T3 was administered at a "physiological" dose of 20 μg/day/m^2 body surface for a period of 96 h in six patients with advanced heart failure and low-T3 syndrome who were undergoing stable conventional treatment and salt intake. L-T3 constant infusion induced a progressive reduction in systemic vascular resistance and an increase in left ventricular ejection fraction and cardiac output, the latter invasively monitored by Swan-Ganz catheter; urinary output also improved, whereas no changes in heart rate and systemic arterial BP were observed [78–80]. An improvement in overall cardiac performance as documented by an increased stroke volume and left ventricular end-diastolic diameter, all noninvasively assessed by cardiac magnetic resonance imaging, was also documented after 3-day L-T3 substitutive infusion in a placebo-controlled study on patients with chronic and clinically stable dilated cardiomyopathy and low-T3 syndrome. Importantly, the improvement in cardiac performance was not associated with an increase in myocardial O2 consumption, nor there was an increase in total cardiac work. Also the concomitant evidence for a neuroendocrine deactivation, probably contributing to the improved hemodynamic conditions, as documented by the decrease in noradrenaline circulating plasma levels and of the counterpart NT-proBNP secondary to L-T3 treatment in patients with heart failure needs further exploration in future studies. In conclusion, overall the above

observations suggest that the modality of the therapeutic approach is likely the key point when testing TH treatment in cardiac patients with nonthyroidal illness.

10.6. Thyroid Hormone Analogues Administration. In 1992, a TH analogue (DITPA) showed cardiac inotropic selectivity comparable to TH itself, accompanied by minimal effects on heart rate and metabolic activity [86]. Long-term administration of DITPA seems also to stimulate coronary arteriolar growth without inducing cardiac hypertrophy, by upregulating key angiogenic growth factors [87]. The rationale of using DITPA was to avoid the detrimental effects of exogenously administered TH, such as an increase in heart rate and body metabolism (3). While the rationale appears logical, the results in human studies do not appear so favourable. In a pilot randomized DITPA versus placebo clinical study, DITPA was administered in patients with NYHA functional class II-III for 2–4 weeks at a dosage of 1.87–3.75 μg/Kg body weight per day. The major hemodynamic effects consisted in improved cardiac index and decreased systemic vascular resistance index. Further, diastolic function was ameliorated as documented by the decrease in the isovolumetric relaxation time which is a parameter of diastolic cardiac relaxation properties [88].

In a very recent placebo-controlled study by Goldman et al. [89]. The TH analogue 3,5 diiodothyropropionic acid (DITPA) was administered in patients with congestive heart failure. DITPA induced an increase in both cardiac index and heart rate associated with a reduction in systemic vascular resistance. However, no improvement on outcome or symptoms was observed, but rather fatigue was more frequent in the DITPA Group. Importantly, weight loss, increased heart rate, fatigue, reduction in serum cholesterol, and suppressed TSH, all well-known signs and symptoms of thyrotoxicosis, suggest an excess of TH action induced by DITPA at the doses utilized in that study [90].

10.7. Genetic Manipulation. Emerging experimental data encourage the adoption of a new target-organ strategy based on genetic manipulation. In this way it is somewhat possible to overcome all the limitations related to a systemic administration of synthetic native hormone or TH analogues. In the rat model of pressure overload induced cardiac dysfunction, the enhancement of T3 signalling by increased deiodinase Type 2 activity or TR expression has been linked to improve cardiac contractile function and prevention of HF [81, 82]. In particular, the mechanism for preventing HF may depend on avoiding the reversion from an adult to a fetal gene-like state, that is, the shift from α-MCH to β-MCH and decreased SERCA2, which has also been demonstrated in the human failing heart in response to hypothyroidism. Moreover, discovery in human VSM cells and in animal microcirculatory system of a deiodinating pathway able to produce local T3 with vasodilating action [91, 92] confirms that the vascular system is also a direct target of TH and may be a potential point of genetic manipulation to modulate vascular peripheral resistance during evolution of HF.

11. Conclusions

Overall available data on patients with cardiac patients treated with synthetic THs suggest that most of the observed effects on cardiac performance can be explained on the basis of both genomic and nongenomic TH direct actions on cardiac and vascular systems, perfectly in line with our knowledge on cellular mechanisms induced by TH.

On the basis of the above-mentioned experimental and clinical findings, in 2009 we started a phase II, randomized, double blind, placebo-controlled study by the use of substitutive doses of synthetic L-triiodothyronine in patients with STEMI (ST-Elevation Myocardial Infarction) and borderline/reduced circulating T3 [93]. Treatment with synthetic TH or placebo starts during the in hospital period in presence of stable haemodynamic conditions (i.e., 48–72 hrs after STEMI) and will be taken for further 6 months after hospital discharge (Chronic phase). Actually, while in-hospital mortality from acute MI has declined in recent years due to improved early intervention, the five-year rate of heart failure has increased in these patient [94]. Furthermore, severe diastolic dysfunction is a strong predictor of one-year rehospitalisation in MI survivors [95]. It is thus likely that TH treatment will specifically target chamber dilatation and diastolic dysfunction by its beneficial effects on cardiac perfusion, architecture and function. Primary objectives of the study are to investigate:

(1) the safety and feasibility of synthetic TH replacement in patients with STEMI;

(2) TH replacement therapy effects on postischemic remodelling and LV function;

(3) the effects of TH replacement therapy on clinical outcome in terms of major (cardiac and non cardiac death, reinfarction) and minor (recurrence of angina, coronary revascularization, and hospital readmission) events.

Secondary objectives of the study are to evaluate the effects of thyroid hormone therapy on

(1) infarct size, regional wall motion abnormalities, systolic, and diastolic myocardial function;

(2) neuroendocrine imbalance;

(3) patients functional capacity, quality of life, cognitive and behavioural status.

When available, data from the above study will help to clarify the potential clinical usefulness of TH treatment as a novel strategy to protect a damaged myocardium from pathological growth and remodelling after an acute ischemic insult.

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Review Article

Thyroid Hormone Action in Cerebellum and Cerebral Cortex Development

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Thyroid hormones (TH, including the prohormone thyroxine (T4) and its active deiodinated derivative 3,3′,5-triiodo-L-thyronine (T3)) are important regulators of vertebrates neurodevelopment. Specific transporters and deiodinases are required to ensure T3 access to the developing brain. T3 activates a number of differentiation processes in neuronal and glial cell types by binding to nuclear receptors, acting directly on transcription. Only few T3 target genes are currently known. Deeper investigations are urgently needed, considering that some chemicals present in food are believed to interfere with T3 signaling with putative neurotoxic consequences.

1. What Is the Level of TH in the Brain?

Congenital hypothyroidism causes mental retardation and has irreversible consequences if not treated soon after birth [1]. Routine testing of newborns allows a rapid diagnostic and permits to initiate a therapeutic intervention, TH supplementation, which usually restores most cognitive functions. However, iodine deficiency is still considered to be one of the most frequent causes of preventable mental retardation in children worldwide [2, 3], prompting to reinforce salt iodization in all countries [4]. Upon thyrotropin (TSH) stimulation, thyroid gland mainly secretes T4, which is deiodinated to produce T3. It is usual to assume that measuring TSH, T4, and T3 levels in blood is sufficient to estimate T3 level in brain. This is, however, ignoring that T3 access in brain is highly regulated. Furthermore, even after the onset of the fetal thyroid gland function, which occurs only at midgestation in humans, fetal brain relies on maternal TH. TH first cross the placenta [5] and then the brain-blood barrier. This transport is thought to favor T4 at the expense of T3 [6]. The predominant function of transport across the brain-blood barrier has been shown by using pregnant female rats, depleted of both maternal and fetal T4 and T3 [6, 7]. In these conditions, administration of excess of T4 during the second half of gestation increases T4 and T3 levels in the maternal serum, and restores the T3 level in both fetal liver and fetal cortex. By contrast, maternal administration of T3 leads to a similar increase in T3 level for the fetal liver, implying its transport through the placenta, but to partial restoration of T3 level in fetal cortex. This confirms the importance of differential T4 and T3 transport, and outlines the requirement of local conversion of T4 by deiodination, performed by type 2 deiodinase (D2), which is already present in glial cells at fetal stage. It has been calculated that, in physiological situation, 80% of T3 in rodent brain might be produced by local deiodination of T4. The differential transport of T4 and T3 has an important consequence: maternal hypothyroxinemia, that is, low T4 level in maternal serum with T3 and TSH levels within normal range, is a cause of neurodevelopmental disorders [3]. Even mild maternal hypothyroxinemia was recently found to be associated with a higher risk of delay in the onset of expressive language of children [8]. This situation is much more frequent than overt hypothyroidism in geographical areas with low iodine uptake. Reporter mice have been made, in which lacZ expression is controlled by a Gal4-TRα1 artificial fusion protein, to visualize T3 signaling in brain [9]. These so-called FINDT3 mice revealed two interesting

features. First T3 visibly activates transgene expression at fetal stage (embryonic day 15.5). Second T3 signaling is highly heterogeneous, and large variations of reporter gene expression are observed depending on brain areas. Midbrain and telencephalon appear to be places of high signaling. This signaling pattern in adults is well correlated with T3 distribution measured in various brain areas [10, 11], suggesting that local T3 concentration is the main determinant of signaling and that unknown mechanisms maintain an heterogeneous distribution of T3 in brain. A number of transporters have been identified, which might participate to the transfer of TH to the brain [12]. However, knockout of individual transporters does not seem to affect neurodevelopment, suggesting a cooperation between several transporters. One transporter that received considerable attention is the monocarboxylate transporter 8, encoded by an X-linked *MCT8 (SLC16A2)* gene. Human mutations of *MCT8* increase rather than suppress TH circulating levels but have dramatic consequences on neurodevelopment, therefore suggesting a predominant function of MCT8 for TH transport across the brain-blood barrier [13]. However, the knockout of MCT8 does not have any obvious influence on mouse brain development and the reasons for this discrepancy are unclear. Two alternative possibilities can be proposed: the first would be that human MCT8 is able to transport not only TH but also some unknown signaling molecule required for proper brain development. This hypothesis is consistent with the clinical observations of patients with MCT8 mutations, which display a syndrome that is apparently distinct and more severe that congenital hypothyroidism. The other possible explanation for the mild consequences of MCT8 mutation in mice could be that other transporters, for example, the OATP14, LAT1, and LAT2 transporters, are present at earlier stages in the rodent brain, and can compensate for MCT8 deficiency in this species [14–16].

Knocking-out the *Dio2* gene, which encodes D2, allowed to precisely address the function of T4 deiodination during fetal brain development, but led to surprisingly mild phenotypic alterations. No major change in T3 level was observed in serum. The reduction of T3 level at postnatal day 15 (P15) was only 50% in cerebellum and hypothalamus, and not significant in cortex [17, 18]. T3 level was normalized in adult brain [19]. Gene expression analysis and neurobehavior testing confirmed a very mild neurodevelopmental phenotype [17]. Crossing with FINDT3 reporter mice indicated that the spatial pattern of TH signaling was not obviously affected throughout development (L. Quignodon unpublished data). Type 1 deiodinase (D1 encoded by *Dio1*) is the other enzyme able to convert T4 into T3, and is mainly expressed in liver. Surprisingly, elimination of both *Dio1* and *Dio2* did not aggravate the neurobehavioral phenotype. Although *Dio1/Dio2* combined knockout increased T4 level, T3 level was maintained in serum [18] and adult brain [19]. *Dio2* knockout was also combined to *MCT8* knockout [19, 20]. For a set of genes that are positively regulated by T3 in cortex, this combination resulted in decreased expression, in both juveniles and adults. This suggests that T3 is produced by local deiodination of T4, or directly transported by MCT8, and that blocking both pathways leads to a status close to hypothyroidism in cortex. Looking at genes that are found to be upregulated in hypothyroid cortex revealed, however, that the situation is more complex, as, for these negatively regulated genes, only *Dio2* knockout had a influence on expression level. Since the molecular mechanism underlying negative regulation of gene expression by T3 is unknown, this observation is difficult to explain, but probably suggests that the entry route of T3 in brain is somehow an important information to define individual gene regulation status.

The third known deiodinase, type 3 deiodinase (D3, encoded by *Dio3*), is responsible for T3 catabolism and is expressed in several brain areas [21]. As *Dio3* gene expression in brain is upregulated in case of hyperthyroidism, local T3 catabolism can act as a safety pathway to protect brain against excess of T3. *Dio3* knockout mice were crossed to FINDT3 reporter mice [22]. This revealed a slow accumulation of T3 in the anterior cortex, indicating that catabolism is required to regulate T3 level in adult cortex. *Dio3* knockout did not eliminate the spatial heterogeneity of T3 signaling, at early and late stages, ruling out a major function of D3 in defining the spatial distribution of T3 in brain. In adult hypothalamus, local control of T3 level by deiodinases appears to be used to sense and react rapidly to external signals. Local TH metabolism participate to hypothalamic response to feeding status [23], inflammation [24], seasonal change [25, 26], and TRH feedback regulation [27]. This example might prompt to reexamine the possibility that rapid metabolism could also influence local environment during development. In conclusion, T3 signaling level greatly varies in brain, depending on brain areas, cell type, and developmental stage and measuring TH levels in blood is poorly informative. Although the system generating this heterogeneity is not understood, it appears as a very robust and insensitive to genetic mutation affecting TH transport and metabolism.

2. Nuclear Receptors Function in Neurodevelopment

The *THRA* and *THRB* genes encode a number of isoforms [28, 29]. Among these only TRα1, TRβ1, and TRβ2 are considered as T3-dependent activators of nuclear transcription. TRα1 mRNA is ubiquitous in brain, both in neuronal and glial cell types whereas TRβ1 expression is more restricted with prominent expression in zones of neuroblasts proliferation such as the germinal trigone and the cortical ventricular zone. TRβ2 expression in brain is limited to the developing hippocampus and striatum [30–32]. To more precisely address *THRA* expression pattern, a mouse strain that expresses TRα1 and green fluorescent protein (Gfp) as a chimeric protein from the *THRA* locus has been recently created by homologous recombination [33]. Immunocytochemistry against Gfp confirmed a broad expression, but also indicates important variations in expression level. Although very reliable, this method is probably of limited sensitivity, and absence of detection should not be taken as a proof for absence of expression. For example, Gfp is detected first at embryonic day 13.5 (E13.5) whereas *in situ* hybridization indicates earlier expression [30]. One interesting case is the Purkinje neurons in cerebellum, in which TRα1 expression

decreases overtime while TRβ1 gradually increases, suggesting a series of postnatal maturation events [34] enrolling the two receptors sequentially.

