MINIREVIEW

Amphibian metamorphosis as a model for studying the developmental actions of thyroid hormone

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ABSTRACT

The thyroid hormones L-thyroxine and triiodo-L-thyronine have profound effects on postembryonic development of most vertebrates. Analysis of their action in mammals is vitiated by the exposure of the developing foetus to a number of maternal factors which do not allow one to specifically define the role of thyroid hormone (TH) or that of other hormones and factors that modulate its action. Amphibian metamorphosis is obligatorily dependent on TH which can initiate all the diverse physiological manifestations of this postembryonic developmental process (morphogenesis, cell death, re-structuring, etc.) in free-living embryos and larvae of most anurans. This article will first describe the salient features of metamorphosis and its control by TH and other hormones. Emphasis will be laid on the key role played by TH receptor (TR), in particular the phenomenon of TR gene autoinduction, in initiating the developmental action of TH. Finally, it will be argued that the findings on the control of amphibian metamorphosis enhance our understanding of the regulation of postembryonic development by TH in other vertebrate species.

Key words: *Thyroid hormone, metamorphosis, postembryonic development, thyroid hormone receptor, autoinduction.*

INTRODUCTION

Well before the chemical identification of L-thyroxine (T_4) and 3, 3' 5-triiodo-L-thyronine (T_3) as thyroid hormones, the secretions of thyroid gland were known to regulate growth and development in a variety of vertebrates[1, 2]. The studies of Evans and colleagues in 1949 on the restoration of growth and maturation of the skeletal system in thyroidectomized and hypophysectomized neonatal rats established experimentally the growth and developmental action of T_4 and its interaction with growth hormone. The demonstration that T_4 is converted intracellularly to T_3 , which is considered to be the active form of thyroid hormone (TH), has made it easier to follow the biochemical and molecular events that underlie the cellular actions of thyroid hormone[2, 3, 5]. Observations on goitre, abnormal development and mental retardation in young children had indicated over two centuries ago the close relationship between thyroid hormone in foetal and prenatal human development and, especially, neuronal maturation[2, 4, 5]. As knowledge of the physiological and biochemical actions of thyroid hormone in different species and tissues progressed, a major characteristic of this hormone emerged, namely the multiplicity of its actions. The rapid advances in gene technology in the last two decades have revealed that thyroid hormone receptors (TRs) are highly conserved and it is generally accepted that the primary intracellular event is the interaction between TR and its ligand in the cell nucleus and that the diversity of actions of TH is generated by species- and tissue-specific factors and mechanisms[6-8]. Whereas the developmental, growth-promoting and metabolic actions of TH in postnatal and adult mammals can be analyzed experimentally with some degree of precision, the same is not possible for determining the role of the hormone in postembryonic and foetal development. The major reason for this obstacle lies in the transplacental transfer of TH to the developing mammalian organism and the multiple and complex interplay between thyroid and other hormones and growth factors during intrauterine development[9]. It has therefore become necessary to examine the developmental actions of TH in organisms in which fertilization occurs externally and the embryos are free-living. The obligatory requirement of thyroid hormone for amphibian metamorphosis, and its many similarities with mammalian postembryonic development, therefore offers a unique opportunity to explore its developmental actions in vertebrates[10, 11, 53]. In this article I shall first describe the salient features of amphibian metamorphosis, followed by the role of TH in regulating morphogenesis, gene and biochemical re-programming and cell death and, finally, the significance of TR gene expression underlying these processes.

Amphibian metamorphosis

During the course of his studies on the nutritional requirements for the growth of frog tadpoles, Gudernatsch[12] found that some of the larvae that had been fed on extracts of mammalian thyroid glands underwent precocious metamorphosis spon-

taneously[12]. Detailed analysis later of the functional role of the developing larval thyroid gland, and the availability of pure T_4 , confirmed that the process of metamorphosis was obligatorily dependent on thyroid hormone. By the 1950s much evidence had accumulated to show that the hormone provokes diverse and multiple morphological, physiological and biochemical responses in virtually every tissue of the amphibian tadpole[10, 13, 14]. Tab 1 lists some of the important responses to TH in different tissues of the pre-metamorphic tadpole, which range for example from *de novo* morphogenesis, re-patterning, functional re-programming and partial and total regression of most tissues. Before extending this article to the biochemical and molecular mechanisms underlying the developmental actions of thyroid hormone, it is useful to briefly consider the special characteristics of the role of TH in amphibian metamorphosis.

