General and Comparative Endocrinology 168 (2010) 190–198

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Peering into molecular mechanisms of action with frogSCOPE

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ARTICLE INFO

Article history:
Received 15 November 2009
Accepted 8 January 2010
Available online 13 January 2010

Keywords:
Amphibian
Thyroid hormone
Transcriptomics
Proteomics
Metabolomics
Environmental contaminants
C-fin assay
iTRAQ
Fourier transform ion cyclotron resonance
Quadrupole time of flight
Endocrine disruption

ABSTRACT

Exposure of critical life stages to harmful chemicals at low, environmentally-relevant concentrations can alter how hormones function, and change metabolic pathways or developmental processes that impact reproduction, behavior, or susceptibility to disease later in life. These alterations can be captured through evaluation of changes to transcriptomes, proteomes, and metabolomes occurring at those critical life stages thereby enabling more effective and earlier identification of mechanism of action, individual susceptibilities and adaptation, and prediction of detrimental sublethal effects. Amphibians are "wet canaries in the coalmine" as indicators for environmental health. There are more than 6000 species living in a variety of ecological niches worldwide yet limited 'omics resources and approaches exist. To provide for a means of addressing this challenge, frogSCOPE (frog *S*entinel species *C*omparative "*O*mics" for the *E*nvironment) combines transcriptomics, proteomics, and metabolomics together to form the foundation for the identification of biological response indicators of harmful effects on a species of wild frog (*Rana catesbeiana*) at a sensitive tadpole stage. Various exposure and sampling methodologies are possible including standard *in vivo* exposures, tail fin biopsies, and the C-fin assay. frogSCOPE establishes methodological and analytical approaches applicable to wildlife by using a uniquely-designed frog cDNA array developed to accommodate cross-species hybridization and quantitative real-time polymerase chain reaction (QPCR) assays on poorly genetically-characterized wildlife species. Combination with proteomics (isobaric tags for relative and absolute protein quantitation; iTRAQ) and metabolomics (mass spectrometry) enable the generation of molecular fingerprints to identify mechanisms of action in a more comprehensive fashion to better define suitable indicators of deleterious biological outcomes to wildlife.

1. Introduction

Amphibians are considered aquatic "canaries in the coalmine". Population declines, and limb, body, and gonadal malformations have featured prominently in popular and scientific literature (Houlahan et al., 2000). They constitute an important food source for wildlife and a natural means of insect control. However, the effects of environmental contaminants on these species are very poorly understood. Many amphibia are particularly vulnerable since they undergo a dramatic life transition: metamorphosis of a tadpole into a frog. This highly coordinated event results in the complete remodeling of the animal and disruption results in death or reduced survival in later developmental stages.

Frog tadpoles are completely dependent upon thyroid hormones (THs) to trigger their metamorphosis into a juvenile frog. Frog metamorphosis has three phases (Fig. 1). Premetamorphosis is where the thyroid gland is not yet functional and is mainly a period of growth. No TH is detected at this time. Prometamorphosis begins with maturation of the thyroid gland and TH levels rise which initiate the first metamorphic changes including limb growth. THs reach maximal levels at metamorphic climax, which is characterized by the overt remodeling of the tadpole. Virtually all tissues are targets for TH action, yet the diversity of response includes complete tail resorption, liver reprogramming, and limb growth. These changes are precociously induced in the premetamorphic tadpole by exogenous administration of TH and therefore TH administration (at physiological levels) to premetamorphic tadpoles serves as a convenient model for prometamorphic tadpoles (Gilbert et al., 1996). If THs are not made at the proper time or if target cells are not able to respond to the hormones, then metamorphosis is altered. Exposure of this critical life stage to harmful chemicals at low, environmentally-relevant concentrations can alter how hormones function and change metabolic pathways or developmental processes that impact reproduction, behavior, or susceptibility to disease later in life. Thus frog tadpoles form the foundation of a highly sensitive bioassay for the detection of endocrine disrupting chemicals (EDCs).