Due to the difficulty to detect endogenous proteins by Western blotting [35] or immunocytochemistry [36], THRA and THRB expression studies in brain have been limited to mRNA analysis. This is regrettable because TRα1 mRNA can encode several proteins, beside the canonical receptor [37]. Among these, the p43 protein is translated from a downstream AUG codon, providing a 43 kDa protein lacking the N-terminus of the TRα1 canonical receptor. This p43 isoform has been proposed to be targeted to mitochondria and to regulate mitochondrial genome transcription, providing a possible complement to the well-known nuclear activation of genes encoding mitochondrial enzymes [38] which is relevant to neurodevelopment [39, 40]. The involvement in neurodevelopment of p43, as well as other so-called nongenomic pathways for T3 action [41] would merit specific investigation. T3 is also a precursor of thyronamines, which are present in brain, and activate a different class of membranes receptors also expressed in brain [42], but which neurodevelopmental function has not yet been evaluated.

Initial studies of THRA and THRB knockouts were surprising. THRA knockout has no visible consequence on cerebellum, a posterior brain structure which postnatal development is highly sensitive to TH deficiency, and is the most classical model to study congenital hypothyroidism in rodents [43]. It induces increased anxiety in adults, which probably involves hippocampus function, but does not necessarily reflect a developmental defect. THRB knockout affects hearing [44] and vision [45] but not the central nervous system development. No developmental defect has been reported after combining THRA and THRB knockout [46, 47]. The inability of THRA/THRB knockout to phenocopy congenital hypothyroidism was explained by depleting THRA knockout newborns of T3 by potassium perchlorate and 1-methyl-2-mercaptoimidazole treatment. In the cerebellum of these mice, the persistence of the external granular layer, a typical sign of congenital hypothyroidism normally induced by T3 depletion, was absent [48]. This strongly suggests that the manifestations of congenital hypothyroidism in brain are mainly due to the presence of unliganded TRα1, which is bound to DNA and represses transcription. Knockin mutations in THRA have been produced to introduce point mutations in the TRα1 reading frame and compromise its ability to transactivate upon T3 binding, without impairing its ability to repress transcription. As expected, such mutations, unlike the THRA knockout, lead to a cerebellum phenotype resembling congenital hypothyroidism [49, 50], and these defects are not limited to cerebellum [51, 52]. Whether TRα1 is the only active T3 receptor during cerebellum development remains unclear. One THRB knockin mutation also produces a cerebellum phenotype resembling hypothyroidism, with some important differences, perhaps due to the associated increase in circulating TH levels [53, 54]. When given to hypothyroid pups, TRβ selective ligands seem to have an effect limited to Purkinje neurons [48, 55]. However, the action of T3 on *in vitro* Purkinje cells dendritogenesis appears to be depending on TRα1 rather that TRβ1 [56]. Finally, many human germline mutations have been reported in the human THRB, but not THRA, gene, responsible for a syndrome of resistance to TH [57]. Although these mutations can be a cause of IQ deficit, they do not have major neurological consequences [58].

3. TH and Neurodevelopment: How Much Is Cell Autonomous?

Most detailed studies are focused on cerebellum postnatal development in rodents, where each cell type is sensitive in some respect to TH deficiency: the inward migration of the granular cell precursors (GCPs) present in the granular layer cells (EGL) is inhibited, the development of the dendritic arborization of Purkinje neurons is impaired, and the maturation of the GABAergic interneurons is delayed [59]. The proliferation and differentiation of glial cells, including astrocytes [60, 61], oligodendrocyte precursors [62], and microglia [63] are affected. The morphology of Bergmann glia, a specific type of radial glia found only in cerebellum, is abnormal [64]. The diversity of these effects raises several possibilities for the mode of action of T3. The first would be that the repertoire of T3 target genes is completely different in different cell types, and that genetic programs governing cell migration, cell proliferation, and cytological maturation are regulated in a cell-specific manner. In this case, the chromatin status in a given cell type would exert a predominant influence to define the repertoire of TR target genes. The alternative would be that T3 exerts similar effects in different cell types, controlling a shared repertoire of TR target genes, but that this cell-autonomous response would represent only a small fraction of the observed effects of T3. TH deficiency is known to affect the level of several neurotrophic and growth factors in brain, which are required for proper development [65, 66]. Cell culture can be used to distinguish between these two possibilities, and to address the cell autonomous response of purified or enriched cell populations to T3, isolated from their natural environment. Purkinje cells [56], oligodendrocyte precursors [67], and astrocytes [68] were all shown to respond to *in vitro* stimulation by T3. By contrast, impaired migration of GCPs, which is a main sign of congenital hypothyroidism, is possibly an indirect effect for several reasons. First, this cell type does not express TRα1 at a high level [33]. Second, microarray analysis, performed on primary cultures of GCPs, identified very limited changes in gene expression after T3 exposure, and failed to establish a direct link between T3 and known mechanism of neuronal migration [69]. Third, according to in situ hybridization, GCPs do not express *THRB*, but their migration is affected by the TR$\beta^{\Delta 337T}$ mutation [54]. Finally, GCPs do not produce the T3-regulated neurotrophic factors (neurotrophin 3, insulin-like growth factor-1, Sonic hedgehog, and brain-derived neurotrophic factor) but need these neurotrophic factors for their proliferation, inward migration, and differentiation [70–72]. As Purkinje cells are the main source of Sonic hedgehog and insulin-like growth factor-1, they probably play a central role in a local network of cellular interactions. However, if this local effect

is plausible, a more indirect, systemic, influence of T3 cannot be ruled out. In that respect, it is remarkable that a metabolic disease, which is clearly restricted to the liver, has been found to impact GCPs inward migration [73]. All these observations suggest that GCPs migration, which is required to give rise to more than 90% of the cerebellum neurons, does not result from a cell autonomous action of T3. A direct genetic demonstration of this hypothesis would be possible using the CRE/loxP technology, for which *THRA* [50] and *THRB* [44] alleles are available.

4. T3 Target Genes

It is striking that genome-wide analysis performed by a number of groups over the years, using a variety of protocols, identified only few direct T3 targets in neural cell types, defined as genes for which transcription is under the direct control of TRα1 or TRβ1 bound to neighboring regulatory sequences. Microarray analysis showed that *in vivo* T3 treatment has a very limited effect on transcriptome of whole cerebellum [74–76], or fetal cortex [77], compared to adult striatum [78] or other tissues [79]. Comparison between these studies also suggests that T3 response is very different in different brain areas and at different developmental stages [80]. The effect of graded degrees of TH deficiency, induced by propyl-thio-uracyl, on gene expression has been studied in postnatal hippocampus and cortex. Interestingly, transcriptome is affected in a dose-dependent manner, specific gene clusters being sensitive to only mild hypothyroidism [81]. Whereas many putative T3 target genes have been listed in these broad surveys, most changes are not rapidly reversed when T3 is given to hypothyroid animals, and might reflect changes in the cell composition of the considered area, or in the differentiation status or the cell types. This is probably the case, for example, for genes expressed in mature oligodendrocytes, as differentiation of this cell type is known to be regulated by T3. The actual demonstration of direct regulation by T3/TR requires deeper investigations, which have been done in only few cases. First, transcriptional regulation in a primary culture system, where environmental parameters can be controlled, can be used to reinforce the hypothesis for a direct regulation. Second, transient expression assays can be used to test the ability of gene promoter regions to confer T3 responsiveness to reporter plasmid constructs. However, this approach is often of little physiological relevance and is hardly feasible in appropriate cell types. Finally, chromatin immunoprecipitation, now considered as a gold standard, can demonstrate actual binding by TRα1 or TRβ1 on putative response elements in their natural genomic environment. This method appears to be much more reliable than *in vitro* protein/DNA interaction assays, which do not account for the complexity of the cellular chromatin context but remains technically challenging for several reasons. First, data obtained in other systems suggest that the distance between TR binding sites and transcription start site can be very large, making it difficult to make a selection among many putative response elements, and to ensure that TR occupancy is responsible for the observed regulation. Second, it is hardly feasible to perform such studies on purified cerebellar cell populations. Preparing chromatin from whole cerebellum [82] mainly addresses chromatin occupancy in granular neurons, which represent a large majority of the cerebellum cells. One attempt has been made to identify new T3 target genes by the so-called chip-on-chip high-throughput analysis [75].

Ironically, *Hairless (Hr),* encoding a transcriptional corepressor of several nuclear receptors, continues to be the best-characterized T3 target gene in neurons, fifteen years after its identification as a T3 target gene in rat postnatal cerebellum [83]. Its expression is rapidly induced in a number of neuronal cell types, and TR occupancy in the promoter region has been confirmed by chromatin immunoprecipitation [69]. Among other likely TRα1/TRβ1 direct target genes in neurodevelopment, are genes encoding a Krüppel-like transcription factor (Klf9) [84], a poorly studied synaptotagmin-related gene (srg1) [85], A kinase (PRKA) anchor protein 1 (Akap1) [69], neurogranin (RC3/Nrgn) [86], and, in the fetal cortex, Ca2+/calmodulin-dependent protein kinase IV (Camk4) [77]. The paucity in well-characterized T3 target genes in brain mainly stems from the high cellular heterogeneity of neural tissues, and the lack of suitable *in vitro* systems. It remains a bottleneck for the analysis of the mechanisms underlying the neurodevelopmental function of T3.

5. A New Class of Neurotoxic Thyroid Hormone Disruptors?

The above considerations have significant consequences for a matter of growing public concern: a number of chemicals found at low concentration in the water and food are suspected to exert a chronic toxicity by interfering with T3 signaling, and might thus be considered as putatively neurotoxic [87]. The main suspected compounds are Bisphenol A [88] flame retardants (tetra-bromo-bisphenol A [89], polybrominated diphenyl ethers [90]) and the persistent polychlorobiphenyls (PCBs) [91]. Acute exposure of rats to these compounds can lower or raise the circulating level of TH. Xenopus tadpole metamorphosis, which is fully dependent on TH, provides a basis for an *in vivo* assay recognized by the Organisation for Cooperation and Development (OECD) to define TH disruptors. The recently developed assay, based on transgenic xenopus tadpoles is a promising alternative, as it is rapid, and might better address TRα1 function [92]. In some cases, transactivation assays performed in transfected cell lines, or primary cultures of neural cells, confirm a possible direct influence of chemicals on T3 signaling [93]. Although some compounds, like coplanar PCBs, display some structural similarities with T3, they do not seem to fit into the T3 binding pocket and are thus unlikely to act as high-affinity TRα1/TRβ1 ligands. Recent data suggests, however, noncompetitive binding to TR, preventing interactions with DNA [94]. In most cases, a systemic influence on T3 metabolism and transport can account for most of the observed *in vivo* toxicity after acute exposure. Few experiments address the possibility that TH disruptors can interfere with the neurodevelopmental function of T3, although significant effects on gene expression

in brain have been reported after exposure to a high dose of PCBs [95, 96] or bisphenol A [97]. One could argue that the powerful compensatory mechanisms, revealed by the knockout of genes encoding transporters and deiodinases, would be sufficient to maintain a stable level of T3 in brain in most cases. Chronic exposure to a low dose of chemicals, acting on T3 transport and metabolism, would thus be unlikely to have neurotoxic consequences, unless other signaling pathways are involved. In fact, T3 level in brain is maintained in case of mild TH deficiency [98], a condition which already requires exposure to high doses of TH disruptors. By contrast, the results of *THRA* knockin mutations suggest that exposure to any chemical that would act as a TRα1 antagonist would be expected to significantly impair neurodevelopment. If such a hypothetical chemical was TRα1 selective, it would not change the circulating level of TH in serum, as this regulation is mainly exerted by TRβ1 and TRβ2. Therefore TRα1 antagonists would not be necessarily recognized by most *in vivo* tests. Defining the neural cell types that respond in a cell autonomous manner to T3 during neurodevelopment, and then identifying direct TRα1 target genes in these cells, appears therefore as a prerequisite to the definition of relevant endpoints for future toxicity assay development.

6. Conclusion

Recent advances in our understanding of the neurodevelopmental function of T3 heavily rely on mouse genetics. A complex landscape in which T3 probably exerts a number of different functions in different cell types has been uncovered. Coordinating the network of interactions, mediated by direct contacts and exchanges of diffusible factors, T3 synchronizes neuronal and glial cells differentiation to ensure the onset of functional neuronal structures. Whereas the transport and metabolism of T3 is a robust system, which enables to maintain T3 level within physiological range in many situations, signal transduction in the developing brain mainly relies on the TRα1 isoform. Single amino-acid substitutions can have dramatic consequences on neurodevelopment and phenocopy congenital hypothyroidism. This is probably the reason why no *THRA* germline mutation has been reported, and why single nucleotide polymorphism in the human locus are only found in noncoding sequences.

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Review Article

Thyroid Hormone Receptors in Two Model Species for Vertebrate Embryonic Development: Chicken and Zebrafish

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Chicken and zebrafish are two model species regularly used to study the role of thyroid hormones in vertebrate development. Similar to mammals, chickens have one thyroid hormone receptor α (TRα) and one TRβ gene, giving rise to three TR isoforms: TRα, TRβ2, and TRβ0, the latter with a very short amino-terminal domain. Zebrafish also have one TRβ gene, providing two TRβ1 variants. The zebrafish TRα gene has been duplicated, and at least three TRα isoforms are expressed: TRαA1-2 and TRαB are very similar, while TRαA1 has a longer carboxy-terminal ligand-binding domain. All these TR isoforms appear to be functional, ligand-binding receptors. As in other vertebrates, the different chicken and zebrafish TR isoforms have a divergent spatiotemporal expression pattern, suggesting that they also have distinct functions. Several isoforms are expressed from the very first stages of embryonic development and early chicken and zebrafish embryos respond to thyroid hormone treatment with changes in gene expression. Future studies in knockdown and mutant animals should allow us to link the different TR isoforms to specific processes in embryonic development.