Absolute requirement for TH

Surgical, chemical or radiation-induced removal or ablation of the thyroid gland of an early tadpole will permanently prevent metamorphosis. The thyroidectomized larva will continue to grow (under the influence of growth hormone and other growth-promoting factors) without it acquiring the adult phenotype. Administration of T_4 or T_3 at any stage after thyroidectomy will cause the resumption of the arrested differentiation and lead to metamorphosis[14]. This finding clearly establishes the obligatory requirement of TH, as well as indicating that the genetic programme for postembryonic development is stable and not determined temporally. A systematic study in which different stages of *Xenopus* embryos and tadpoles were exposed to exogenous T_3 , and a number of indices of morphological and biochemical responses measured, showed that the competence was established well before the tadpole's thyroid gland had developed functionally[15]. This finding is of particular relevance in understanding the role of TH receptor since it means that TR is expressed constitutively early in development.

Diversity of genetic programmes under TH control

Tab 1 illustrates that no two tissues of the amphibian larva exhibit the same response to TH. Some of the responses are the manifestation of a new genetic programme activated by the hormone, whereas others represent the acceleration or slowing down of the expression of a programme that had already been initiated before the action of the hormone[16]. What is of particular interest are the direct response genes, namely those whose transcription is initiated in the presence of inhibitors of protein synthesis. Using a "gene screen" procedure, Brown, Shi and their colleagues have been able to classify genes in *Xenopus* tadpoles which are up- or down-regulated by T_3 and those that are direct response genes[17, 18]. Most direct response genes are up-regulated during metamorphosis, even in tissues programmed for total tissue regression.

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Tab 1. Diversity of morphological and biochemical responses to thyroid hormone during amphibian metamorphosis

Tissue	Response	
	Morphological	Biochemical
Brain	Re-structuring; axon guidance and growth; cell turnover	Cell division; apoptosis; protein synthesis
Liver	Functional differentiation; re-structuring	Induction of albumin and urea cycle enzymes; larval adult haemoglobin switch
Eye	Re-positioning; new retinal neurones; altered lens	Visual pigment switch; induction of β -crystallin
Skin	Re-structuring; keratinisation; granular gland formation	Induction of collagen, 63 kDa keratin, magainin
Limb bud, lung	<i>De novo</i> morphogenesis of bone, skin, muscle, nerve, etc.	Cell proliferation; gene expression
Tail, gills	Total tissue regression and removal	Programmed cell death; induction of lytic enzymes
Intestine, pancreas	Major re-modelling of tissues	New structural and functional constituents
Immune system	Re-distribution of immune cell populations	Acquisition of new immunocompetence
Muscle	Growth, differentiation, apoptosis	Induction of myosin heavy chain

TH acts directly and locally

Transplantation studies with early tadpole tissues had already indicated that changing the location of tissue or organ did not alter its response later on to endogenous or exogenous TH[14], thus proving that the hormone does not determine the developmental programme but only initiates it. More convincing evidence for a non-systemic action of TH comes from tissue culture experiments which show that the hormone acts directly on each target tissue to set into motion the same predetermined genetic programme as in whole tadpoles. This has been demonstrated for organ cultures of larval skin, liver, limb buds, intestine and tail[19, 20]. In a study in which the tail and limb buds from the same tadpole were cultured in parallel, T_3 induced morphogenesis in one tissue but cell death in the other[19], thus demonstrating that the hormone initiates diametrically opposite developmental changes in these two tissues.

