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Chapter 10

Crossregulation of the Thyroid Hormone and Corticosteroids in Amphibians and Fish: The Effects of Endocrine Disruption

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Additional information is available at the end of the chapter

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1. Introduction

Thyroid hormones are involved in many physiological processes, during growth, development, behaviour, stress. Their actions are mediated by TH receptors (TR-alpha and TR-beta), which are members of the nuclear receptor (NR) superfamily and function as ligand-activated transcription factors. In amphibians, TR-alpha is expressed shortly after hatching and is maintained at a relatively constant level throughout tadpole life and metamorphosis. Then amphibian metamorphosis is dependent on thyroid hormone (TH) changes, which induces the suite of molecular and cellular changes that cause a tadpole to transform into a frog.

Hormones other than TH play important roles in amphibian metamorphosis, in part by modifying the production and actions of TH. Corticosteroids (CS), hormones produced by adrenocortical cells (interrenal glands in frogs and in fish), synergize with TH at target tissues to promote morphogenesis [1,2]. The production of CS changes with development, rising throughout metamorphosis and reaching a peak at metamorphic climax [2]. Like TH, CS actions are mediated by NRs encoded by two different genes: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR).

In the current study we examine molecular and physiological mechanisms involved in TH and CS axes regulation. We investigate the synergy between TH and CS not only during amphibian metamorphosis, but also in fish. Indeed TH play important role in fish development. TH level is especially high in the eggs and larvae of several fish species, including the Japanese flounder (*Paralichthys olivaceus*), the zebrafish (*Danio rerio*) and the seabream (*Sparus aurata*) [3]. Darras *et al*. [4] showed that exogenous T3 increased TH levels in zebrafish embryos and accelerated development and hatching, and it seems that

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elevated T3 levels in culture medium regulates TR expression (for review see reference 4). Glucocorticoids are also key endocrine factors in teleost fishes, involved in metabolism, growth, reproduction [5], and GR can also regulate fish development. Hillegass *et al*. [6] showed that the embryonic zebrafish corticosteroids activate GR and modulate expression of matrix metalloproteinases during development. It seems that TH and CS can regulate each other, in a positive or negative way depending on molecular, cellular and physiologic context. This cross-regulation is important to amplify hormone signals, regulate hormone activity, and coordinate hormone action.

Due to their aqueous exposure, fish and amphibians can be used as indicators for ecotoxicological studies and for detection of endocrine disruptors (ED) *in vivo*. Endocrine disruption has become one of the major topics in environmental research, but also in public health since the ED is known to affect reproductive biology, metabolism, growth and development. The aquatic environment exposes developing embryos to all compounds present in water. After hatching, penetration of ED into aquatic organism is even easier, resulting in high bioavailability and bioaccumulation of chemicals. Amphibians (*Xenopus laevis*) and fish models (zebrafish and medaka (*Oryzias latipes*)) are species widely used for the development of new tools for ED detection and screening of chemicals in the environment. They were firstly used to study endocrine disruption of estrogenic and androgenic systems. But now, more and more studies involve chemicals in thyroid and corticosteroid disruption. Indeed, the thyroid function and corticosteroid axis are both targets for ED. Being given the involvement of both TH and CS axis in amphibian and fish development, these two species were largely used to study TH and CS disruption. The abundant knowledge about endocrinology and developmental biology in amphibian and fish, and the general scientific interest about TH and CS disruption provide evidence for using these animal models for the study of ED of these two endocrine systems [7]. For that reason, we propose to summarize the impact of endocrine disruption on TH and CS axes.