1. Introduction

Thyroid hormones (THs) play an important role in development by controlling the growth and differentiation of almost every organ in the vertebrate body. They act mainly, although not exclusively, by binding to intracellular TH receptors (TRs), members of the nuclear receptor superfamily. TRs are ligand-inducible transcription factors that bind 3,5,3′-triiodothyronine (T_3) or with a lower affinity also 3,5,3′,5′-tetraiodothyronine or thyroxine (T_4). They function as homodimers or preferentially as heterodimers with other members of the same receptor family, notably the retinoid X receptors (RXRs). TRs recognize specific DNA sequences in the promoter region of TH-responsive genes and can bind to these TH response elements (TREs) even in the absence of ligand. Generally, unliganded TRs are bound to a set of corepressors leading to active repression of gene transcription. Ligand binding induces a conformational change, resulting in release of the corepressors and recruitment of coactivators and stimulation of gene transcription. The molecular mechanisms involved in TR-mediated gene transcription have recently been reviewed in more detail by other authors (e.g., [1–4]).

The first clear evidence for the need of THs in vertebrate development came from frogs, where THs control the transition from an aquatic larva to a terrestrial juvenile during metamorphosis. Since that time, THs have been shown to be involved not only in postnatal/posthatch development but also in earlier stages, both in mammals and in nonmammalian species. All vertebrate embryos have access to THs long before the embryonic thyroid gland starts hormone secretion, either by transplacental transfer in mammals [5] or by TH deposition in the yolk in other vertebrates [6, 7]. Whether or not these THs can influence early development largely depends on the presence and tissue-specific distribution of TRs in the species investigated. In

this paper we try to summarize the information available for two nonmammalian model species for vertebrate embryonic development. The chicken is a long-established model for the study of early development. It has been a major model in embryology for more than a century and has recently become even more powerful thanks to the possibilities of gain- and loss-of-function technologies [8]. The zebrafish emerged more recently but became a mainstream model organism for the molecular aspects of development very rapidly, because it combines an external development and a relatively short generation time with several possibilities of genetic manipulation [9].

2. Different Isoforms of Nuclear TH Receptors

Vertebrates generally have two TR genes located on different chromosomes, encoding, respectively, thyroid hormone receptor alpha (TRα) and thyroid hormone receptor beta (TRβ). Due to ancestral gene duplication, some nonmammalian vertebrate species, including several fish, have two TRα-encoding genes [10]. Each TR consists of an amino-terminal regulatory domain, a central DNA-binding domain, and a carboxy-terminal hormone-binding domain. The latter not only binds THs, but is also involved in the interaction with corepressors and coactivators, and in the dimerisation of the receptors. The structure and function of TRs has been very well conserved throughout vertebrate evolution. They seem to originate from a single TR gene that has a common ancestry with a TR gene found in the cephalochordate *Amphioxus* and the sea squirt *Ciona*, and interestingly even in the trematode *Schistosoma*. This suggests that the origin of the TR gene goes back early in animal evolution [11, 12]. Each vertebrate TR gene typically gives rise to several variants through alternative splicing and the use of different transcription start sites. In rodents, this leads to three hormone-binding TRβ variants (TRβ1, TRβ2, and TRβ3) differing in their amino-terminal domain, one hormone-binding TRα variant (TRα1) and two TRα variants (TRα2 and TRα3) that have a different carboxy-terminus and are not capable of hormone binding. In addition, some truncated TRs have been identified (TR$\Delta\beta$3, TR$\Delta\alpha$1, and TR$\Delta\alpha$2) that have the capacity to bind THs but that cannot bind to TREs [1]. So far the number of TR isoforms identified in chicken and zebrafish is more restricted, but a more thorough investigation may considerably increase their number as happened in rodents over the last decades.

The presence of nuclear binding sites for T_3 was first shown in rat in the early seventies [13, 14]. Approximately ten years later, similar studies in chicken embryos showed that such binding sites were already present early in embryonic development, in liver, brain, and lung tissue [15–17]. In addition, these studies showed that shifts occurred in the K_a values for T_3 binding during development and that while nuclei purified from brain of 9-day-old embryos (E9) bound T_3 twice as good as T_4, this shifted to a 5-fold better binding of T_3 at E17 [16]. This led to the suggestion that maybe more than one type of binding site was present and that their relative abundance might change during development.

The first step to the identification of the molecular structure of TRs was made when it was shown in chicken and in rat that the cellular counterpart of the *v-erb-A* gene coded for a protein capable of binding T_3 with the same affinity as the previously identified nuclear binding sites [18, 19]. As a result, it became clear that the previously described chicken *c-erb-A* gene [20] was the gene for chicken TRα [18]. A few years later, a cDNA encoding a chicken TRβ was characterised. This cTRβ closely resembled the human and rat TRβ sequences identified at that time, but it had a much shorter amino-terminal domain [21, 22]. Shortly thereafter another TRβ with a longer amino-terminal domain was identified [23]. It closely resembled rat TRβ2 and was therefore named cTRβ2, while the previously identified shorter TRβ was named TRβ0 [23]. It was shown that TRβ2 was more efficient in transactivation of a reporter gene than TRβ0 [23, 24]. All three chicken TR isoforms have a functional hormone-binding domain and bind T_3 with higher affinity than T_4. No truncated or non-ligand-binding variants have been described so far. A comparison of the structure of the chicken and mouse TR variants is given in Figure 1.

Two TR genes were originally identified in zebrafish, TRα and TRβ, giving rise to the transcripts TRα1 and TRβ1 [26, 27]. Zebrafish TRα1 showed a high similarity with TRαs from other vertebrates, but it had 14 additional carboxy-terminal amino acids that were not found in any other known TR [26, 28]. Zebrafish TRβ1 had the typical structure of all other TRβs including the short amino-terminal domain [27, 29]. One year later, a second TRβ isoform was described that had a 9-amino acid insert in the hinge region between the DNA- and the ligand-binding domain [28], a feature found in several teleost TRβs but not in other vertebrate classes [30]. Comparison of the activity of the TRα and TRβ proteins suggested that TRβ1 transactivating activity was ligand-dependent and repressed in the absence of T_3, while TRα had constitutive transactivating activity in the absence of ligand [27, 31].

Only recently it was shown that due to ancestral gene duplication, zebrafish has two TRα genes and that they are both expressed [10, 32]. The originally identified TRα gene has therefore been renamed *thraa*, while the second one is called *thrab*. The *thraa* gene gives rise to at least two proteins: TRαA1 and TRαA1-2. TRαA1 corresponds to the original TRα1 with the carboxy-terminal extension while TRαA1-2 does not have this extension [32]. The so-called F domain extension does not alter the overall structure of TRαA1, but it reduces the transcriptional activity of the receptor by changing its affinity for the zebrafish coactivator NCoA2 [32]. The sequence of the ligand-binding domain of TRαB, the transcript encoded by the *thrab* gene, is very similar to the one of TRαA1-2, but based on the predicted sequence, it probably has a shorter amino-terminal domain and some splice variations that might have functional consequences [32]. All the zebrafish TRs mentioned above have a functional hormone-binding domain. However, there is some evidence for the presence of a TRβ2-like transcript that could encode a truncated TR with a complete DNA-binding domain but no ligand-binding domain [33]. A

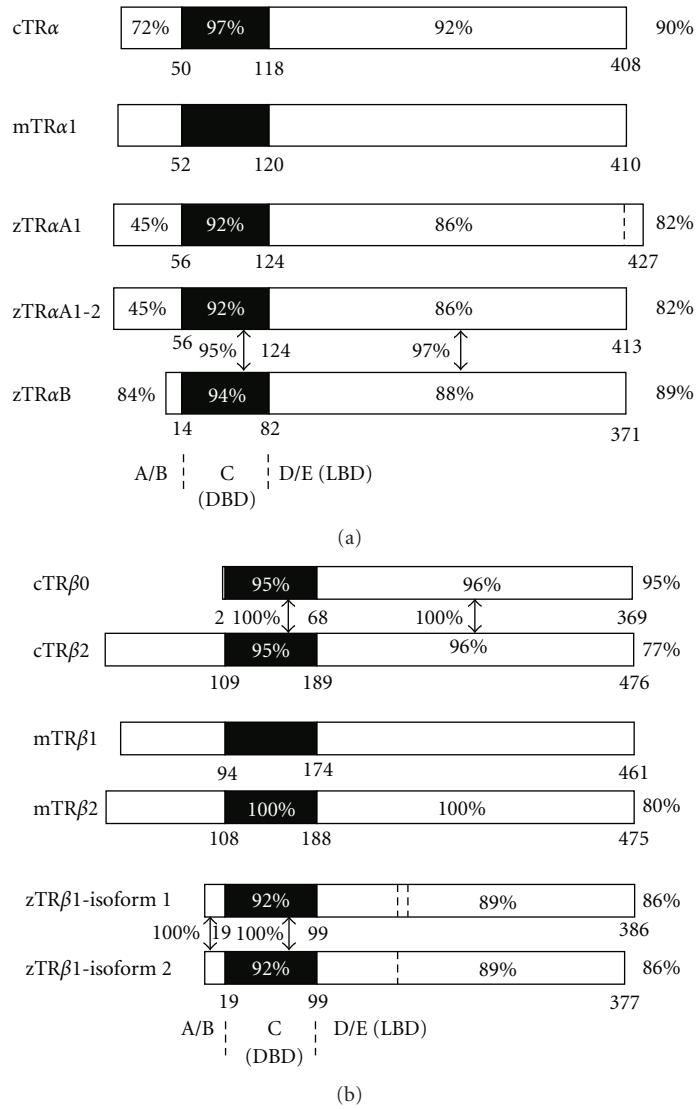

Figure 1: Comparison of mouse (m), chicken (c), and zebrafish (z) TR isoforms. (a) TRα variants and (b) TRβ variants. Truncated TR isoforms that do not bind T_3 are not included, nor are the TRβ3 variants that have only been found in rats. Numbering of amino acids is represented under each bar. Percentages within the bars are identities of that domain with the homologous domain of mTRα1 or mTRβ1, respectively. Percentages right of the bars give the overall similarity of the entire protein with the canonical mouse homolog. Percentages in between bars show the similarity between the respective domains. A/B: A and B domain; C (DBD): C domain or DNA-binding domain; D/E (LBD): D and E domain or ligand-binding domain. The dotted line in the D/E domain of zTRαA1 marks the first of the 14 additional carboxy-terminal amino acids not found in the other TRs. The dotted lines in the D/E domain of zTRβ1 isoform 1 delineate a 9-amino-acid insert that is missing in the equivalent location in zTRβ1 isoform 2 (also indicated with a dotted line). Comparisons were based on the following UniProtKB sequences: mTRα1 (P63058-2), mTRβ1 (P37242-1), mTRβ2 (P37242-2), cTRα1 (P04625), cTRβ0 (P68306), cTRβ2 (Q91003), zTRαA1 (Q98867-1), zTRα1-2 (Q98867-2), zTRαB (A0ST48), zTRβ1-isoform 1 (Q9PVE4-1), and zTRβ1-isoform 2 (Q9PVE4-2).

comparison of the structure of the zebrafish and mouse TR variants is given in Figure 1.

3. Expression and Distribution of TRs during Embryonic Development

The expression pattern of chicken and zebrafish TRs has so far been studied predominantly, if not exclusively, at the mRNA level using techniques like Northern blot analysis, *in situ* hybridisation (ISH), and quantitative reverse transcription polymerase chain reaction (qRT-PCR). While ISH is the only technique providing information on the cell-specific distribution pattern, qRT-PCR is by far the most sensitive one. This difference in detection limit has to be taken into account when comparing the results from different groups published over the years.

Chicken embryonic development takes three weeks from the beginning of incubation to hatching. Studies in early chick embryos have shown that TRα is expressed earlier

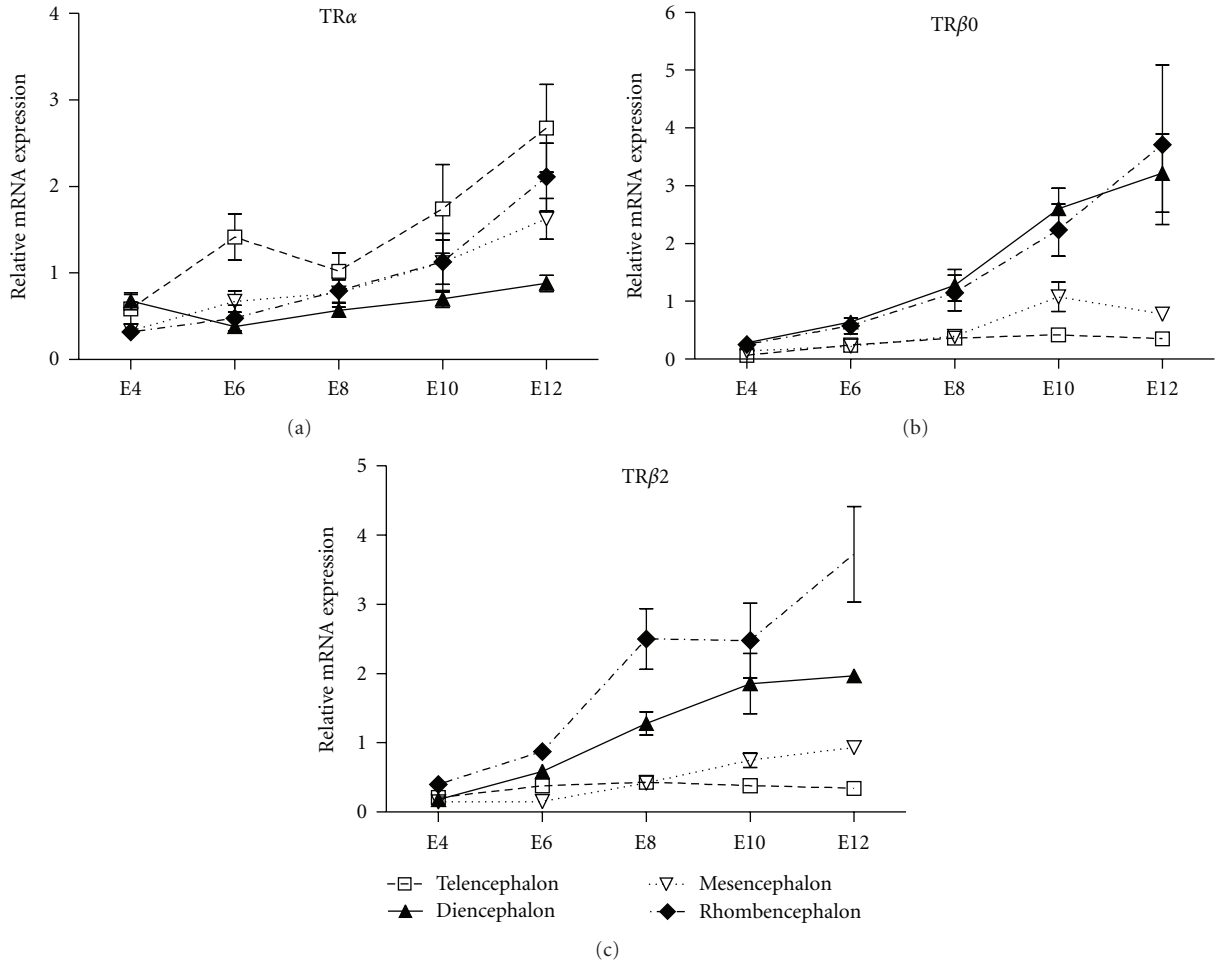

Figure 2: Ontogenetic pattern of TRα, TRβ0, and TRβ2 expression in different brain regions of 4- to 12-day-old chick embryos. Specific mRNA levels were measured by qRT-PCR and normalised against a combination of four housekeeping genes: β actin, GAPDH, β2 microglobulin, and cyclophilin A. Values represent the mean ± SEM for 6 independent samples per stage.