Fig 1. Schematic representation of the hormonal regulation of amphibian metamorphosis. In response to environmental cues, the dormant thyroid gland of the tadpole is activated to produce the thyroid hormones T_4 and T_3 by the hypothalamic and pituitary hormones TRH, CRF and TSH. Thyroid hormone (TH) is obligatorily required to initiate and maintain the metamorphosis, its action being potentiated by glucocorticoid hormone and retarded by prolactin. Nutr., nutritional factors; TRH, thyrotrophin-releasing hormone; CRF, corticotrophin-releasing factor; TSH, thyroid stimulating hormone; T_4 , L-thyroxine; T_3 , triiodo-L-thyronine; GC, glucocorticoid hormone.

TH action can be modulated by other hormones

Although the culture studies confirmed that TH acts directly on its target tissues, it is also known that other hormones, factors released by neighbouring tissues or environmentally transmitted signals can modify the response of a given tissue to TH [10, 11]. Among important endocrine factors are glucocorticoid hormone, CRF (corticotrophin-releasing hormone) and prolactin (PRL) (see Fig 1). Exogenous glucocorticoids, ACTH and CRF are known to potentiate, while PRL blocks both natural and TH-induced metamorphosis [21, 22]. These hormones will also accel-

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erate or inhibit the action of TH in cultures of individual tissues. Although the interplay between TH and PRL and glucocorticoids and the cellular and molecular mechanisms of this interplay are poorly understood, the modulation of TH action by glucocorticoids and PRL has provided a useful tool in analyzing the action of TH, especially in organ cultures of tadpole tissues.

Programmed cell death is an important feature of amphibian metamorphosis

A major characteristic of metamorphosis is the restructuring and further differentiation of tissues such as the brain, limb buds, intestine and pancreas[16, 23]. The first studies on isolated tadpole tails in culture and the use of inhibitors of protein synthesis demonstrated that cell death is dependent on the de novo synthesis of new proteins[24]. This requirement for new protein synthesis for the onset of apoptosis is a common feature of programmed cell death during development. With the recent advances in the characterization of genes encoding cell death and cell survival proteins, the identification of hormonally regulated "early" proteins responsible for initiating apoptosis is now a most interesting prospect.

Thyroid hormone receptors

Thyroid hormone receptors are the key to understanding how the hormone controls its diverse metabolic and developmental functions, including metamorphosis. Although transport across the cell membrane and interactions with cytoplasmic proteins determine the dynamics of TH distribution within the target cell, it is the interaction with TRs in the cell nucleus which is the crucial step that initiates the molecular and biochemical progression leading to the physiological response to the hormone.

General characteristics of thyroid hormone receptors

TRs are members of an evolutionarily highly conserved supergene family of steroid/thyroid hormone/retinoid nuclear receptors that function as ligand-inducible transcription factors[6-8, 25-27]. In all vertebrates examined so far they are encoded by two genes termed TR α and β from which are generated multiple isoforms according to the tissue and species. The modular structure of nuclear receptors comprising the N-terminus, DNA-binding and ligand-binding domains is now well-known. TRs belong to the subgroup that includes nuclear receptors for retinoic and 9-cis retinoic acids (RARs and RXRs, respectively), vitamin D3 (VDR) and peroxisome proliferators (PPAR)[7]. Unliganded TRs are constitutively located in the nucleus as components of chromatin and, unlike steroid receptors, the ligand is not involved in their translocation from the cytoplasm into the nucleus[27]. It is generally accepted that liganded TRs activate the transcription of their target genes by interacting with thyroid response elements (TREs) in their promoters, the most common motif being AGGTCAAnnnnAGGTCA and known as direct repeat plus 4 (DR+4)[7, 25].