The tadpole metamorphosis assay for detection of TH-disrupting chemicals has several advantages over other animal models

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doi:10.1016/j.ygcen.2010.01.012

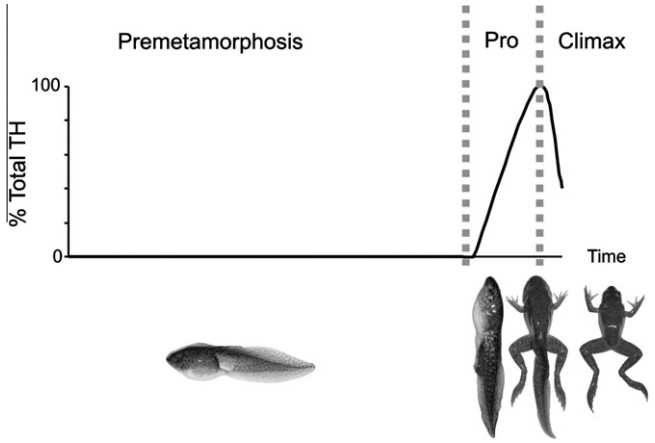

Fig. 1. Stages of metamorphosis, developmental timing, and the relationship to endogenous TH levels. Metamorphosis represents a developmental period that is particularly sensitive to sublethal effects of pollutants. Premetamorphic tadpoles are sensitive to exogenous TH and will undergo a precocious metamorphosis. Prometamorphic tadpoles (Pro) have increasing levels of endogenous TH accompanied by some morphological changes. Metamorphic climax is reached when endogenous TH levels are maximal and the tadpole undergoes rapid morphological changes. The TH levels are based upon (White and Nicoll, 1981).

or cell-based *in vitro* assays. First, it displays a temporal coupling of TH exposure to subsequent observable and measurable morphological outcomes to a degree unmatched by any other known animal process. Second, because anurans have aquatic and terrestrial life stages, permeable skin, fewer active detoxifying enzyme systems than humans (Waring and Harris, 2005), and lack protective membranes (such as egg shells or amniotic membranes) during the critical stages of embryonic and larval development, they are exceptionally susceptible to damage by environmental chemicals. As such, they can serve as excellent sentinel species for the detection of TH-disrupting environmental contaminants (Kloas, 2002) as well as other EDCs (Kloas et al., 1999; Mayer et al., 2003). In particular, premetamorphic tadpoles are optimally poised for *in vivo* detection of TH-disruption, as they are fully competent to respond to exogenous TH, yet have virtually no endogenous production of the hormone (Shi, 2000; Tata, 1993). This eliminates a potential confounding variable in establishing causal links between contaminants and effects. Not only is premetamorphosis the simplest phase during which to assess TH-disruption, but it is also the period during which tadpoles show the greatest sensitivity to TH (Degitz et al., 2005; Opitz et al., 2006b). A third advantage of this assay stems from the ecological niche of the larval anuran at this most sensitive point in its life cycle: the entire metamorphic process occurs in aquatic environments, where the risk of EDC contamination is highest (Kloas and Lutz, 2006). The critical timeframe of TH-sensitivity in anuran larvae resembles that of the perinatal period in mammals (Kanamori and Brown, 1996; Zoeller and Rovet, 2004; Zoeller et al., 2002). In addition, anuran metamorphosis displays many other marked similarities to mammalian post-embryonic and perinatal development, both in the general processes involved (such as cell proliferation, differentiation, and apoptosis) and at the level of cellular effectors of such functions (Shi, 2000). Hence, the use of tadpole metamorphic endpoints in screening for TH-disruption offers a sound framework for between-species predictions of specific cellular endocrine effects by environmental contaminants.