than TRβ. TRα mRNA was already detected on the first day of incubation and whole-mount ISH in embryos after 18 to 33 hours of incubation showed the highest expression in the neurectoderm [34]. At that time, TRβ expression was found to be extremely low [34]. This predominance of TRα at early stages was confirmed by Northern blot analysis showing the presence of TRα mRNA in brain, red blood cells and yolk sac at E4, while TRβ0 mRNA could only be detected from E7 onwards in yolk sac [21, 35]. Using an RNase protection assay, TRβ2 mRNA was first detected at E6, specifically in the retina [23]. Recently, qRT-PCR analysis by our own group demonstrated that all three known chicken TR variants are already expressed in brain at E4. Analysis of different brain regions from E4 up to E12 showed a clear and gradual increase of TRα mRNA levels in telencephalon, mesencephalon, and rhombencephalon (including cerebellum, pons, and myelencephalon), while the increase was marginal in diencephalon. In contrast, expression of both TRβ0 and TRβ2 clearly increased in diencephalon and rhombencephalon, whereas there was only a small increase in telencephalon and mesencephalon (Figure 2).

Several studies in older embryos have confirmed that TRα and TRβ are expressed in a spatiotemporal divergent pattern and that TRα is the most widely distributed isoform. Northern blot analysis in embryonic tissues from the second week of incubation onwards showed TRα expression in brain, eye, lung, kidney, heart, liver, intestine, muscle, spleen, red blood cells, and yolk sac [21]. The high expression in embryonic red blood cells confirmed earlier findings obtained by ISH [35]. The TRβ0 signal on Northern blot was restricted to brain, eye, lung, kidney, and yolk sac [21]. ISH on embryonic brain samples showed that both TRα and TRβ were predominantly expressed in cerebellum. However, while TRα was abundantly present in E15 and E19 cerebellum, TRβ0 expression was still faint at E19 and increased after hatching [36]. The same research group also found that during eye development, there was a shift from a relatively high TRβ2 expression to a predominance of TRβ0 towards the last days of embryonic development [21]. Our group again used qRT-PCR to analyse the expression of TRβ2 in tissues of late-stage embryos and early posthatch chicks. It was found, as in mammals, that TRβ2 expression was very restricted in peripheral tissues. At E18 this receptor was

mainly expressed in brain, thyroid gland, pineal gland, pituitary gland, and retina, with a clear predominance in retina [37, 38]. More detailed analysis in diencephalon, pituitary, and thyroid gland showed a steady increase in expression from E14 up to E20. Then levels stabilised in diencephalon and pituitary, but they continued to increase in thyroid gland [38].

Zebrafish development from fertilisation to hatching only takes 3 days. The embryos are small and in most studies complete embryos have been pooled for RNA extraction and quantification of TR mRNA levels. Northern blot studies showed that TRαA1 mRNA was clearly present at the start of development but those levels rapidly dropped towards the early gastrula stage. This probably reflects the disappearance of maternal transcripts, since TRαA1 is only present in high amounts in ovary and testis of adult zebrafish [26, 32]. Zygotic expression of TRαA1 could be demonstrated after the mid-blastula transition about 3 hours postfertilisation (hpf) using RNase protection and qRT-PCR, but levels remained very low throughout embryonic and larval development [26, 32]. In contrast, after the disappearance of maternal TRαA1-2 and TRαB mRNA, zygotic expression of these TR isoforms was increased 5- and 28-fold, respectively, in larvae at 4 days post fertilisation (dpf) compared to embryos at 1 dpf [32]. Except for the recent study of Takayama and colleagues [32], the available RT-PCR expression data were all obtained using primers based exclusively on the *thraa* sequence and also do not allow to distinguish the different TRαA transcripts. Transcription levels for TRαA and TRβ have been compared during the first 12 cell cycles of the zebrafish zygote (0–4 hpf) using semiquantitative RT-PCR. The results showed that the level of maternal TRαA transcripts was higher than the level of TRβ1 transcripts, which were already degraded by the 2-cell stage. Zygotic expression of TRαA and TRβ1 transcripts could already be shown at the 8- to 16-cell stage, well before the mid-blastula transition and the increase in TRαA appeared to precede the increase in TRβ1 [27]. The same research group continued their studies at later stages, showing more or less stable expression of TRα at 1, 2, and 3 dpf, while TRβ, expression increased substantially between the early gastrula stage (5-6 hpf) and 2 dpf [39]. We measured TRαA expression by qRT-PCR at regular intervals during embryonic development, confirming relatively high mRNA levels at 8 hpf followed by low levels up till hatching [7]. For TRβ we found more or less stable mRNA levels throughout embryonic development, followed by a rapid increase around hatching [25] (Figure 3). Data of an ISH study for a wide range of nuclear receptors showed no or only baseline signal for TRα in zebrafish embryos, while TRβ was expressed from approximately 30 hpf onwards in the retina and from approximately 40 hpf onwards also in the mid- and hindbrain [10, 40].

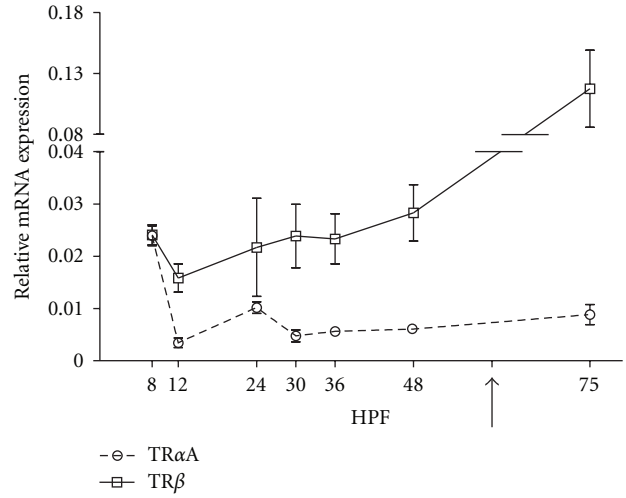

Figure 3: Expression of TRαA and TRβ in whole zebrafish embryos during the first three days post fertilisation. Specific mRNA levels were measured by qRT-PCR and normalised against the housekeeping gene Elongation factor 1α. Values represent the mean ± SEM for 3 independent samples (pools of 50–100 embryos) per stage. The arrow indicates the average time of hatching. HPF: hours post fertilisation. Data were taken from [7, 25].

4. TR-Mediated Actions of TH during Embryonic Development

Thyroid hormones play a major role in the development and maturation of most chicken organs, as is the case in all vertebrates. In addition THs are important in chicken for yolk sac retraction and hatching [41]. While it is clear that nuclear TR-mediated gene transcription is extremely important in the control of these developmental processes, little is known about the specific role of each of the different TR isoforms. In mammals, studies in transgenic mice have contributed substantially to our understanding of the role of each hormone-binding TR variant, leading to the conclusion that all of them seem to have both unique and redundant functions [42–44]. Given the high similarity of chicken TRα, TRβ0, and TRβ2 with mammalian TRα1, TRβ1, and TRβ2, this is probably also the case in chicken and a number of studies indeed point in that direction.

The relatively high expression of TRα in neurectoderm during the first two days of incubation suggests that this receptor plays an important role in the early development of the nervous system [34]. At this moment, it is still unclear whether the main function of TRs at that stage is repression of gene transcription in the unliganded state or whether the low amount of TH in the embryo is already controlling ligand-dependent stimulation of gene transcription. The latter is possible since T_3 was shown to be present in the embryo from the first day of incubation and administration of a high dose of T_3 disturbed the development of neural tube and brain [34]. We showed that E4 embryos efficiently take up THs from the yolk and injection of surplus hormone at that stage is capable of changing the expression of some TH-responsive genes, including TRs, in the embryonic brain, indicating that ligand-dependent control of gene transcription is possible at these early stages [45, and own unpublished results]. The upregulation of TR protein by T_3 found in hypothalamic neurons from E6 embryos kept in culture also points to the early effects of TH-dependent physiological actions of TRs [46].

The relatively high expression levels of TRβ2 at E6 and its progressive decrease later on suggest that this specific TR isoform is important for the early stages of retina development [23]. *In ovo* treatment of E7 to E12 embryos with T_4 accelerates the maturation of the cornea [47], but since all three TR isoforms are expressed in chicken eye by E9 [21, 23], this effect cannot be linked to a specific receptor subtype. Later in development, TRβ2 seems to be important in feedback regulation of the thyrotropic axis. We found that its expression level in diencephalon and in pituitary closely paralleled the increase in plasma T_4 from E14 towards a maximum at E20 and the decrease thereafter. Moreover, a 30 min *in vitro* exposure of E18 pituitaries to 10 or 100 nM T_4 or T_3 reduced TRβ2 expression by more than half [38]. This would agree with the role of TRβ2 described in the regulation of TSH and TRH production in mice [48, 49] and the negative effect of T_3 on the TRβ-mediated TRH transcription found in primary cultures of embryonic chick hypothalamic neurons transiently transfected with TRα or TRβ [50].

Describing the role of the different zebrafish TRs in embryonic development is hampered by the lack of information on their tissue-specific distribution in these early stages since the primers or probes used in many studies, including our own, do not allow to unequivocally identify the distinct transcripts. Moreover, while a multitude of genes have been knocked down using specific morpholino antisense oligomers to study their role in zebrafish embryonic development, the TR genes seem to have escaped notice. It has been suggested that at the beginning of development, in the absence of TH, TRαA1 functions mainly as a transcriptional repressor and that it may repress retinoic acid signalling in blastula- and gastrula-stage embryos [26]. Overexpression of TRαA1 in early development interfered with the role of retinoic acid in the establishment of the anteroposterior axis in the central nervous system and resulted in severe disruption of the rostral hindbrain [51]. However, although the zebrafish thyroid gland only starts hormone secretion around the time of hatching, zebrafish embryos take up THs from the egg yolk, and hormone-dependent stimulation of gene transcription may occur even in early embryos. We showed that when embryos were reared in medium containing 5 nM T_3, hormone levels in the embryos increased dramatically, concomitant with an acceleration of developmental rate and hatching. We also observed an increase in the expression of TRα in T_3-treated embryos at 48 hpf compared to controls, while TRβ expression was not altered [7]. Other investigators found that immersion in 5 nM T_3 first downregulated TRαA and TRβ1 levels, while continued treatment up to 72 hpf resulted in upregulation of expression of both genes [27]. The same group showed that T_3 treatment of zebrafish embryos starting at 48 hpf upregulated TRα and TRβ expression, whereas the drug amiodarone that can bind to TRs and antagonise their action strongly inhibited TRα and TRβ expression. This suggests that, as in early chicken embryos, TH can exert a positive autoregulatory feedback control on the transcription of its receptors [39]. This agrees with our studies where we knocked down the type 2 iodothyronine deiodinase, the enzyme converting T_4 to the receptor-active T_3. In these embryos, TRα expression was slightly lower at 24 hpf and 31 hpf compared to controls, and TRβ expression was clearly reduced [52].

5. Conclusions

As in other vertebrates, several TR isoforms have been identified in chicken and zebrafish. All of them appear to be fully functional receptors and so far no truncated TRs have been characterised. The different TR variants are expressed throughout embryonic development in spatiotemporal divergent patterns. As in mammals, there seems to be a predominance of TRα over TRβ expression at the early stages of embryonic development in both species. Before the embryonic thyroid gland becomes active, chicken and zebrafish embryos have access to THs from a maternal deposit in the yolk, and it has been shown that TH administration to early embryos can stimulate transcription of TH-dependent genes. However, as in mammals, it remains unclear whether the main action of TRs in the first stages of development is the repression of gene transcription in the unliganded form or the stimulation of gene transcription following ligand binding. We also need more data to be able to link the different TR isoforms to specific processes in embryonic development, particularly in zebrafish. In combination with data available from frogs and mammals, this will allow identifying isoform-specific actions that have been conserved throughout vertebrate evolution. Gene knockdown studies and the use of mutant embryos can certainly contribute to solve these questions in the near future. This will even further increase the attractiveness of these externally developing model species for functional genomics studies in relation to the role of THs and their receptors in human development and health.

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Review Article

Thyroid Hormone Receptor Mutations in Cancer and Resistance to Thyroid Hormone: Perspective and Prognosis

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Thyroid hormone, operating through its receptors, plays crucial roles in the control of normal human physiology and development; deviations from the norm can give rise to disease. Clinical endocrinologists often must confront and correct the consequences of inappropriately high or low thyroid hormone synthesis. Although more rare, disruptions in thyroid hormone endocrinology due to aberrations in the receptor also have severe medical consequences. This review will focus on the afflictions that are caused by, or are closely associated with, mutated thyroid hormone receptors. These include Resistance to Thyroid Hormone Syndrome, erythroleukemia, hepatocellular carcinoma, renal clear cell carcinoma, and thyroid cancer. We will describe current views on the molecular bases of these diseases, and what distinguishes the neoplastic from the non-neoplastic. We will also touch on studies that implicate alterations in receptor expression, and thyroid hormone levels, in certain oncogenic processes.