Interestingly, the same direct repeat separated by 1, 2, 3 or 5 nucleotides acts as response elements for RXR, PPAR, RAR and VDR, respectively. The mechanism by which TR regulates transcription is not fully understood, but three important features of the receptor are relevant. First, unlike other nuclear receptors, unliganded TR acts as a strong repressor, the repression being relieved by the ligand[7, 28, 29]. Second, although TR monomer and homodimer can interact with TRE, the physiologically active form is the heterodimer formed with RXR, a property also shared by other members of its subgroup of nuclear receptors[6, 7, 25]. Third, two groups of proteins that have recently been identified as co-repressors (CoR) or co-activators (CoAc) are thought to be essential for the transactivation function of the receptor[7, 29, 30]. The role of hormone binding to the ligand-binding domain of TR would be to cause the dissociation of CoR from its inactive complex with the receptor while at the same time facilitating the recruitment of CoAc to form a transactivational complex[7]. Also recently, much interest has been generated by the finding that co-repressors and co-activators have histone acetylase or deacetylase activities[31, 32]. It will not be surprising that more components participating in such complexes and structurally organized as chromatin[33, 34] are discovered in the near future. These considerations are important for understanding the role of TH in regulating amphibian metamorphosis, since it is quite likely that different combinations of nuclear receptors, transcription factors, co-repressors, co-activators and other chromatin constituents may determine the diversity of tissue-specific manifestation of differential gene expression programme seen in amphibian metamorphosis (see Tab 1).

Developmental regulation of amphibian TRs

In *Xenopus* tadpoles normal metamorphosis does not begin until the larval thyroid gland becomes functional, which can be about 6 weeks after fertilization. The onset and rapid acceleration of metamorphosis correlates well with the build-up of circulating T_3 [16, 35]. The fact that competence to respond to exogenous TH is established as early as 1 week after fertilization[15] means that functional TR is present well before the secretion of the hormone. Hence it was not surprising that both TR α and β mRNA and protein can be detected in most tissues of the early premetamorphic larvae[22, 36-40], indicating the constitutive expression of TRs at low levels. There is also strong correlation between the accumulation of TR mRNA and protein and the rising levels of TH. Upon completion of metamorphosis the concentrations of all three constituents decline very sharply. This correlation raised the question as to whether the hormone itself regulates the expression of its own receptor genes.

Autoregulation of TRs during metamorphosis

Biochemical, in situ hybridisation and immunocytochemical analyses of TR mR-

NAs and proteins have clearly shown that exogenous TH can precociously upregulate TR gene expression[36-41]. The autoinduction of TR correlates well with the increased sensitivity of *Xenopus* tadpoles to T_3 as development progresses and the process occurs in all tissues irrespective of whether or not they undergo de novo morphogenesis, total regression or re-structuring. The upregulation of TR genes is among the most rapid responses to TH in amphibia, is more marked for the β than the α gene and is the result of direct activation of their transcription[16, 17, 22, 35]. It can also be reproduced in *Xenopus* cell lines with similar kinetics to those seen in whole tadpoles[35, 42, 43]. The advantage of studying receptor autoinduction in tissue culture is not only a greater precision of establishing its kinetics but it also allows one to investigate the mechanism of the process by DNA transfection.

In order to determine if TR interacts with its own gene, it was first necessary to identify TRE sequences in the promoter of the amphibian TR genes. The promoter of *Xenopus* TR gene has been cloned[44, 45] and several regulatory elements identified, including two DR+4 sites upstream of the transcription initiation site[45]. Mutational analysis and transfection of two *Xenopus* cell lines (XTC-2 and XL-177) with promoter constructs showed that both sites responded to T_3 . Furthermore, overexpression of unliganded TRs reduced the basal transcriptional activities of these cells which was overcome by T_3 , as in mammalian cells[7, 28, 29]. Using different isoforms of recombinant TRs and RXRs in electrophoretic mobility shift assays Machuca and collaborators[45] were able to demonstrate that TR-RXR heterodimers, but not TR monomers or homodimers, specifically interacted with both TREs in the promoter of the *Xenopus* TR β gene, thus suggesting that the heterodimer could regulate the transcription of TR.

