General and Comparative Endocrinology 162 (2009) 265–275

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Seasonal changes in gonadal expression of gonadotropin receptors, steroidogenic acute regulatory protein and steroidogenic enzymes in the European sea bass

Ana Rocha, Silvia Zanuy, Manuel Carrillo *, Ana Gómez

Department of Fish Physiology and Biotechnology, Instituto de Acuicultura de Torre la Sal, Consejo Superior de Investigaciones Cientificas (CSIC), Torre la Sal 12595, Ribera de Cabanes s/n, Castellón, Spain

ARTICLE INFO

Article history:
Received 22 September 2008
Revised 12 February 2009
Accepted 26 March 2009
Available online 5 April 2009

Keywords:
Follicle-stimulating hormone receptor
Luteinizing hormone receptor
Gametogenesis
P450 11β-hydroxylase
P450 aromatase
Teleost fish

ABSTRACT

The endocrine regulation of gametogenesis, and particularly the roles of gonadotropins, is still poorly understood in teleost fish. This study aimed to investigate transcript levels of both gonadotropin receptors (*FSHR* and *LHR*) during an entire reproductive cycle in male and female sea bass (*Dicentrarchus labrax*). To have a more comprehensive understanding of how different key factors interact to control sea bass gonadal function, changes in the transcript abundance of two important steroidogenic enzymes, P450 11β-hydroxylase (*CYP11B1*) and P450 aromatase (*CYP19A1*), and the steroidogenic acute regulatory protein (*StAR*), were also studied. These expression profiles were analysed in relation to changes in the plasma levels of important reproductive hormones and histological data. Expression of the *FSHR* was connected with early stages of gonadal development, but also with the spermiation/maturation–ovulation periods. The expression profile of the *LHR* seen in both sexes supports the involvement of LH in the regulation of the final stages of gamete maturation and spermiation/ovulation. In both sexes *StAR* expression was strongly correlated with *LHR* expression. In females high magnitude increments of *StAR* expression levels were observed during the maturation–ovulation stage. In males, gonadotropin receptors and *CYP11B1* mRNA levels were found to be correlated. In females, the expression profiles of *FSHR* and *CYP19A1* and the changes in plasma estradiol (E2) indicate that the follicular production of E2 could be under control of FSH through the regulation of aromatase expression. This study supports the idea that FSH and LH may have different roles in the control of sea bass gonadal function.

1. Introduction

In teleost fish, as in mammals, gametogenesis is regulated by the interplay of systemic and intragonadal factors and the importance of each type of regulation varies depending on the developmental stage of the gonad (Patiño and Sullivan, 2002; Schulz and Miura, 2002). The pituitary-derived gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are primary mediators of gonadal steroidogenesis and gametogenesis. They bind and activate specific receptors (FSH receptor (FSHR) and LH receptor (LHR)), present on the surface of gonadal somatic cells, regulating the expression and activity of key steroidogenic enzymes (Themmen and Huhtaniemi, 2000). Although deeply studied in mammals, the precise function of each gonadotropin in teleosts is still largely unknown (Swanson et al., 2003). In the salmonid model, complementary functions of the gonadotropins were suggested by assessment of their transcript and plasma levels. FSH is considered to be involved in the initiation and early stages of gametogenesis, such as vitellogenesis and spermatogenesis, to some extent through the synthesis of estradiol-17β (E2) and 11-ketotestosterone (11-KT), respectively. LH is linked to final maturation and ovulation/spermiation, in part by stimulating the production of maturation inducing hormones (MIHs, the progestins 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 17α,20β,21-trihydroxy-4-pregnen-3-one (20βS)) (Nagahama, 1994; Swanson et al., 2003).

Synthesis of steroids involves a complex cascade of oxidative enzymes that convert cholesterol into different functional steroids. The cytochrome P450 11β-hydroxylase, encoded by the *CYP11B1* gene is necessary for the final steps of the synthesis of 11-KT (Jiang et al., 1996) whereas cytochrome P450 aromatase (P450arom, encoded by the *CYP19A1* gene), catalyzes the conversion of testosterone (T) to estradiol (E2) (Simpson et al., 1994). The cDNAs encoding these cytochromes have been cloned and characterized in several fish species including the sea bass (*Dicentrarchus labrax* L.) (Socorro et al., 2007; Dalla Valle et al., 2002). Nevertheless, their expression during gametogenesis of this Perciform has not yet been investigated.

* Corresponding author. Fax: +34 964 319509.
E-mail addresses: carrillo@iats.csic.es, anarocha@iats.csic.es (M. Carrillo).

0016-6480/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2009.03.023

In teleosts, final gamete maturation is initiated by a rapid shift from the synthesis of androgen/estrogen to the synthesis of MIHs (Nagahama, 1994). This steroidogenic shift is typically accompanied by an increase in steroid synthesis. Biosynthesis of steroid hormones has an acute and a chronic hormonal regulation. Whereas chronic, long-term regulation of steroidogenic capacity involves increased transcription/translation of the genes encoding steroidogenic enzymes, the acute regulation of steroidogenesis depends on cholesterol transport into the mitochondria (Miller, 1988; Stocco and Clark, 1996). In mammals, it has been proven that this transport is mediated by the steroidogenic acute regulatory (StAR) protein (Manna and Stocco, 2005). In addition there is evidence of a positive regulation of *StAR* expression by tropic hormones such as FSH and LH in granulosa cells (Balasubramanian et al., 1997; Sekar et al., 2000) and by LH in Leydig cells (Manna et al., 1999).