1. Preface

More than two thousand years ago, Aristotle discovered a link between castration and disruption of male maturation. Through extensive experimentation on bird and beast, he hypothesized that the testes were vital to the development of secondary male sex characteristics [1]. Excision of these organs drastically altered body size and behavior, as well as hair, feather, and horn growth [2]. These experiments were the earliest seeds of what would eventually become our current understanding of endocrinology. And from these same beginnings arose the recognition that aberrant endocrine signaling, through intentional intervention, accident, or pathogenic processes, could lead to disease.

Comprehension of endocrine signaling grew slowly over the next two millennia until the mid-19th century, which oversaw a dramatic expansion of research into endocrine glands and their secretions. With these studies came the first hints of methods to clinically intervene when normal endocrine homeostasis was disturbed. In 1849, Berthold discovered how to undo the deed of Aristotle, showing that castrated roosters regained their comb and wattle if the testes were surgically transplanted back into the abdominal cavity; Berthold correctly reasoned that the growth-enhancing compound in the testes must be soluble and blood-borne [3]. Similarly, the roles of the thyroid gland came to focus when Murray, in 1891, determined that a patient's symptoms (now known to be due to hypothyroidism) disappeared after grafting half of a sheep's thyroid beneath her skin. Because the patient's symptoms disappeared quickly after the operation, Murray surmised his patient's improvement could not be attributed to regained function of the sheep's gland but rather must be "due to the absorption of the juice of the healthy thyroid gland by the tissues of the patient" [4]. He later suggested that injections of thyroid gland extract would likely produce the same effect, a prediction subsequently confirmed by Baumann and Roos [5]. Graves reciprocally demonstrated that excessive thyroid gland activity leads to the pathological process now denoted hyperthyroidism [6]. In 1915, Kendall reported the successful isolation of thyroid hormone [7].

As more and more endocrine hormones were identified between the mid-19th to mid-20th centuries, interest turned toward understanding not only their synthesis and chemical structures, but also their mechanisms of action within their

target tissues. In the 1960s, Jensen et al. demonstrated that radiolabeled estrogen injected into female rats localized, in part, to reproductive target tissues, hinting at the existence of a tissue-specific receptor for this hormone [8, 9]. In 1973, Jensen et al. demonstrated that the estrogen/estrogen receptor (ER) complex shuttled from the cytoplasm to the nucleus and enhanced RNA synthesis in uterine tissue (Jensen et al. referred to it as an "alleviation of a deficiency in RNA synthesis") [10]. This was one of the first indications that nuclear receptors could influence transcription, foreshadowing both the appellation of "nuclear" to the term "receptor" and the role of these receptors in gene regulation. Additional evidence for the participation of nuclear receptors in transcription control soon accumulated, extending this paradigm to glucocorticoids and thyroid hormones [11–19]. The molecular cloning of the cDNA for glucocorticoid receptor (GR) was reported in 1985, and, just a year later, the cDNAs for the human estrogen receptor and thyroid hormone receptors (TRs) were isolated and described [20–25]. Today, 48 members of the nuclear receptor family have been identified in humans, 49 in mice, 21 in flies, and 270 in worms [26–28].

This work ultimately led to the current model of endocrine signaling wherein minute amounts of potent compounds are carried from their site of synthesis through the blood to mediate distal physiological changes. In the cases of interest to us here, these compounds are small, lipophilic molecules derived from cholesterol (the androgens of Aristotle's observations), highly modified amino acids (the thyroid hormones), or a variety of other greasy compounds. Nuclear receptors within the target tissues are the regulatory ambassadors in this endocrine diplomacy: they receive extracellular information in the form of their cognate hormone, bind to specific target genes, collaborate with coregulatory partners, and initiate phenotypic change by altering the regulation of a broad array of gene targets [10, 29, 30]. We now know that nuclear receptors have a pervasive reach into nearly all aspects of animal biology and play key roles not only in endocrine signaling but also in metabolic and xenobiotic sensing [31–33]. In humans, frogs, flies, and likely every other form of metazoan life, nuclear receptors are key regulators of development, growth, metabolism, reproduction, homeostasis, and circadian rhythm. A recent hierarchical clustering analysis based on nuclear receptor expression, function, and physiology organized the known mouse nuclear receptors into six distinct clades that span steroidogenesis, reproduction, development, metabolism, and energy homeostasis [34].

Not surprisingly, departures from this normal pathway of endocrine signaling in humans have the potential to wreak developmental or physiological disorder and can require medical intervention. In the day-to-day routine of the clinical endocrinologist, these departures are most commonly the consequence of too little or too much hormone production. Although we will touch on these hormone deficiencies and excesses, the main topic of this paper lies on the other side of the equation: mutations in the nuclear receptors that receive the hormone signals, rather than defects in the hormone signals *per se*. This paper will introduce thyroid hormone endocrinology and discuss how thyroid hormone receptors function as members of the larger nuclear receptor family. We will then discuss the role of TR signaling in human disease, with an emphasis on endocrine and neoplastic disorders.

2. Normal Thyroid Hormone Endocrinology

2.1. The Signal. In a healthy individual, thyroid hormone is produced in response to a cascade of signals originating in the hypothalamus, which synthesizes thyrotropin-releasing hormone (TRH) (Figure 1). TRH induces expression of thyroid-stimulating hormone (TSH) in the anterior pituitary, which induces, in turn, synthesis and release of T3/T4 thyronine by the follicular cells of the thyroid gland. T3 and T4 are the most abundant forms of thyroid hormone and are carried in the circulation chiefly as complexes with transthyretin, serum albumin, and thyroxine-binding globulin (TBG) [42, 43]. On arrival at a responsive cell, T3 and T4 are transported across the cell membrane primarily by monocarboxylate anion transporters 8 and 10 (MCT8 and MCT10) [44, 45] (Figure 1). T4 can be converted to T3 by deiodinase type 2 (DIO2) found in a variety of other responsive tissues [46]. Although both T3 and T4 can bind to, and modulate the activity of, intracellular TRs, T3 is considerably more active than T4, leading many to view the latter as a prohormone [46]. Deiodination of T3 or T4 on their inner ring by deiodinase type 3 (DIO3) leads to their inactivation. Interestingly, DIO1, a third deiodinase found primarily in the liver and kidney, can remove iodines from either the outer or inner ring and therefore can alternatively generate or inactivate T3 [46]. It should be noted that several metabolic derivatives of thyroid hormone can signal through membrane-associated G-protein coupled receptors such as TAAR1 [47]; however, the TRs appear to represent the key receptors for T3 and T4 and are the focus of the remainder of this paper.

2.2. The Receptor. Once in a target cell, T3 and T4 bind to the TR subfamily of nuclear receptors. In common with virtually all members of the nuclear receptor family, TRs are composed of a shared architecture consisting of an N-terminal (A/B) domain that contains binding sites for transcriptional coregulators, a central DNA binding domain C responsible for target gene recognition, an intervening "hinge" domain (D), and a C-terminal, hormone-binding domain (E/F) (Figure 2).

2.2.1. The "A/B" Domain. The "A/B" domains of the TRs recruit an assortment of coregulatory proteins that can participate in ligand-independent transcription regulation and/or modify the hormone-dependent transcriptional properties of the E/F domain (see below) [48–51]. This region is also a target of a variety of phosphorylation events that modulate TR function [52]. Interestingly, the (A/B) domain of many nuclear receptors appears to posses little inherent secondary or tertiary structure but is thought

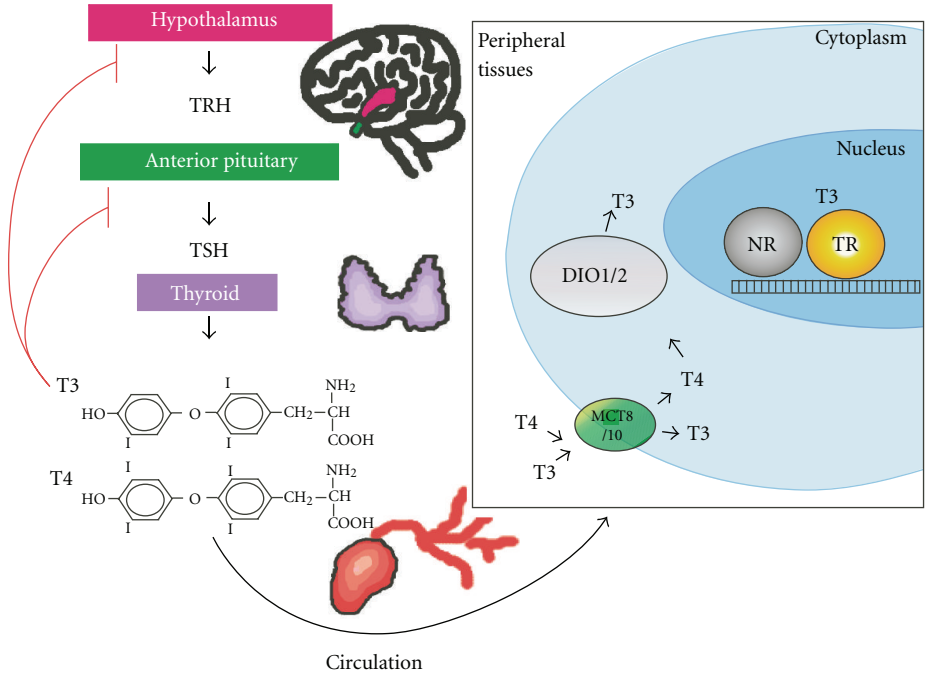

Figure 1: Regulation of thyroid hormone synthesis and activity. TRH is produced in the hypothalamus (shown in pink) and stimulates the anterior pituitary (shown in green) to create TSH, which stimulates the follicular cells of the thyroid gland (purple) to produce T3 and T4. T3 and T4 circulate through the blood to the peripheral tissues (see box at right), where they are transported across the cell membrane into the cytoplasm by MCT8/MCT10 (green oval). T4 can be converted to T3 by deiodinase type 1 and deiodinase type 2 (DIO1/2, gray sphere). Both T3 and T4 can enter the nucleus and regulate TR activity. TR is shown here as a yellow sphere bound to DNA. On most sites, TRs can dimerize, either as homodimers or as heterodimers, with another nuclear receptor partner (NR, dark gray sphere).

instead to assume more ordered conformations on interaction with other proteins; it has been suggested that this induced fit phenomenon allows the (A/B) domain to adapt to different coregulators and to different cellular environments [53–57].

2.2.2. The "C" Domain. The "C" domain in TRs, in common with virtually all other nuclear receptors, is comprised of two, highly conserved α-helical domains that are oriented and stabilized through interactions with coordinated zinc atoms [58–61]. The first α-helix tucks into the major groove of DNA and interacts intimately with a cognate hexanucleotide sequence on the DNA [62–64] (Figure 2). The most crucial base-specific contacts are made by the "P-box" amino acids within this first α-helix, and nuclear receptors with different P-box amino acids recognize different hexanucleotide sequences [65–67]. TRs possess an EGKG P-box and bind most tightly to consensus AGGTCA DNA sequences *in vitro* but can recognize a variety of variations on this theme; the presence of nonconsensus sequences in nature are likely to contribute to the specificity of target gene recognition by TRs *in vivo* [68].

The second α-helix in the "C" domain lies orthogonal to the first α-helix and stabilizes the receptor-DNA interaction through both direct and water-mediated contacts with the DNA phosphodiester backbone [60]. Amino acids within or flanking the second a-helix (the D-box) also can serve as a receptor dimerization interface [60, 69]. In fact TRs can bind to DNA as receptor monomers, homodimers, or heterodimers with retinoid X receptors (RXRs) or other members of the nuclear receptor family [70–74]. The best characterized TR DNA binding sites ("thyroid hormone response elements" or TREs) consist of two hexanucleotide sequences (half-sites) and bind a TR-TR or TR-RXR receptor dimer. The sequence, orientation, and spacing of the half-sites all contribute to proper TR recognition. In TRs, the second α-helix is followed by a short, flexible loop of amino acids and a third α-helix; this "C-terminal extension" helix both makes additional dimerization contacts and can contact the minor groove of the DNA, permitting recognition of an extended DNA sequence that includes bases 5′ to the historically defined hexanucleotide half-site [61, 75]. In addition to its role in DNA binding, the "C" domain also represents a docking surface for several known coregulatory proteins [76].

2.2.3. The "D" Domain. The "D" domain is thought to act as a flexible linker joining together the more conformationally and evolutionarily constrained "C" and "E/F" domains. TRs can recognize a surprising variety of half-site orientations, and the receptor "D" domain has been proposed to provide the rotational flexibility to accommodate the necessary twists and turns [70–74]. Consistent with this concept, different crystal structures of TR reveal different structural options for

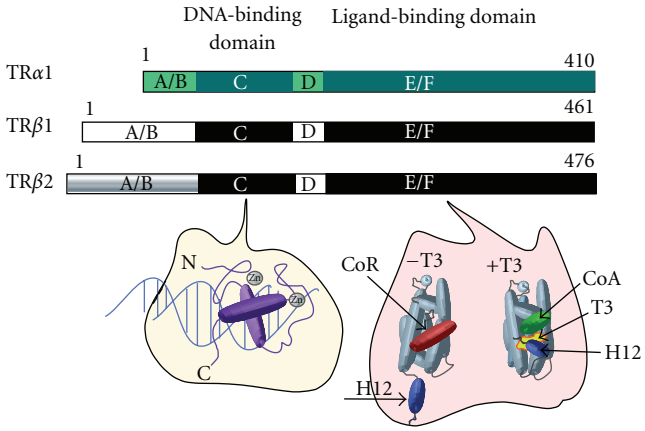

Figure 2: Domain comparison of different TR isoforms and schematic of DNA- and ligand-binding domain crystal structures. Each TR isoform is represented as a horizontal bar, from N to C termini. Total amino acid length is indicated at right [35, 36]. Within a given isoform, the location of each domain is lettered (A/B, C, D, and E/F). Identical domains of TRβ1 and TRβ2 are shown in matching colors. Note the unique A/B domain of TRβ2. Below left depicts the structure of the TR DNA-binding domain. α-helical domains are represented as purple cylinders and coordinating zinc atoms (Zn) as silver spheres. Below right depicts two conformations (−T3 and +T3) of the TR ligand-binding domain, which is composed of 12 α helices; the 12th helix (dark blue cylinder, labeled "H12") contains the ligand-dependent activation domain. In the −T3 conformation, helix 12 is in an extended position and the corepressor binding groove is filled with the CoRNR-box helical motifs found in SMRT and NCoR (red cylinder, labeled "CoR"). In the +T3 conformation, helix 12 has rotated to close around T3 hormone ligand (shown in yellow), and a novel docking surface for the LXXLL motifs of a transcriptional coactivator has formed (green cylinder, labeled "CoA").