2. Thyroid hormone and corticosteroid endocrine systems: current knowledge

2.1. Thyroid hormone

Thyroid hormones (thyroxine T4 and tri-iodothyronine T3) play an important role in development, differentiation, and metabolism [8]. The lack of T3 in early human development results in growth disturbances and severe mental retardation, a disease called cretinism. TH action is also primary for developmental changes in the nervous system that occur during amphibian metamorphosis. Later in life, T3 plays an important role in metabolic balance [9]. T3 action is mediated by nuclear T3 receptors (TRs) that can bind T3 with high affinity [8]. TRs belong to the nuclear receptor superfamily that also includes the receptors for retinoids, vitamin D, fatty acids, and prostaglandins, as well as "orphan receptors" with no identified ligands [10-13]. TR is encoded by two separate genes, which are designated TR-alpha and TR-beta, located in different chromosomes (17

and 3, respectively, in humans). Like other nuclear receptors, TRs have modular structures with six regions (A–F) and three functional domains.

TR is considered as a transcription factor: it regulates target genes expression directly through DNA response elements. The thyroid hormone response element (TRE) is composed of repeated DNA sequences [14]. Although TRs can bind to TREs as monomers or homodimers, the major form of TR bound to the TRE is the heterodimer with Retinoid X Receptor (RXR). An important property of TRs is their ability to bind TREs constitutively independent of ligand occupancy [8,10,12,13]. Unliganded TR generally represses basal transcription. Ligand binding triggers a conformational change in the TR, resulting in activated transcription of its target gene. In the past few years, great progress in biochemical, functional, and structural studies has clarified the molecular mechanism of TR action.

A classical vertebrate model for thyroid hormone action in development is the amphibian tadpole. Thyroid hormone controls amphibian metamorphosis and thus plays an important role in the developmental changes in the nervous system that occur during metamorphosis. In anuran amphibians, thyroid function regulates the metamorphic process so these are one of the most commonly used *in vivo* systems for studying TH function [14]. TR mRNAs are present at very low levels in the oocyte and during embryogenesis [15-17]. High levels of TR-alpha mRNA are present after hatching and until the end of metamorphosis when levels decrease markedly, staying low in juveniles and adults [18]. TR-beta mRNA levels increase in parallel with endogenous TH levels. The promoter of TR-beta gene contains a thyroid response element, and its expression is induced by thyroid hormone itself in *X. laevis* [19]. As for TR-alpha mRNA, TR-beta mRNA levels decrease in juveniles and adults [18]. Similar profiles were observed when analyzing protein expression [20]. Furthermore, the proteins are functionally active as shown by T3 treatment inducing precocious metamorphosis [18].

Although TH effects have been mainly studied in mammals and amphibians for metamorphosis process, more and more data show that TH play important role in fish development. TH level is especially high in the eggs and larvae of several fish species [3]. In zebrafish, the thyroid gland begins to develop during early embryogenesis and begins to be active around 55 hours post fertilization (hpf) [21]. Before this developmental stage, TH comes from the maternal stock in the egg [3]. The TH receptors (TR-alpha and TR-beta) are both present in prehatch fish embryos, and allow TH functions. Prehatch embryos possess all TH function components: TR-alpha and TR-beta and TH from the maternal stock [3]. TH are synthesized and secreted by the thyroid gland after TSH stimulation. TSH is produced by thyrotropes present in the fish adenopituitary. Terminal differentiation of thyrotropes in the zebrafish adenopituitary occurs around 48hpf. The thyroid gland is active later, about 55 h post fertilization. Concerning the thyroid gland tissue organization, the zebrafish thyroid gland derives from precursor cells located in the endoderm prior to pharynx formation. During two morphogenetic phases, the thyroid primordium first adopts a position close to the cardiac outflow tract, with the first differentiated thyroid follicle, that grows afterwards along the ventral pharyngeal midline. The thyroid gland in the adult zebrafish is a loose aggregation of follicles close to the ventral aorta.

Zebrafish genome encodes two TR-alpha genes and one TR-beta gene, which are expressed at different developmental stages, suggesting that they have different function during development (for review see reference 4). Essner *et al* [22] showed that TR-alpha functions mainly as a transcriptional repressor and may repress retinoic acid signalling in zebrafish early development, and its overexpression results in a loss of the midbrain-hindbrain border and a severe disruption of the rostral hindbrain, suggesting an important role for THs in brain development. Some studies showed that a T3 exposure of embryos up-regulates TR expression and accelerates developmental rate and hatching [4,23]. This suggests that TH can exert a positive auto-regulatory feedback control on the transcription of its receptors.