Further evidence that TRs could act directly on the promoters of their own genes has come from studies on dominant-negative (d-n) TRs in whole *Xenopus* tadpole tissues and cell lines[46]. A large number of d-n TR β 1 mutant receptors in man, associated with the syndrome of generalized thyroid hormone resistance, have been shown to bind TREs, but not TH, and inhibit transactivation by wild-type TRs in a dominant-negative manner[7, 16, 25, 47]. It is therefore significant that human mutant TR β s and a synthetic mutant *Xenopus* TR β were able to inhibit autoinduction of wild-type TR β when transfected into XTC-2 cells[46]. The strength of the dominant-negative effect of the mutant TRs correlated well with heterodimerization with RXR and the binding of the heterodimers with various TREs. More importantly, transfection of tadpole tail muscle in vivo[48] showed that d-n mutant TRs prevented wild-type TR β autoinduction in premetamorphic *Xenopus* tadpole tissues. These results now lead to the important question of how relevant is the autoinduction of TR to the process of metamorphosis itself. While awaiting the outcome of experiments designed to answer the above question there is good indirect evidence from two separate observations of an intimate relationship between TR gene expression and the regulation of amphibian metamorphosis by thyroid hormone. First, there is a good correlation between the inhibition and potentiation of

metamorphosis by PRL and glucocorticoid, respectively, and the inhibition or enhancement of autoinduction of TR and RXR genes in several tadpole tissues during natural or T_3 -induced metamorphosis[19, 39, 41, 49]. This correlation is particularly marked for the antagonism between TH and PRL, as can be discerned from Tab 2. PRL blocks the upregulation of both TR α and β mRNAs induced by T_3 , but does not affect the constitutive expression of the receptor genes, as determined by different analytical procedures in whole premetamorphic tadpoles and in organ cultures. The conclusion that TR autoinduction is necessary for the activation of downstream TR target genes is supported by the inhibition by PRL of the activation of the downstream genes encoding albumin, 63 kDa keratin and stromelysin-3 genes[39, 49]. Interestingly, the action of PRL is specific for TR since T_3 lowered the accumulation of RXR mRNA but PRL did not block this action of T_3 as it does for TRs. On the other hand, the synthetic glucocorticoid dexamethasone which potentiates T_3 -induced metamorphosis elevates the levels of both TR and RXR mRNAs but PRL would only suppress that of TR when all three hormones are added to organ cultures of premetamorphic *Xenopus* tadpole tails[49]. The mechanism of the anti-metamorphic action of PRL in amphibia remains unknown, although recently some progress has been made in elucidating the molecular action of this hormone in mammals[50].

Tab 2. Relative accumulation of TR α and β mRNAs in pre-metamorphic *Xenopus* tadpoles treated with T_3 and prolactin (PRL)

Treatment	Relative Units	
	TR α	TR β
None	505	24
T_3	1290	368
T_3 + PRL	799	10
PRL	405	43

Batches of 20 stage 50 *Xenopus* tadpoles were treated with 2×10^{-9} M T_3 with or without 0.1 iu PRL/ml for 4 d before relative amounts of TR mRNAs were measured in total larval RNA by RNase protection assay. See ref. [39] for details.

Another indirect indication that the autoinduction of TR genes is closely linked to amphibian metamorphosis comes from comparative studies of TR in neotenic amphibia, i.e. those that do not undergo metamorphosis spontaneously[35, 36, 51]. Facultatively neotenic amphibia such as the mexican axolotl or the tiger salamander (*Ambystoma*), which do not go through metamorphosis normally, will do so if exogenous TH is given, while obligatory neotenic amphibia such as *Necturus* and *Proteus* do not respond to TH. As can be seen in Tab 3, low levels of TR mRNAs can be detected in *Ambystoma* tissues which can be upregulated by the administration of T_3 in parallel with a partial metamorphic response (loss of tail fin, growth of limbs, excretion of nitrogen as urea), as in *Xenopus*. In contrast, only TR α transcripts

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could be detected in tissues of *Necturus* in which T_3 failed to upregulate the expression of TR α or β mRNA.