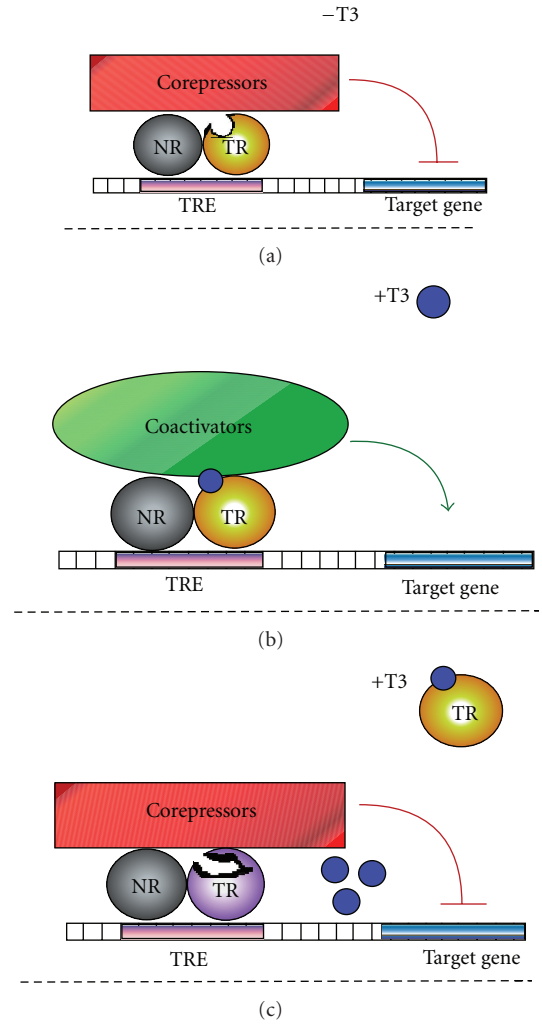

Figure 3: Transcriptional activity of wild-type and dominant-negative TRs. (a) In the absence of T3, wild-type TR (orange sphere plus a grey homo- or heterodimer partner) binds to thyroid hormone response elements (TREs-, shown as pink rectangle on DNA), recruits a cohort of corepressor proteins (shown as a red rectangle), and represses transcription of a given target gene (blue rectangle). (b) In the presence of T3 (dark blue sphere), wild-type TRs undergo a conformational change and exchange corepressor proteins for coactivators (green oval) to activate transcription of a target gene. (c) Dominant-negative TR mutants (shown here as a disfigured lavender sphere) have defects in hormone binding, corepressor release, or coactivator recruitment and consequently repress transcription even in the presence of hormone and other wild-type TRs.

the "D" domain, either a flexible loop or a short α-helix, as it exists from the "C" domain [77]. The "D" domain also possesses key nuclear localization motifs and can participate in recruitment of several regulatory proteins, either alone or in conjunction with the other nuclear receptor domains [77–80].

2.2.4. The "E/F" Domain. The "E/F" domain of TRs binds the thyroid hormone. It also forms a second receptor dimerization surface and is a major site of coregulator interaction (Figure 2). Although less than 35% sequence identity is conserved among the "E/F" domains of different nuclear receptors, structural analysis reveals a highly shared canonical architecture composed of a triple laminate of α-helices surrounding a variable-sized hollow pocket lined with hydrophobic residues (Figure 2) [81–88]. This pocket varies in size and shape for different nuclear receptors, thereby defining their ligand specificity. A C-terminal α-helix (denoted helix 12 or H12) exists from this triple helical stack and forms a short, pivoting structure that can adopt different conformations depending on presence and character of the hormone ligand. Binding to hormone induces a "mouse-trap mechanism" whereby portions of the "E/F" domain constrict around the hormone, and H12 swings shut to close off the pocket [81, 89].

These hormone-driven conformational changes are the principal means by which ligand regulates TR-mediated transcriptional regulation (Figure 3). For example, the TR "E/F" domain possess a hydrophobic surface groove composed of portions of helices H3, H4, and H5 [90]. In the absence of hormone, this surface groove can interact with

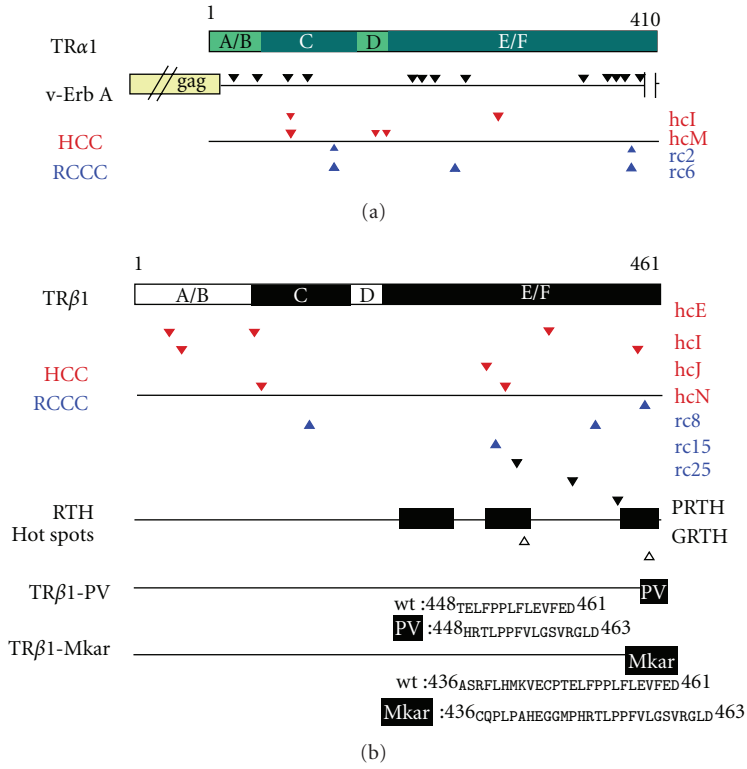

FIGURE 4: Oncogenic- and RTH-associated mutations in different TR isoforms. (a) A schematic of wild-type TRα1 is shown as a horizontal bar as in Figure 1; beneath, horizontal lines depict v-Erb A and several representative HCC/RCCC TRα1 mutants. As a result of fusion of retroviral gag-sequences, the N-terminus of v-erb A is 12 amino acids shorter than TRα1. V-erb A's 13 mutations are indicated by black arrowheads. From left to right, they are R24H, Y44C, G73S, K90T, K186R, P191L, P203L, K233N, T342S, P363S, T370A, C378Y, and F395S. A 9 amino-acid C-terminal deletion is indicated by vertical lines. All mutations and deletions are in relation to the avian TRα1 sequence [24]. Under the schematic for v-Erb A, red and blue arrowheads indicate mutations found in representative HCC and RCCC mutants, respectively, [37–39]. The nomenclature for each mutant is provided at the far right of the figure. For HCC, these mutants are hcI-TRα1 (K74E, A264V) and hcM-TRα1 (K74R, M150T, and E159K). For RCCC, these mutants are rc2-TRα1 (I116N and M388I) and rc6-TRα1 (I116N, A225T, and M388I). (b) A schematic of wild-type TRβ1 is shown as a horizontal bar as in Figure 1; beneath, horizontal lines depict several representative HCC/RCCC TRβ mutants, RTH hot spots, and the RTH mutant, TRβ1-PV. As above, red and blue arrowheads indicate representative mutations found in HCC and RCCC [37–39]. For HCC, these mutants are hcE-TRβ1 (M32I, C107R, and T368N), hcI-TRβ1 (S43L, C446R), hcJ-TRβ1 (M313I), and hcN-TRβ1 (K113N and T329P). For RCCC, these mutants are rc8-TRβ1 (F451S), rc15-TRβ1 (K155E, K411E), and rc25-TRβ1 (Y321H). Below the schematic for HCC/RCCC mutants, the locations of RTH hot spots are shown (amino acids 234–282, 310–353, and 429–460 [36]). Representative mutants for PRTH are: R338L, R383H, and R429Q. For GRTH, these mutations are G345S and P453S. The TRβ1-PV mutant has undergone a C-insertion at codon 448 that results in a frameshift at the C-terminus of the receptor [40]. The location of the 16 new PV-specific amino acids is indicated by a black box on the TRβ1-PV schematic, and the identities of these amino acids (and their wild-type TRβ1 counterparts) are shown below. The TRβ1-Mkar mutant has a T insertion at codon 436 that results in a frameshift at the C-terminus of the receptor. The locations of these new 28 amino acids are indicated by a black box on the TRβ1-Mkar schematic, and their identities are shown below. Note that Mkar shares with PV the amino acid sequence from codons 448 to 463 [41].

CoRNR-box helical motifs found in the SMRT and NCoR family of corepressors, resulting in recruitment of these corepressors. The corepressors, in turn, recruit deacetylases and additional histone modifiers that, by altering the chromatin template, lead to repression of transcription [91–94]. The reorientation of H12 that occurs in response to binding of hormone agonist occludes this corepressor docking surface, releasing corepressor and simultaneously forming a novel docking surface for the LXXLL motifs that are found in many transcriptional coactivators, such as SRC1 [90, 95–99] (Figure 2). These coactivators typically possesess associated histone acetyl and methyl transferase activities that, by appropriately modifying the chromatin, enhance transcription. Other coactivators include the Mediator complex (which helps recruit the general transcriptional machinery) and ATP-dependent chromatin remodelers (which regulate nucleosomal packaging). Differences in the shape and size of the hormone ligand can operate the H12 conformational toggle switch in different fashions; hormone antagonists, for example, induce H12 conformations that further stabilize

corepressor binding and/or destabilize coactivator binding [100–102].

Although this H12-driven mechanism by which TRs bind corepressors in the absence of hormone and release corepressors and bind coactivators on binding to T3 is the best worked out paradigm (Figure 3), a substantial number of genes are regulated by TRs in the inverse fashion (activated in the absence and repressed in the presence of T3) [103–105]. Additional genes appear to be constitutively regulated up or down by TRs in a hormone-independent manner [37]. The precise basis for this diversity in the transcriptional response is incompletely understood, but it presumably reflects mechanisms by which the nature of the DNA binding site, and/or the presence of additional transcription factors on the target gene, can alter coregulator recruitment or function. It should be noted that thyroid hormone receptors not only operate as transcription factors but also mediate nonnuclear effects by interacting with other proteins; although not the focus of this review, this aspect of TR function will arise again in our discussion of the TRβ-PV mutant (Figure 4) [106].

3. Diversification of Signal Reception: The TR Isoforms

TRs in humans are encoded by two distinct genetic loci: TRα on chromosome 17 and TRβ on chromosome 3. Alternative splicing and promoter usage produces additional diversity, leading to the synthesis of a series of TR "isoforms," the most studied of which are TRα1, TRβ1, and TRβ2 [35, 106] (Figure 2). All three bind T3 and can modulate expression of target genes in response to this hormone (not all splice variants do so; the TRα2 splice form, e.g., does not bind T3 and appears to mediate a hormone-independent mode of transcriptional regulation [35, 106]). Though virtually all cells express some form of TR, the ratios of the different isoforms vary in different tissue types and during development [106, 107]. TRα1 is expressed in the early stages of embryonic development, and is widely distributed, although particularly abundant in skeletal muscle and brown fat. TRβ1, in contrast, appears later in development and is present at the highest levels in the liver and kidney. TRβ2 is restricted to the pituitary, hypothalamus, sensory cells in the inner ear, and in the cone cells of the retina [35, 106–110].

TR knockout mice have helped delineate each isoform's role in thyroid hormone action. Mice missing the TRα1 isoform, for example, have cardiac abnormalities and lower body temperatures, whereas TR$\beta^{-/-}$ animals have hearing defects and a loss of negative feedback regulation of the hypothalamus/pituitary/thyroid axis (e.g., high T3/T4 and unsuppressed TSH and TRH levels) [111–114]. Of note, mice bearing genetic disruption of all TR isoforms also present with high circulating T3/T4 and unsuppressed TSH levels (apparently due to the loss of TRβ2 in the hypothalamus and pituitary) but otherwise display fewer systemic abnormalities than do the TRβ-specific isoform knockouts. Presumably the loss of the peripheral TRα1 and TRβ1 response in these combined knockout mice renders them resistant to the otherwise detrimental effects of their elevated T3/T4 levels [115, 116]. In fact, chemically or genetically induced hypothyroidism also presents as a much more severe syndrome than does the TRα/TRβ combined receptor knockout, indicating that the presence of unliganded TRs is more disruptive physiologically than is the complete lack of TR function. Taken as a whole, these genetic studies indicate that the different isoforms mediate both shared, and specific physiological and developmental functions and that TRs play major biological roles even in the absence of T3.

Although there appears to be significant overlap between the target genes regulated by the different TR isoforms, the detailed transcriptional response on a given gene can differ for each isoform [37, 117, 118]. For example, TRα1 can induce expression of certain genes more strongly than does TRβ1, whereas these isoforms confer nearly equal activity on other genes [37]. Similarly, TRβ2 fails to repress and instead activates certain genes under T3 conditions that confer repression by TRβ1 or TRα1 [50, 119–122]. These gene- and isoform-specific transcriptional responses are likely to reflect differences in the coregulatory factors that are recruited by each isoform once bound to a given target gene.

4. A Failed Response: TR Mutations and Resistance to Thyroid Hormone (RTH) Syndrome

Circulating T3/T4 levels are tightly controlled by a negative feedback loop wherein surges of thyroid hormone bind to TRs in the hypothalamus and pituitary, which then suppress TRH and TSH production and, as a consequence, repress further release of T3/T4 (Figure 1). Production of too much or too little thyroid hormone causes a number of clinically important endocrine disorders. In Graves' disease, for example, a hyperstimulated thyroid overproduces T3 leading to cardiac abnormalities, palpitations, fatigue, weight loss, dyspnea, myxedema, and muscle wasting [123, 124]. Conversely, insufficient T3 (hypothyroidism) produces depression, weight gain, edema, thickened speech, reduced cognition, cold intolerance, and, in a neonate, cretinism (a disorder marked by retarded physical and mental development) [124–126].