2.2. Corticosteroid

Corticosteroids are implicated in many physiological process including osmoregulation, respiration, immunity, reproduction, growth and metabolism. Like thyroid hormone, corticosteroids production and action has been studied in amphibian models, for their implication in the positive control of metamorphosis [24]. In bony fishes, corticosteroids are secreted from the interrenal tissue located in the head kidney region. Cortisol is the major corticosteroid in teleost fish and its release involves the coordinated activation of the hypothalamus-pituitary-interrenal (HPI) axis. The key mediators include the release of corticotrophin-releasing factor (CRF) from the hypothalamus, and stimulating the release of adrenocorticotropic hormone (ACTH) from the pituitary. Circulating ACTH binds to melanocortin receptor 2 (MC2R) on the steroidogenic cells and activates the signalling pathway leading to cortisol biosynthesis [25].

In bony fishes, the corticosteroid receptor is a ligand-dependant transcription factor, with two major classes of receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Cortisol is the physiological ligand for GR. The molecular characterization of fish GR began with the cloning of rainbow trout (*Onchorynchus mykiss*) GR1 cDNA, followed by cloning of partial GR cDNA from tilapia (*Oreochromis mossambicus*) and full length GR cDNAs from Japanese flounder (*Paralichthys olivaceus*), rainbow trout GR2 and multiple GRs from cichlid fish (*Haplochromis burtoni*). The alignment of fish GR polypeptide sequences with mammalian GR showed very high homology with both C domain (DNA-binding) and E domain (hormone-binding), but the transcriptional transactivation domain has little homology. The two rainbow trout GR (rtGR1 and rtGR2) showed high sequence homology, and it has been shown phylogenetically that these two GR are a result of gene duplication common to most of teleost fishes. Expression of trout GR transcript has been found in many tissues. Fish GR showed a wide distribution in the brain, gill, and liver.

Little is known about GR and MR function in fish development. Recently, zebrafish has been used to investigate the role of corticosteroid signalling in development. It was shown that both of these receptors are present during embryogenesis [26]. Indeed, during embryogenesis, GR transcripts drops from 1.5 to 25 hpf, and then increases after hatching to the level at 1.5 hpf and was significantly higher at 25 hpf, and this level was maintained until 6 days. This study suggested a more important role for this receptor after hatch in zebrafish. It was also shown that cortisol synthesis occurred only after hatching and that maternal cortisol

contributes to early developmental programming [26]. Further, Pikulkaew *et al*. [27] demonstrated that knocking down maternal GR leads to developmental defects in mesoderm formation in zebrafish. Recently, it has also been shown that GR signalling is essential for zebrafish muscle development [28].

In mammals and non-mammalian vertebrates such as amphibians, the major mineralocorticosteroid is aldosterone. However, aldosterone is not detected in fish, and deoxycorticosterone (DOC) is considered as MR ligand in fish. Like aldosterone, DOC is a selective MR agonist, that does not activate trout GR. Cortisol is also a high-affinity ligand for MR. Fish MR is distributed beyond the tissue involved in salt and water balance, especially in gills and intestine, and MR mRNA is also high in rainbow trout brain. The role of MR and its ligand remains less clear than GR, especially concerning its implication during development. MR transcripts continuously increased between 1.5 and 97 hpf and remained at the same high level at 6 days. MR has been suggested as responsible for corticosteroid signalling just after hatching, since it could be activated by maternal cortisol [26].

2.3. Relationship between TH and CR

The corticosteroid and thyroid hormone receptors possess equivalent transactivation domains and have some structural functional similarity [10,29], suggesting that these nuclear receptors may enhance transcription of target genes by similar mechanisms as summarized above. The thyroid and corticosteroid systems interact at multiple levels to influence several physiological processes like development, growth or behaviour.