Amphibian metamorphosis and mammalian postembryonic development

Unlike the abrupt changes in response to thyroid hormone that occur during amphibian metamorphosis, mammalian postembryonic or foetal development is characterized by a relatively slow and continuous progression of developmental changes during which the foetus is exposed to multiple hormonal and other developmental signals of both maternal and foetal origin[2, 9, 52, 53]. Nevertheless, there are some remarkable similarities between the developmental changes induced by thyroid hormone during metamorphosis (Tab 1) and those seen during intrauterine and perinatal development of mammals (Tab 4). Good examples of the similarities are the switch from larval or foetal to adult haemoglobin, induction of albumin genes, skin keratinization, bone and limb maturation and the appearance of urea cycle enzymes.

Tab 3. Association between TR autoinduction and response to thyroid hormones (T_3 , T_4) of spontaneously metamorphosing and facultatively or obligatorily neotenic amphibia

Specie	Metamorphosis	Endogenous T_3 , T_4	TR Genes	
			Expressed*	Autoinduced#
<i>Xenopus</i>	Spontaneous	Yes	Yes	Yes
<i>Ambystoma</i>	Facultatively neotenic	No	Yes	Yes
<i>Necturus</i>	Obligatorily neotenic	Yes	Yes	No

* Only TR α detectable in *Necturus* # By exogenous TH

Tab 4. Some examples of mammalian postembryonic developmental expression of genes and processes specifying the adult phenotype

Process	Genes and tissues
Gene switching	α -Foetoprotein to albumin and foetal to adult haemoglobin in liver; immunoglobulin genes
Morphogenesis	Maturation and differentiation of limb, bone, lung, skin
Neurogenesis	Neural cell turnover; axonal growth, aquisition of new sensory functions
Tissue re-modelling	Keratinization of epidermis; connective tissue formation; re-structuring of intestine
New functions	Nitrogen metabolism and urea synthesis new cell adhesion molecules
Cell death	Removal of tissues or organs by induction of lytic enzymes and expression of cell death and survival genes; digit formation

Prominent among the similarities are the parallelism between structural and functional changes that occur in the larval or foetal brain and limb development during amphibian metamorphosis and mid- to late foetal (or perinatal) development in mammals[2, 9, 54-58], where TH also plays an important role. Perhaps the best documented illustration of the importance of TH during human foetal or perinatal development is cretinism or severe mental retardation and abnormal skeletal differentiation caused by a deficiency of TH at mid- late stages of pregnancy[4, 5, 59, 60]. Experimental hypothyroidism induced in rodents during this period has been shown to cause severe impairment of many sensory functions (taste, hearing, vision) whose normal establishment coincides with a substantial surge in the level of TH in foetal blood. Tissue culture studies have shown a direct action of TH on the differentiation of several types of neuronal cells[4, 8, 54, 59, 60]. Recently, Forrest and his colleagues have demonstrated a deficient hearing ability in mice in which the $TR\beta$ gene had been "knocked-out" by homologous recombination[61]. It is therefore most significant that TH exerts a strong influence on olfactory and optical patterning and function during amphibian metamorphosis[56, 62]. Also, in the context of autoinduction of TRs during amphibian metamorphosis, it is equally significant that the level of TRs increases in human foetal tissues during the period of gestation coinciding with the rapid rise in foetal TH levels[53].

Finally, an intriguing question to consider is to determine if the action of TH in mammalian foetal development is retarded or potentiated by some other factor(s), similar to the interplay between PRL, glucocorticoid and TH in metamorphosis. While the maturational influences of glucocorticoids is known for mammalian perinatal period, virtually nothing is known about the possible antagonism between PRL and TH in foetal development. It is worth noting that the mammalian foetus is exposed during the second half of gestation to extraordinarily high concentrations of placental lactogen, the product of a PRL-like gene, followed by an abrupt fall in its concentration[9, 52, 53, 63]. This fact raises several important questions concerning the major role of placental lactogen in foetal development as, for example, what are the factors regulating its production, its receptors in foetal tissues and its biochemical and molecular actions. It would be particularly relevant, in the light of the discussion in this article about hormonal interactions during amphibian metamorphosis, to resolve these questions if we are to better understand the mechanisms underlying the timing and acquisition of the adult phenotype in the crucial period of late foetal and perinatal development.

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