The consequences of over- or underproduction of circulating T3/T4 had been recognized for over a century when Refetoff et al., in 1967, reported an intriguing paradox in a study of two siblings with goiter, short stature, deafness, mutism, and bone deformations [127]. Although these symptoms shared several characteristics with hypothyroidism, both patients had high concentrations of thyroid hormone in the blood. Refetoff et al. suggested that the patients' tissues might be deficient in their ability to sense T3 and coined the phrase "Resistance to Thyroid Hormone (RTH) Syndrome: [127, 128]. This was soon confirmed, and, since then, RTH syndrome has been recognized as an autosomal dominant genetic disease that affects approximately 1 in 40,000 people worldwide [36, 129].

The vast majority of RTH cases have been traced to mutations in the TRβ isoform (Figure 4) [130–134]. As of 2010, at least 137 different RTH-TRβ mutations have been identified,

distributed among more than 300 families [36, 128, 135–138]. Despite this genetic diversity, virtually all of these RTH-TRβ mutations appear to share one key property: they encode mutant receptors that function as dominant-negative inhibitors of wild-type TR function [36] (Figure 3). RTH syndrome is, in fact, largely a disease of heterozygotes, and it is believed that RTH-TR mutant receptors interfere with normal T3 signaling by competing with the wild-type TRs expressed in the same cells from the unaffected TR alleles. Only two cases of patients homozygous/hemizygous for the TRβ mutation have been published: one was the product of a cousin marriage, and the other was born to a mother with goiter and a father of indeterminable genotype [139, 140].

RTH-TRβ mutants can interfere with both wt TRα1 and wt TRβ1 functions and are likely to mediate both isoform-specific and nonspecific effects *in vivo*, depending on the tissue and on the target gene. Interestingly, no RTH mutations have been mapped to TRα in humans, and, when TRβ RTH mutations are artificially targeted to TRα1 in mice, they do not produce RTH but generate instead a distinct slew of neoplastic and metabolic defects [141–145]. Although less frequently cataloged, and presenting with distinct symptoms, genetic defects in the MCT8 transporter, or in the incorporation of selenocysteines into the active sites of deiodinases, can also lead to defects in thyroid hormone signaling [36, 44]. This paper, however, will focus on RTH syndromes that arise due to lesions in the TRβ gene.

The genetic lesions responsible for RTH syndrome cluster in several "hot spots" mapping within the "D" and "E/F" domains of TRβ and result in defects in the hormone-driven release of corepressors and acquisition of coactivators (Figure 4) [79, 146–149]. In many cases, these mutations map to the hormone binding pocket and impair or eliminate the ability of the RTH-TRβ mutant to bind T3/T4 [36]. Although somewhat more rare, additional RTH mutants have been identified that retain a near wild-type affinity for T3/T4 but are defective in the conformational machinery that couples hormone binding to corepressor release and/or coactivator recruitment [150]. For example, proline 453 in TRβ1 is an important pivot on which H12 reorients in response to hormone agonist (Figure 4). Different amino acid substitutions at P453 have been identified in multiple human RTH syndrome kindreds; RTH-TR mutants bearing these substitutions retain significant T3 binding, but nonetheless exhibit defects in corepressor release, presumably due to a failure of H12 to properly reorient in response to bound hormone [151–155].

It is important to note that the symptoms of RTH syndrome are not identical to those of either a homozygous or heterozygous null mutation of TRβ. Instead it is the ability of the RTH syndrome TRβ mutants to function as dominant-negatives that plays a critical role in producing the disease phenotype. Is it the failure of the mutant TRβ to release corepressor, or to bind coactivator, that leads to this dominant-negative phenotype? In most RTH mutants tested, experimental inhibition of corepressor binding by biochemical or genetic manipulation reduces dominant-negative activity [150, 156]. Consistent with these findings, RTH patients with TR mutants that interact weakly with corepressors generally have more minimal disease symptoms than those with a strong corepressor interaction [157]. Nonetheless, a defect in coactivator binding (rather than in corepressor release) represents the primary defect in at least one RTH-TR mutant [147] and appears to contribute to the dominant-negative phenotype exerted by several other RTH-TR mutants (see Pituitary Resistance, below). It is also important to note that there are multiple forms of corepressor, and RTH mutants can display alterations in corepressor selectivity, rather than global defects in corepressor release. For example, NCoR and SMRT are closely related corepressor paralogs found in many cells. Wild-type TRs preferentially interact with NCoR, whereas the Mkar RTH mutant of TRβ (representing a C-terminal frame shift mutation), significantly reduces NCoR binding, but results in an increase in the SMRT interaction (Figure 4) [41]. NCoR and SMRT also undergo alternative mRNA splicing, and several RTH-TRβ mutants differ from wtTRβs in their ability to bind to these different corepressor splice variants [38, 158, 159]. This point will be addressed again in our discussion of oncogenic versions of TR (below).

5. Different Paths to Resistance: Generalized versus Pituitary RTH Disease

RTH has been divided clinically into two main subtypes, generalized (GRTH) versus pituitary (PRTH) [118, 130, 160–164]. GRTH is characterized by a broad insensitivity to thyroid hormone; as a result GRTH patients display some characteristics suggestive of hypothyroidism (e.g., short stature, goiter, and hearing impairments, reflecting an impaired T3 hormone response in peripheral tissues) but also have inappropriately high circulating levels of T3 and T4 and nonsuppressed TSH (a consequence of a loss of negative feedback in the hypothalamus/pituitary/thyroid gland axis) [35]. In essence, GRTH patients make more T3 and T4 than normal, but "do not know it," and present in some fashion as if they make too little. In contrast, in PRTH patients, negative feedback sensing in the hypothalamus/pituitary/thyroid gland is selectively impaired (resulting in high levels of circulated T3/T4), whereas the peripheral tissue response remains relatively intact (resulting in symptoms of hyperthyroidism, such as cardiac palpitations, heat intolerance, and nervousness) [35, 165, 166]. Thus, PRTH patients make too much T3 and T4, and "do know it," often to the point of peripheral thyrotoxicity.

These subtypes are not completely discrete: a given mutation can manifest as either GRTH or PRTH in different individuals, or within a given individual at different times [36]. Nonetheless certain RTH mutations are more often associated with one or the other form of disease, an observation that has been recently confirmed in a mouse knock-in model of PRTH syndrome [167]. Notably, the mutations most often associated with GRTH typically map to amino acid substitutions in the hormone binding or pivot/H12 domains of TRβ and can be explained conceptually through their potential to interfere with hormone

binding, corepressor release, or coactivator recruitment. In contrast, the most extensively characterized PRTH mutations map to a set of three arginines that form charged clusters on the surface of the TR "E" domain. In normal TRs, these arginines have been implicated in stabilizing the overall conformation of the "E/F" domain and also as important contacts in receptor homodimerization [168, 169].

Several explanations have been advanced for how PRTH mutations might impair T3 negative feedback in the hypothalamus/pituitary/thyroid axis while sparing the T3 response in the peripheral tissues. One proposal focuses on the observations that (a) TRβ1 forms homodimers more efficiently than does TRβ2, (b) TR homodimers recruit corepressors more efficiently than do TR/RXR heterodimers, and (c) many PRTH mutations impair homodimerization but retain the ability to form heterodimers with RXRs [94, 167, 170–177]. By this scenario, the diminished homodimerization properties of the PRTH mutants would favor TR-mediated activation over TR-mediated repression, resulting in a loss of repression of T3 synthesis in the hypothalamus and pituitary (producing increases in circulating T3 levels), yet enhancing T3-mediated positive gene regulation, resulting in the symptoms of peripheral thyrotoxicity characteristic of PRTH.

Alternatively, it is known that the hypothalamus and pituitary express primarily the TRβ2 splice form, whereas most peripheral tissues, such as liver, muscles, and kidneys, express primarily TRβ1 [35, 106, 178–183]. TRβ2 displays an enhanced ability to respond to T3 than does TRβ1, a phenomenon that may permit the hypothalamus and pituitary to sense, and suppress, surges of T3 before these elevated hormone levels saturate the more widely distributed TRβ1 isoforms [122, 184]. TRβ1 and TRβ2 share the same "C," "D," and "E/F" domains, and so RTH mutations are expressed as both splice forms. We have suggested that PRTH mutations have a more severe impact on the T3 response of TRβ2 compared to their impact on TRβ1, resulting in an increase in thyroid hormone levels (due to the impaired TRβ2-mediated negative feedback response in the hypothalamus/pituitary) while nonetheless conferring a thyrotoxic effect in peripheral tissues (mediated by the less-impaired TRβ1 splice form) [122]. As is most often the case with competing scientific theories, it is likely that both models play a role in the actual genesis of PRTH disease.

6. A Still Darker Side to Aberrant T3 Sensing: TRs and Their Mutations in Oncogenesis

In an ironic twist of history, TRs were linked to cancer before they were ever recognized as endocrine receptors. The avian erythroblastosis retrovirus (AEV) was first identified in 1935 as a retrovirus that could induce erythroleukemias and fibrosarcomas in infected chickens [185]. By the early 1980s it was realized that the oncogenic proclivities of AEV mapped to two viral oncogenes, v-Erb A and v-Erb B, that worked together to induce oncogenic transformation [186–188]. In 1986, v-Erb A was shown to be a retrovirally acquired, mutated version of avian TRα1 (Figure 4) [24, 25], establishing the precedent that mutated versions of TR can participate in the initiation or progression of oncogenesis. Mutated versions of TRs have been subsequently linked to hepatocellular carcinoma (HCC), renal clear cell carcinoma (RCCC), pituitary adenomas, and thyroid malignancies (Figure 4) [189–192]. Conversely, wt TRs can function as tumor suppressors, and loss of wt TR expression has been associated with these and other tumors [193]. We will discuss these malignancies in turn.

6.1. V-Erb A. Acutely transforming retroviruses cause neoplasia by acquiring, mutating, and inappropriately expressing host cell genes involved in the control of normal cell proliferation or differentiation. AEV represents a model by which two virally acquired cell genes, v-Erb A and v-Erb B, cooperate to induce neoplasia [187, 194–196]. V-Erb B is a mutated version of the avian epidermal growth factor (EGF) receptor, a cell surface tyrosine kinase that induces a cascade of mitogenic signals in response to extracellular EGF [188, 197]. Through loss of its extracellular regulatory and C-terminal domain, compounded by internal point mutations, v-Erb B has acquired a constitutive kinase activity that can induce proliferation of immature erythroid cells and fibroblasts even in the absence of EGF. V-Erb A is, as noted above, a mutated version of chicken TRα1. However, in contrast to the constitutive activation seen for v-Erb B, the mutations in v-Erb A have turned the latter into a constitutive repressor [198–201]. V-Erb A cooperates with v-Erb B in oncogenesis by suppressing differentiation of AEV-infected erythroid cells and by promoting the growth and life span of AEV-infected fibroblasts.

The basis of the dominant-negative activity of v-Erb A is obvious on inspection: the H12 helix toggle switch critical for corepressor release and coactivator recruitment by the wt TRα1 is deleted from the v-Erb A coding region (Figure 4) [24, 25]. In addition to this C-terminal deletion, v-Erb A has sustained a fusion at its N-terminus with sequences derived from the retroviral "gag" protein and 13 internal amino acid substitutions (Figure 4) [24, 25]. Several of these substitutions map to the hormone binding pocket, virtually abolishing the ability to bind T3 and further favoring corepressor over coactivator binding, whereas others map to the "A/B" and "C" domains.

Thus, in many ways, one would expect v-Erb A to operate as a particularly virulent version of an RTH mutant. Why then does v-Erb A function in neoplasia, whereas the RTH mutants induce primarily endocrine disorders? Neither the avian origin nor the TRα1 isoform backbone of v-Erb A fully explains this phenomenon. Instead, the acquisition of oncogenesis by v-Erb A appears to result in large part from changes in its DNA recognition domains. V-Erb A has sustained two amino acid substitutions within the P- and D-boxes of the "C" domain that play crucial roles in DNA binding specificity, as well as two additional amino acid substitutions in the "A/B" domain that can modify

DNA recognition by the adjacent "C" domain [202]. As a consequence, v-Erb A possesses an altered specificity for artificial DNA response elements *in vitro* compared to wt TRα1 and an altered target gene specificity in transfected cells [196, 203–207]. It is likely that the oncogenic properties of v-Erb A reflect these changes in DNA recognition, permitting the viral protein to target a distinct set of "neoplastic" genes that differ from the "endocrine" genes normally targeted by TRα1. These novel v-Erb A targets may include those regulated by other nuclear receptors (such as retinoic acid receptor), or by other, nonreceptor transcription factors [194]. Consistent with this proposal, replacement of portions of the "C" domain of v-Erb A with the corresponding wt TRα1 sequences severely inhibits oncogenic transformation by AEV [208]. It should be noted that these DNA binding domain mutations probably work together with the other mutations in v-Erb A that favor repression by deleting H12, inhibiting T3 binding, enhancing homodimer formation, and widening the ability of v-Erb A to bind to both SMRT and NCoR forms of corepressor [205].

6.2. Hepatocellular Carcinoma. The neoplastic properties of v-Erb A were viewed as an obscure tidbit of avian retrovirology exotica until eerily analogous TR mutants were discovered in a variety of human tumors. The first among these was human hepatocellular carcinoma (HCC). Worldwide, HCC ranks 5th out of all neoplasias for number of cases and third for number of deaths [209]. HCC can manifest as a medley of symptoms, including upper abdominal pain, weakness, weight loss, and jaundice [210]. Infection with hepatitis B or C virus is one of the major risk factors for HCC, along with cirrhosis, and exposure to aflatoxin, a highly mutagenic fungal compound often found in stores of contaminated grains or nuts [211].

Though the risk factors for HCC are known, the molecular mechanisms responsible for subsequent tumor initiation and progression are not fully understood. Alterations in a variety of tumor suppressors and oncogenes have been identified in HCC, as have a variety of chromosomal losses, gains, and translocations [212–216]. Most provocatively for the topic of this paper, however, is that TR mutants have been identified at high incidence in both HCC cell lines and in solid tumors [189, 217]. One study found that 65% of examined tumors had mutations in TRα and 76% had mutations in TRβ, with a significant subgroup of these tumors bearing mutations in both loci [189].