Thus, the hypothalamo-pituitary-interrenal axis modulates the thyroid axis in fishes and other vertebrates. Indeed heterologous CRH potently stimulated the release of TSH from cultured pituitary cells *in vitro* [30]. Cortisol exposure also downregulates T4 plasma level in European eel (*Anguilla anguilla)*, but not in trout [31,32].

Conversely, T3 seems to be involved in corticosteroid receptors regulation. Recently, Terrien *et al*. showed that 48 hours of T3 exposure increased MR and GR genes expression in one day-old zebrafish embryos [23]. In another study in common carp (*Cyprinus carpio*), experimentally induced hyperthyroidism downregulates plasma cortisol level. Kelly *et al*. [33] demonstrated a synergistic effect of thyroid hormone and cortisol on cultured *O. mykiss* pavement cell epithelia *in vitro*.

Other studies have suggested a coincident expression and synergetic action of TH and corticosteroids in other vertebrate models [34-37]. Indeed, corticosteroids and thyroid hormones act synergistically during some physiological processes such as amphibian metamorphosis, which is one of the most relevant biological models the most studied for TH and CR crossregulation [34]. Thus, it was shown that corticosteroids can synergize with thyroid hormone to accelerate tadpole metamorphosis, whether corticosteroids increase T3 binding capacity [2], or corticosteroids can increase the conversion of active T3 from T4, and decrease the degradation of T3 [38,39]. Moreover, corticosterone treatment upregulates TR-beta expression in the intestine of premetamorphic tadpoles and in tail explants cultures [37]. It is also known that GR suppresses TSH expression [40].

Like in fish models, TH seems to regulate CS in amphibians. Krain and Denver [37] showed that T3 upregulated the glucocorticoid receptor expression in tadpoles tail, and this regulation might be consistent with a physiological regulatory relationship, given the developmental pattern of thyroid hormone production and GR mRNA in the tail. In contrast, T3 has been shown to downregulate GR expression in the brain.

The coincident increase in cortisol and TH during flounder metamorphosis [35] and the regulation of GR mRNA expression after T3 treatment in *X. laevis* [37] support the idea of possible crosstalk between the thyroid hormones and the corticoid signalling system and are in agreement with previous studies highlighting the relationship between TH and the corticoid signalling system. More recently, corticosteroids have been shown to synergize with TH to promote morphogenesis [1], and that the synergistic actions of TH and corticosteroids occurs at the level of the TR-beta expression and deiodinase type 2, which converts T4 to T3 [34].

3. Use of aquatic organisms to investigate endocrine disruption

Many natural or synthetic chemicals are now routinely observed in water. Evidence revealed that these compounds might interfere with the endogenous endocrine systems of wildlife and humans. Thus, it is now essential to monitor their presence in the environment. Aquatic organisms as amphibians and fish models (zebrafish and medaka) are species widely used in ecotoxicology and for the development of transgenic techniques. These techniques allow development of new tools to detect and screen chemicals in the environment. The zebrafish has numerous technical advantages, so that it can be considered as a model organism: its complete embryogenesis occurs during the first 72 hours post-fertilization and most of the internal organs develop rapidly in the first 24-48 hours. They are easy to observe because embryos are transparent, which allows to easily track their development and expression of fluorescent proteins in transgenic fishes *in vivo*, until advanced stages [41]. In addition to rapid development (adult zebrafish can start to breed after 4 months), there are several other advantages of using zebrafish for assay development, including: small size, low cost to maintain, and easily bred in large numbers. Furthermore, a pair of zebrafish can produce over 100-200 eggs per day. Finally, single embryos can be maintained in small volumes during first days of development until hatching so that zebrafish can be used for automatic reading in 96-well plates. Chemicals can then be tested directly in the solution in which the embryos develop, facilitating high throughput screening [42]. These significant advantages over other species are making the zebrafish as a fully relevant biological model for detecting the effects of pollutants present in the water.