The anuran metamorphic program requires individual tissues and organs to respond differentially to TH, with effects ranging from *de novo* organogenesis of limbs and lungs, to functional reprogramming of existing organs such as the intestine, brain, and liver, to total tissue resorption as seen in the tadpole tail and gills (Shi, 2000). These transformations are organ-autonomous, and can be seen in specific tadpole organ cultures treated with TH *in vitro* (Ishizuya-Oka and Shimozawa, 1991; Tata et al., 1991). Endocrine disruption effects notwithstanding, the metamorphic program of anurans is likely the most extreme example of the multiplicity of TH action, hence it lends itself well as a model in attempting to answer a central question in developmental endocrinology, namely: "How does a single chemical signal initiate such disparate responses?"

The temporal synchronicity of tissue-specific events during metamorphosis is mediated at the cellular level through the interaction of TH with the thyroid hormone receptors (TRs), members of the nuclear receptor superfamily of transcription factors. More specifically, TRs belong to a subgroup of this family that heterodimerize with 9-cis retinoid acid receptor (RXRs) and display ligand-dependent transcriptional modulation of target genes (Kanamori and Brown, 1996; Mangelsdorf et al., 1995; Zhang et al., 1992). In contrast to the other members of this subfamily, such as vitamin D_3 receptor (VDR) and the peroxisome proliferator-activated receptors (PPARs), TR-RXR heterodimers bind target genes regardless of the presence or absence of TH (Wong et al., 1995). Binding of TRs to target genes occurs *via* thyroid response elements (TREs) located in the promoter or enhancer regions of target genes (Buchholz et al., 2006).

Tissue-specificity of differential TH responses might be partially explained by several features of TRs and their interactions with target genes. There are two families of TRs present in all species characterized thus far; these are TRα and TRβ isoforms, first cloned in humans (Weinberger et al., 1986) and chickens (Sap et al., 1986). Both TR isoforms display tightly regulated temporal and tissue-specific gene expression profiles and are known to undergo alternative splicing in some species (Forrest et al., 1990). All three of these mechanisms have been hypothesized to account for some of the multiplicity of effector responses under the control of TH. Also, the existence of three RXR receptors isoforms: RXRα, β, and γ (Mangelsdorf and Evans, 1995) raises the possibility that combinatorial effects of TR and RXR could contribute to some of the tissue-specificity of TH actions. However, given the limited of number of possible permutations within TR-RXR heterodimers, it is unlikely that different receptors alone could account for the large variety of developmental effects of TH observed during anuran metamorphosis (Kanamori and Brown, 1996).

The structure of TH itself plays a role in response-specificity, as it is endogenously found in two forms. It is produced primarily as thyroxine (T_4) in the thyroid. Subsequently, T_4 is converted to the more bioactive form of TH, 3,5,3′-triiodothyronine (T_3), a transformation catalyzed by deiodinases located in peripheral tissues (Bianco et al., 2002). Traditionally, T_4 has been viewed as the 'transport' form and T_3 as the 'effector form' of TH, but we recently uncovered genes in *Xenopus laevis* brain that are selectively up-regulated by T_4 (Zhang et al., 2006) suggesting tissue-specific sensitivity differences to the two forms. Other factors that may also contribute to the pleiotropic effects of TH include the reciprocal roles of liganded and unliganded TR-RXR dimers in transcription modulation and TH-dependent differential recruitment of corepressors and coactivators of transcription and their functions (reviewed in Shi, 2009).

Though much progress has occurred toward elucidating genomic mechanisms by which tissue-specific TH actions are achieved, much remains to be discovered. Hormone signalling pathways include both genomic (nuclear receptor-mediated) and non-genomic (membrane receptor-mediated) mechanisms (Bassett et al., 2003; Ordoñez-Moran and Muñoz, 2009; Yen et al., 2006) that have differing influences depending upon the tissue context and propensity for influence of signalling pathway crosstalk (Hogan et al., 2007, 2008). These factors necessitate the need for development