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Aromatase, steroid-5-alpha-reductase type 1 and type 2 mRNA expression in gonads and in brain of *Xenopus laevis* during ontogeny

R. Urbatzka [a,*], I. Lutz [a], W. Kloas [a,b]

[a] *Department of Inland Fisheries, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, 12587 Berlin, Germany*
[b] *Department of Endocrinology, Institute of Biology, Humboldt-University, Unter den Linden 6, 10099 Berlin, Germany*

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Abstract

The key enzymes involved in the production of endogenous sex steroids are steroid-5-alpha-reductase and aromatase converting testosterone (T) into dihydrotestosterone (DHT) and into estradiol (E2), respectively. To gain more insights into the molecular mechanisms of sexual differentiation of amphibians, we determined the mRNA expression of steroid-5-alpha-reductase type1 (Srd5a1), type2 (Srd5a2) and aromatase (Aro) during ontogeny starting from the egg and ending after completion of metamorphosis in *Xenopus laevis*. Expression of all three enzymes was measured by means of semi-quantitative RT-PCR, determining for the first time Srd5a1 and Srd5a2 mRNA expression in amphibians. mRNA was analyzed in whole body homogenates from stage 12 to 48, while brain and gonads with kidney were studied separately from stage 48 to 66. Different ontogenetic mRNA expression patterns were observed for all genes analyzed, revealing early mRNA expression of Srd5a1 already in the egg at stage 12 whereas Srd5a2 and Aro was detected at stage 39. Sex-specific mRNA expressions of Srd5a2 and of Aro were determined in the gonads with kidney but not in brain. Srd5a2 was two-fold higher expressed in testes than in ovaries while Aro mRNA was ten-fold higher in ovaries. No gender-specific mRNA expression was observed for Srd5a1 in gonads and in brain. The ontogenetic patterns of Aro, Srd5a1 and Srd5a2 suggest that these genes are involved in sexual differentiation of gonads and brain already in early developmental stages. Especially in gonads Srd5a2 seems to be important for physiological regulation of testis development while Aro is associated with the development of ovaries.
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1. Introduction

Sexual differentiation is a complex interplay of different processes regulating the development of an organism towards a male or female phenotype. An important aspect of sexual differentiation is the process of gonadal differentiation that is regulated by genetic sex determination and steroid signalling pathways triggering the bipotential gonads to develop into a testis or an ovary. In mammals, the SRY gene located on the Y-chromosome is responsible for the genetic sex determination. If the SRY gene is expressed in the gonads, a gene cascade is started including Sox9 and Dmrt1 that trigger the development of the testis whereas otherwise an ovary develops (Hughes, 2001; Brennan and Capel, 2004). The testis developmental pathway involves the subsequent emergence of Sertoli and Leydig cells. Sertoli cells secrete the anti-Müllerian hormone inducing the regression of the Müllerian duct and promote the emergence of the Leydig cells that secrete testosterone (T), responsible for development and maintenance of the male secondary sex characteristics (Nef and Parada, 2000). If the ovary developmental pathway is triggered by the absence of the SRY gene, a different set of genes is expressed including follistatin, Wnt-4 and Dax-1. In the following follicular and theca cells develop, the latter one secreting mainly

* Corresponding author. Address: Department of Inland Fisheries, Leibniz-Institute of Freshwater Biology and Inland Fisheries, Müggelseedamm 301, 12587 Berlin, Germany. Fax: +49 (0) 30 64181 682.
E-mail address: ralph.urbatzka@igb-berlin.de (R. Urbatzka).

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17β-estradiol (E2), leading to the development of ovaries (Hughes 2001; Ross and Capel, 2005). In lower vertebrates, some of these genes have been also discovered (Torres Maldonado et al., 2002; Kamata et al., 2004; Osawa et al., 2005; Oshima et al., 2005), however, like in mammals the complex processes of early regulation of sexual differentiation is only partly understood and not all players and their complex interplay have been identified yet.

In the later processes of sexual differentiation, endogenous steroids have a key function since the different hormonal environment of females and males influence the development of secondary sex characteristics. T is derived by the steroidogenic pathway from cholesterol and is the precursor for two antagonistic steroidal secretagogues: T can either be converted by the enzyme steroid-5-alpha-reductase into dihydrotestosterone (DHT) or by the enzyme aromatase into E2. DHT and E2 have opposite effects, masculinizing and feminizing ones, respectively. Therefore, the regulation of the production of endogenous steroids is a crucial step for the differentiation of an organism into male or female phenotype. T, DHT and E2 are mainly produced in the gonads, released into the blood stream and transported to peripheral tissues, where they induce steroid specific gene expression. In peripheral tissues containing corresponding steroidogenic enzymes, T can be also converted into DHT or E2. From mammals, it is well known that the enzyme steroid-5-alpha-reductase has two isoforms named type 1 (Srd5a1) and type 2 (Srd5a2). Srd5a1 is distributed in androgenic and in non-androgenic target tissues while Srd5a2 predominates in androgenic target tissues in rat (Torres et al., 2003).