Zebrafish embryo bioassay has been extensively employed in drug and chemical screening [43,44] and the advantages promoting the use of the embryo assay for those purposes should also promote its use for endocrine disruptors phenotypic screening. Zebrafish embryos and larvae express hormones and receptors, and they possess all molecular actors to respond to exposure to endocrine disruptors.

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Moreover, transgenesis in zebrafish is fast and routinely used. For these reasons, the zebrafish is now used to develop simple, rapid, cost-effective and innovative methods for screening environmental pollutants [45,46]. Some stable transgenic zebrafish lines have recently been used for screening chemicals that can mimic the action of estrogens [47], and for developing automated image acquisition and analysis in 96-well plates [46]. Recently, a fluorescent transient fluorescent transgenic zebrafish model has been developed to easily and rapidly screen compounds capable of disrupting thyroid function [23].

Another fish species, the medaka (*Oryzias latipes*) has evolved as an alternative model organism for rapid screening of endocrine activities. Indeed, the medaka has the same advantages than zebrafish (transparent embryos, fishes easy to breed, a short life cycle, easy reproduction and regular renewal of a large number of experiments). In addition to these technical advantages, the medaka has been widely used as a vertebrate model for the study of organogenesis and embryogenesis, and is now increasingly used as a model for conducting toxicity studies [48]. The growing interest in studies on the model of medaka was accompanied by a strong development of tools and methods available to study genetic aspects of such development. In this context, it is not surprising that medaka is now used to study the impact of endocrine disruptors, particularly for the characterization of the stress response and its genetic components [49]. Unlike other models of fish (zebrafish, stickelback), the medaka is more robust, easily exposable to environmental samples, while also the tools of transgenesis are more effective in this model. It is also one of the fish models referenced by the OECD for the detection of estrogenic disruptors.

Finally, amphibians are also used as indicators for ecotoxicological potencies of several environmental stressors. The aquatic larvae are continuously exposed to chemicals compounds present in water because the eggs are lacking a protective eggshell or membrane. After hatching, the skin of amphibians' larvae is still very permeable, allowing an easy penetration of all compounds leading to high bioavailability and bioaccumulation of endocrine disruptors. This development stage of amphibians is the most sensitive and the most used to study effects of environmental pollutants. Thus, Fini *et al*. used *in vivo X. laevis* tadpoles to monitor heavy metal pollution in water in continuous flow systems [50]. The same team already used *X. laevis* to detect the thyroid disrupting effect of BPA *in vivo* [51].

4. Impact of endocrine disruptors on thyroid hormone and corticosteroids systems in fish

Many natural and man-made chemicals (plasticizers, pesticides, detergents and pharmaceuticals) interfere with the endocrine system and can result in adverse health effects in humans, mammals and fish. Wildlife living in or in closer association with the aquatic environment are especially impacted by these endocrine disruptors, because water act as sinks for chemical discharges. Thus, fish and amphibians are the main potential targets for endocrine disruption at multiple levels, either direct or indirect, through ingestion and accumulation of endocrine disruptor, the exposition or through

the food chain. Chronic exposure to endocrine disruptors, such as the oestrogenic compounds used in birth control, can feminize male fish and decrease their capacity to reproduce. In the opposite, masculinised female specimens were found in effluent containing androgenic chemicals [52]. Endocrine disruption on thyroid hormone and corticosteroids in fish was also studied.

Endocrine disruptors may impact corticosteroid signalling system in a direct manner (competition with endogenous ligand) or indirect manner (alteration in accessory proteins, kinases, cytoskeleton...). Among corticosteroids disruptors, chemicals including Polychlorinated Biphenyls (PCB) and heavy metals were the most studied. For example, it has been shown that Arsenic affects GR signalling and one mechanism involves the downregulation of GR content in trout hepatocytes [53]. Another study showed that Copper exposure during 5 days *in vivo* reduced GR-immunoreactivity in rainbow trout gill cells [54].