The HCC-TR mutants, when analyzed, resemble in many of their properties the RTH paradigm: they are impaired for transcriptional activation, many display defects in T3-driven corepressor release and/or coactivator binding, and the majority can function as dominant negative inhibitors of wild-type receptor activity in reporter gene assays (Figure 4) [39]. Unlike RTH syndrome, however, the TR mutations in HCC are not inherited, but instead arise *de novo* during the progression of the HCC tumors [189]. Also in stark contrast to RTH syndrome, the vast majority of HCC-TR mutants analyzed had sustained two or more genetic lesions, with at least one lesion located so as to impact DNA recognition (i.e., in the "A/B" or "C" domains). Indeed, two of the HCC-TR mutants studied were able to bind *in vitro* to DNA sequences not recognized by the wild-type receptors [39].

This suite of molecular defects suggested a potential role for these HCC-TR mutants in the mismanagement of transcription of genes not normally under T3 regulation. Gene expression analysis of hepatoma cell lines expressing specific HCC-TR mutants confirmed this supposition by demonstrating that these mutants regulate a distinct set of genes from that regulated by the corresponding wild-type receptors [37]. Analysis of the HCC-TR target gene set revealed several provocative features. A subset of genes normally regulated by wt TRs were not targeted by the HCC-TR mutants tested; conversely, the HCC-TR mutants regulated a panel of novel genes that were not targets of wt TR regulation. Several genes were targeted by each of the HCC-TR mutants, such as AGR2, DKK1, CDC7AL, and SLC2A2 and were repressed in both the absence and presence of hormone compared to the wild-type receptors [37]. Interestingly, HCC-TR target genes included not only genes that were constitutively repressed by the mutant receptors, as expected from prior reporter gene assays, but also genes that were constitutively activated, including GNG12, GPC3, and KCNAB2 [37]. At least several of these aberrantly regulated genes have been previously implicated in cancer [37]. Therefore, although the TR mutations associated with HCC appear to impede the ability of the receptor to respond to T3, they do not necessarily prevent the receptor from mediating hormone-independent transcriptional effects, both down and up.

Although the role of many of the HCC target genes in oncogenesis remains to be determined, it was notable that the HCC-TR mutants gained the ability to activate several genes known to play proproliferative roles (CSF1, NRCAM, and CX3CR1) and to repress several genes known to function as tumor suppressors (DKK1, TIMP3). Conversely, several potential proproliferative genes repressed by wt TRs were not repressed by the HCC-TR mutant (e.g., GPC3, expression of which has been linked to cell proliferation in liver), and several potential tumor suppressor genes activated by wt TR were not activated by the HCC-TR mutant (e.g., TIMP3) [37].

These findings further extended the conceptual model first put forward for v-Erb A: TR mutants associated with disease act, at least in part, as dominant-negative inhibitors of normal TR action. In the absence of any additional changes, these TR mutants can cause endocrine disorders such as RTH syndrome. Acquisition of yet-additional lesions that impact the DNA recognition domains of the receptor, as observed for v-Erb A and for the HCC-TR mutants described above, appears to unleash a previously cryptic oncogenic function in the TRs, permitting the mutant receptors to extend their regulatory reach to genes capable of contributing to leukemogenesis and hepatocellular carcinogenesis. Drawing this conceptual link between v-Erb A and the HCC-TR mutants tantalizingly closer, systemic expression of v-Erb A in transgenic mice under

a β-actin promoter results in a high incidence of HCC [218].

Given the evidence that multiply mutated TRs contribute to multiple neoplastic diseases, are there other forms of cancer in which TRs might play a role? To address this question, we next turn our discussion to renal clear cell carcinomas.

7. The Internist's Tumor: Renal Cell Carcinoma (RCCC)

RCC accounts for ~3% of all adult malignant diseases [219]. In men, it is the 7th most commonly occurring cancer; in women, it is the 9th [220]. Once known as "the internist's tumor" for its ability to produce an assortment of internal maladies and symptoms (flank pain, blood in the urine, fever, and palatable abdominal masses, to name a few), RCC actually encompasses a diverse assortment of tumor subtypes [221]. The most common of these subtypes (~75–80%) is of the clear cell variety and is abbreviated RCCC (or ccRCC) [219]. The name is derived from the appearance of the cytoplasm after histological prep of cancer tissue: high lipid content results in a clear solution [219]. Risk factors for RCC include tobacco use, high body mass index, and hypertension [219, 222–226]. Though methods of detecting renal tumors have improved in recent years, worldwide incidence and mortality rates are on the rise [220]. Metastatic RCC is highly resistant to conventional treatments (chemotherapy, radiation, and hormone therapy) and survival outcomes after diagnosis are typically less than one year [220, 227]. Though understanding the molecular basis of this disease has greatly advanced treatment options, therapy-refractory tumors typically develop 6–15 months after initial clinical intervention [228].

Approximately 80% of RCCCs bear inactivating mutations in the von Hippel Lindau gene (*VHL*) [229]. *VHL* encodes the targeting component of an E3 ubiquitin ligase complex, which marks the hypoxia inducible factor (HIF) for degradation. Normally, HIF functions as an oxygen-sensing transcription factor; under hypoxic conditions it activates an array of genes involved in the formation of new blood vessels [230–232]. When VHL is inactivated, HIF accumulates and proangiogenic factors are transcribed unchecked; this contributes to the highly vascular tumors characteristic of RCCC [233]. Additionally, VHL has been implicated in spindle misorientation and chromosome instability; a defective VHL protein may, therefore, drive formation of additional tumor-promoting mutations [234]. In RCCCs with this genetic root, one defective *VHL* allele is typically inherited, and the other is deleted or mutated somatically.

Although VHL inactivation is considered the predominant molecular change associated with development of RCCC, it alone is not sufficient to cause cancer in mice [235, 236]. It is likely that VHL inactivation serves as the first step towards tumorigenesis and that additional steps, or "hits," are required for tumor progression [237]. In fact, an intriguing diversity of TR mutations, deletions, and aberrant mRNA expression patterns have been observed in RCCC. For example, an analysis of 71 RCCC tumors found characteristic deletions at 3p26 and 3p24, which are home to *VHL* and *TR*β, respectively [238]. Analysis of TR mRNA expression in RCCC tumor tissues revealed a significant reduction of TRβ mRNA in the majority of samples tested (although paradoxically, TRβ mRNA was overexpressed in several samples) [239]. Reduction of TRα mRNA was also observed in several RCCC tumors, although complete loss of the TRα locus on chromosome 17 was rare [238–240]. And, of greatest relevance to the topic of this paper, mutations in both TR isoforms have been identified in ~40% of RCCC tumors examined, in TRα, TRβ, or both [190]. It is therefore likely that defects in TR function can serve as a 2nd hit that triggers, or participates in, the transition from renal cyst to clear cell carcinoma.

Ten different RCCC-TR mutants have been studied in molecular detail [190]. In common with HCC, the majority of these RCCC mutants contain more than one genetic lesion each, with at least one or more of these lesions frequently mapping to the "A/B" or "C" domains; nonetheless, no two identical TR mutations have been isolated to date from the two different forms of neoplasia. The majority of the RCCC-TR mutants tested display hormone binding and coregulator release/acquisition defects *in vitro* and can function as dominant negatives in reporter gene assays (Figure 4) [38]. Several RCCC-TR mutants also display a gain in their specificity for certain splice forms of SMRT and NCoR compared to the wild-type receptor [38]. The multiple genetic lesions carried by a given mutant receptor can work together to contribute to the overall dominant-negative phenotype [38].

Do the mutations in the "A/B" and "C" domains of the RCCC-TR mutants alter their DNA specificity? Consistent with this idea, nuclear extracts from RCCC tumors were found to be impaired in their ability to bind to consensus TREs compared to extracts from wt tissues [190]. Expression array analyses of cells stably transfected with RCCC mutant receptors are in progress to determine if there are changes in target gene specificity (Rosen, Chan, and Privalsky unpublished observations).

8. Thyroid Neoplasia

A third example of an association of a human neoplasia with mutations in the TR loci was revealed by studies of papillary thyroid malignancies. Almost 63% of these malignancies were found to have mutations in TRα, and a remarkable 94% in TRβ; in contrast 22% and 11% of thyroid adenomas bore mutations in these isoforms, respectively, and no mutations were found in normal thyroid controls [191, 241]. This pattern is most consistent with a role of the TR mutants in cancer progression, rather than initiation. Further analysis demonstrated that the majority of these mutated TRs lost transcriptional activation function and displayed dominant-negative activity when coexpressed with their normal TR counterparts [191, 241]. Many, but not all, of these mutants contained multiple genetic lesions, with one tumor possessing 5 different lesions within a single TRβ1 allele and another possessing 6 in TRα1

and 2 in TRβ1 [191]. In many of these mutants, lesions included at least one mutation within the "A/B" or "C" domains. The effects of these mutations on DNA binding *in vitro*, or target gene specificity in cells, have not been reported.

9. Potential Cracks in the Wall Separating RTH Syndrome from HCC, RCC, and Thyroid Malignancy

The narrative to this point may have led the unwary reader to the conclusion that the absence or presence of DNA binding domain mutations determines if a given dominant-negative TR mutant induces endocrine or neoplastic disease. However, there is some evidence that this phenomenon may not be absolute. Although not associated with overt neoplasia, RTH-TR mutations in humans often lead to goiter, a nonneoplastic hyperplasia of the thyroid gland in response to the loss of T3/T4 feedback regulation. Further, a very strong dominant-negative RTH-TRβ mutant, denoted PV and representing a frameshift at the C-terminus of the receptor, causes not only severe disruption of the pituitary-thyroid axis and goiter, but also TSH-omas, and metastatic follicular thyroid carcinoma in homozygous-mutant mice [40, 242–245]. The "A/B" and "C" domains of the PV mutant are fully wild type in sequence (Figure 4), suggesting that strong, dominant-negative RTH-TR mutants may have an inherent oncogenic potential that is rarely displayed in humans (where homozygosity for the RTH mutation is very unusual) but can uncloak when presented with an appropriate opportunity.

Subsequent analysis of the PV/PV mutant mice revealed several mechanisms by which the mutant receptor appears to be mediating oncogenesis; significantly, none of these involved the classic mode of direct binding of the TR mutant receptors to DNA [163]. The PV mutant was found to heterodimerize with, and inhibit, another member of the nuclear receptor family, peroxisome proliferator-activated receptor-γ, removing an antiproliferative signal [246, 247]. Many nuclear receptors exert nongenomic functions outside of the nucleus, and the PV mutant also induced one of these: the phosphatidyl-inositol 3 kinase/AKT pathway [248, 249]. The PV mutant also makes protein-protein interactions with β-catenin and pituitary tumor transforming gene protein, increasing levels of these proteins by inhibiting their degradation [249–252]. Finally, through protein-protein interactions with the CREB transcription factor, the PV-TRβ mutant was able to induce cyclin D1 [245]. These PV-TR studies raise the possibility that similar TR signaling pathways, unrelated to DNA recognition *per se*, may also play a role in HCC-TR and RCCC-TR oncogenesis.

10. Ups and Downs of Wild-Type TR Expression in Oncogenesis

The impact of TRs on neoplasia is not restricted to scenarios involving receptor mutants. Wild-type TRs can act as tumor suppressors in many contexts, and losses in wild-type receptor expression appear to precipitate, or otherwise contribute to, several classes of neoplasia. For example, a double knockout of both TRα and TRβ in mice results in a higher incidence of follicular thyroid carcinoma and increased aggressiveness in a skin cancer model [253, 254]. Changes in TRα1 levels have been shown in 49% of human gastric cancers analyzed by immunoblotting [193]. Reduction in TRβ1 levels or changes in subcellular localization have been reported in colorectal cancers [255]. In several cases these changes in TR expression levels were associated with alterations in the restriction pattern of the TR gene, suggesting that loss of expression might reflect an underlying genetic event. In other cases, TR expression appears to be suppressed epigenetically by hypermethylation of the promoter region of the TR gene; for example, biallelic inactivation of TRβ expression by promoter methylation has been found in human breast cancers [192, 256]. Notably, reintroduction of wild-type TRβ into HCC or mammary carcinoma cell lines that have lost endogenous TR expression retards proliferation, results in partial mesenchymal to epithelial transitions, and suppresses invasiveness, extravasation, and metastasis in nude mice [253, 257].

11. Thyroid Hormone Status and Cancer

As noted above, changes in TR expression and function are associated with a wide variety of neoplastic events. Can changes in thyroid hormone levels exert similar effects? Answering this question has proven to be complex and somewhat contentious. In clinical studies, hypothyroidism has been reported to correlate with a lower risk of primary mammary carcinoma and a reduction in progression to invasive disease [258]. Pharmacologically induced hypothyroidism has similarly been reported to yield an improved survival in glioblastoma when used together with tamoxifen [259]. Consistent with hypothyroidism being beneficial, T3 has been reported to induce the proliferation and invasiveness of several types of tumor-derived cells in culture or in xenograft models, including HCC-derived cells [253].

In contrast, however, other studies indicate that low thyroid hormone levels increase the risk of HCC in humans, and high T3/T4 are therapeutic [260]. Dating back to the late 18th century, administration of thyroid extract was often used in conjunction with oophorectomy as a treatment for breast cancer [261–263] though its efficacy was not well established [264]. More recently, T3, operating through TRβ1, has been shown to retard the proliferation, anchorage-independent growth, and invasiveness of mammary cancer cells in culture [265]. Similarly, long-term hypothyroidism in women has been associated with an elevated risk of HCC [266], whereas T3 administration can reduce HCC progression in animal studies [267], and T4 has shown some success in reducing the risk of colorectal cancer [268].

Clearly "results may vary!" It is likely that the impact of thyroid hormone differs in different types of cancer and may control different aspects of the same cancer (proliferation, differentiation, invasion, metastasis, apoptosis, and

senescence) differently. For example, the investigators that have shown T3 to be promitogenic in rodent liver have also shown that T3 suppresses formation of preneoplastic nodules in a diethylnitrosamine rat model of HCC; T3 is therefore likely to be exerting both proproliferative and prodifferentiation effects on liver [269]. It is worth noting that T3 also induces both differentiation and proliferation in several other contexts, such as the gut [270]. As is virtually always true in science, more studies will be required to fully reveal all of the intricate web of biological processes regulated by T3 and its receptors.

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