The important role of sex steroids has been impressively shown in lower vertebrates that have been exposed to exogenous sex steroids leading to sex reversal in fish (Gimeno et al., 1998; Orn et al., 2003), amphibians (Bögi et al., 2002; Kloas et al., 2002) and reptiles (Crews et al., 1991). The inhibition of endogenous E2 biosynthesis by the inhibition of the enzyme aromatase caused masculinization in fish (Kitano et al., 2000) and in amphibians (Yu et al., 1993; Chardard and Dournon, 1999) while the inhibition of the enzyme steroid-5-alpha-reductase led to suppressed spermatogenesis in rat (O'Donnell et al., 1996) or accelerated ovarian development in female gonads of amphibians (Zaccanti et al., 1994).

In anurans such as the South African clawed toad (*Xenopus laevis)*, most potent androgens are DHT, and less effective T, whereas E2 is the active estrogen and exposure to these steroids led to significant masculinization and feminization, respectively (Bögi et al., 2002; Kloas et al., 2002). Therefore it is obvious that genes triggering estrogen and androgen steroid biosynthesis will influence the sexual differentiation of *X. laevis*. The stages 44–50 according to Nieuwkoop and Faber (1994) were determined as sensitive for complete gonadal sex-reversal, while between stage 51 and 54 incomplete feminization and some partial hermaphroditism emerged in response to estrogen exposure (Villalpando and Merchant-Larios, 1990). However, occurrence of aromatase mRNA expression was reported to be present first at stage 51 and to be increased at stage 56 (Miyashita et al., 2000). Consequently, a hypothesis was raised suggesting a central role for steroid-5-alpha-reductase during gonadal differentiation (Bögi et al., 2002; Kloas, 2002), since aromatase mRNA seemed to be not expressed during the decisive stages for sex reversal.

To gain more insights into the regulation of sexual differentiation of *X. laevis*, in the present study the ontogenetic mRNA expression patterns of steroid-5-alpha-reductase type1 (Srd5a1), type2 (Srd5a2) and aromatase (Aro) in whole body homogenates and starting at stage 48 in gonads with kidney and in the brain were determined during the complete development starting from the egg and ending after completion of metamorphosis. Special emphasis was taken to obtain the first data for a potential involvement of steroid-5-alpha-reductases in the processes of sexual differentiation of the gonads in comparison with aromatase. The mRNA expression of these steroidogenic enzymes were analyzed in the brain to get more information about their potential involvement in brain sexual differentiation including organisation of behavioural and neuroendocrine functions, which may also regulate gonadal differentiation via the hypothalamus-pituitary-gonadal axis (Urbatzka et al., 2006).

2. Materials and methods

2.1. Animals

Xenopus laevis were taken from the breeding stock of the Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany. Adult *X. laevis* were injected with human chorionic gonadotropin (SIGMA, Deisenhofen, Germany) into the dorsal lymph sac to induce spawning as described previously (Kloas et al., 1999). Fertilized eggs and tadpoles were reared in 50 L tanks containing reconstituted tap water using deionised water supplemented with 2.5 g marine salt (Tropic Marin Meersalz, Tagis, Dreieich, Germany) per 10 L. Tanks were aerated and temperature was adjusted to 22 ± 1 °C. The light:dark cycle was 12:12 h. Ten days after fertilization tadpoles were transferred to 10 L glass aquaria at a density of 25 tadpoles per tank. Water was renewed every Monday, Wednesday and Friday and tadpoles were fed daily with Sera Micron (Sera GmbH, Heinsberg, Germany).

2.2. Sampling

The developmental stages of the tadpoles were determined according to the Normal Table of *X. laevis* (Nieuwkoop and Faber, 1994). Tadpoles were collected at distinct developmental stages (stage 12, 39, 44, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66-1, 66-2). Stage 66-1 referred to tadpoles that finished metamorphosis and were directly collected while stage 66-2 frogs were sampled two weeks after reaching the stage 66. All samples were directly snap-frozen in liquid nitrogen. The experiment started with the sampling of eggs at stage 12. Eight eggs were pooled per sample to ensure that the amount of total RNA is sufficient for the RNA isolation procedure. At stages 39, 44 and 48 whole larvae were sampled. Additionally, at stage 48 tadpoles were dissected and brain, gonads with kidney and the remaining tissues were collected. Starting at stage 48, brain and gonads with the attached kidney tissue were sampled separately since the amount of tissue was sufficient for RNA isolation. Males and females can be distinguished by gross morphology at stage 56 and brain and gonad samples were taken separately for both sexes from stage 56 to 66-2. At least ten tadpoles at the undifferentiated stages (12–54) and at least six male and six female tadpoles at the sexual differentiated stages (56–66-2) were collected.

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