Cadmium is another metal that is widely distributed in the aquatic environment and is toxic to fish at sublethal concentrations [55]. Due to its long half-life and low excretion rate, Cadmium can also accumulate in various organs, primarily within the liver, kidney and reproductive and respiratory systems in fish [56]. This metal is known to disrupt head kidney corticosteroid production in fish. Vijayan *et al* [57] showed recently that Cadmium exposure leads to suppression of the ACTH-stimulated cortisol production. This study suggested that MC2R signalling, the primary step in ACTH-induced cortocosteroidogenesis, is a key target for Cadmium-mediated disruption of cortisol production in trout.

Because Polybrominated Diphenyl Ethers (PBDE), used as flame retardants, are similar in structure to thyroxine T4 and tri-iodothyronine T3 [58], several teams have studied their effect on thyroid function. Biologic effects of PBDEs in rodent are similar to those of PCB, increasing risks for reproductive and endocrine disruption. In 2011, Yu *et al*. [59] exposed zebrafish to low levels of PBDEs for most their lives. While the fish did accumulate different types of PBDE in their tissues, there was no toxicity. But the PBDEs were present in the eggs, and the chemicals and their associated toxic effects passed along the progeny and reduced hatch rates and altered thyroid hormone system of the next generation. This study is important because it shows that PBDEs trigger thyroid hormone disruption not only in the exposed population but also in the subsequent generation.

Due to its structural homology with thyroid hormone, Bisphenol A (BPA) is also frequently studied as endocrine disruptor for thyroid function. Is has been shown that BPA can interact with TR and it can be considered as a TR antagonist [60,61]. First, transgenic *X. laevis* were used to test *in vivo* whether BPA interferes with TH and to create an *in vivo* detection system for BPA endocrine disruptive properties [62]. More recently, Terrien *et al*. [23] used zebrafish receiving transient transgenesis of TR-sensitive reporter systems to study BPA disrupting effects. In this fish model, the green fluorescent protein (GFP) is expressed under the control of the TH/bZip promoter from *X. laevis* known to contain two thyroid hormone responsive elements (TRE). Exposure to T3 increased the GFP fluorescence in these transient transgenic fish. When tested alone, Bisphenol A did not modify fluorescence, but when tested with T3,

it significantly reduced T3-induced fluorescence suggesting disruption of the thyroid function by BPA [23]. Many other natural (Rotenone) or synthetic (Malathion, Endosulfan) chemicals present in the environment produce thyroid disruption in fish, with varied responses (for review see reference 7).

Because of the close relationship between thyroid and corticosteroid axis in fish and amphibians, alteration of one of these axes could affect the other one. Indeed endocrine disruption due to chemicals present in the environment affects these two endocrine systems, separately or concomitant. Further studies have to investigate the final effect of disruption of one of these endocrine axes on the other one.

5. Conclusion

Nuclear receptor crossregulation are important mechanisms for amplifying hormone signals, regulating hormone activity through negative feedback, and coordinating hormone action in a temporal and tissue-specific-manner. In this chapter, we were interested in crossregulation between thyroid hormone and corticosteroids. These two endocrine systems are keys actors of many physiological processes. Their coincident expression and synergetic action were studied in different models (amphibian metamorphosis, stress response in fish). Corticosteroids are known to synergize with thyroid hormone to promote metamorphosis, and links between the thyroid and corticosteroid axes are present at multiple levels. Understanding the interactions between TH and CS will allow us to better understand the effects of endocrine disruptors.

The use of fish species as model organism for research on endocrine disruption is interesting the identification of potential new endocrine disruptors because of endocrine system and hormone signalling pathways are sufficiently similar to other vertebrates. In this context, we should observe more and more studies leading to a large development of screening tools based on these aquatic animals in a next future. However the consequences of the molecular, physiological or organisms' effects for the population may be different between species. Confirmation in higher vertebrates or in humans of the effects observed in fish is necessary if we want to clearly identify new endocrine disruptors. For instance, the use of aquatic organisms in endocrine disruption studies is relevant, because of their closeness with water-soluble chemicals. And the vast technical possibilities offered by the zebrafish and the medaka models for functional genomics studies justify their use in ED research.

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