As mentioned above, most of the available information regarding physiological aspects of fish gonadotropins refers to salmonid species whose germ cells develop in a synchronous fashion. The fish species selected for this study is the European sea bass that presents a group-synchronous type of ovarian development (successive clutches of germ cells that will mature and be spawned are recruited from a population of vitellogenic oocytes), producing 3–4 consecutive spawns during a 1–2 months spawning period that is repeated once a year during the winter (Asturiano et al., 2000). It is then difficult the extrapolation of salmonid findings to sea bass (or other fish with a non-synchronous type of gonadal development). Contrary to what was described for salmonids (reviewed in Swanson et al., 2003), the expression of the gonadotropin subunits during the reproductive cycle of male sea bass shows overlapping profiles, suggesting that both hormones could be involved in the control of all stages of gonadal development (Mateos et al., 2003).

Recently, we have described the molecular characterization of sea bass gonadotropin receptors (Rocha et al., 2007a). In the present study, we aimed to investigate their temporal expression patterns during an entire reproductive cycle in both male and female sea bass; To have a more holistic understanding of how different key factors interact to control sea bass gonadal function, changes in the expression of *CYP11B1*, *CYP19A1* and *StAR* genes were also evaluated in relation with sex steroid and LH plasma titers as well as gonadal development.

2. Materials and methods

2.1. Animals and sample collection

Male and female sea bass (*D. labrax*) were obtained from the stock raised at the Instituto de Acuicultura de Torre la Sal (Castellón, Spain, 40°N) facilities. They were sampled monthly during their first sexual maturation period (puberty), which generally occurs during the second year of life in males and in the third year of life in females. At each sampling point, five fish of each sex were anesthetized, weighed, sized and sacrificed in accordance with the Spanish legislation concerning the protection of animals used for experimentation or other scientific purposes. Blood was collected via the caudal vein using heparinized syringes, centrifuged at 2500*g* for 25 min at 4 °C and the obtained plasma was stored at −20 °C until analysis. Gonads were dissected, weighed and one portion was flash frozen in liquid nitrogen and stored at −70 °C. The other portion was fixed by immersion in 4% formaldehyde: 1% glutaraldehyde (McDowell and Trump, 1976), embedded in 2-hydroxyethyl methacrylate polymer resin (Technovit 7100, Heraeus Kultzer, Germany), sectioned (3 μm) and stained according to Bennett et al. (1976) for histological analysis. The stages of testicular development were classified by light microscopy, following previously established criteria (Begtashi et al., 2004): stage I, the immature stage; stage II, early recrudescence; stage III, mid recrudescence; stage IV, late recrudescence; stage V, full spermiating testes and stage VI, post-spawning. The ovarian stages were as follows: previtellogenesis (prevtg); early vitellogenesis (evtg); late-vitellogenesis and post-vitellogenesis (lat-postvtg); maturation–ovulation (mat–ovul) and atresia (atre) (Asturiano et al., 2000). Representative sections showing the different gonadal developmental stages of the animals used in this work can be found in Rocha et al. (2007b). Gonadosomatic index (GSI) was determined by the following formula: gonad weight/body weight × 100.

2.2. Hormone analysis

Plasma E2 was measured by a conventional enzyme immunoassay (EIA), validated for its use on the sea bass in our laboratory (B Crespo, JM Navas, A Rocha, S Zanuy, M Carrillo, unpublished). The assay uses a rabbit antiserum against E2 whose specificity is shown in (Prat et al., 1990). The EIA protocol was similar to that previously developed for testosterone determination (Rodriguez et al., 2000a). Briefly, plasma was extracted with methanol. The organic solvent was evaporated and the dry extract was reconstituted in assay buffer (EIA buffer, Cayman Chemical MI, USA). Each component, E2-acetylcholinesterase tracer, anti-E2 rabbit antiserum and E2 standards (Sigma–Aldrich, Inc.) or samples, were added to 96-well microtiter plates coated with mouse anti-rabbit IgG monoclonal antibodies (Clone RG-16, Sigma–Aldrich, Inc.) and incubated overnight at 37 °C. Then, plates were rinsed and colour development was performed by addition of Ellman's reagent and incubation for 2 h at 20 °C in the dark. Optical density was read at 405 nm using a microplate reader (Bio-Rad microplate reader model 3550).The sensitivity of the assay was around 0.156 ng/ml (Bi/B0 = 90%).

The plasma levels of 11-KT were determined by an EIA developed for the Siberian sturgeon (Cuisset et al., 1994) and modified for its use in sea bass (Rodriguez et al., 2005). The assay sensitivity of 11-KT was 0.0012 ng/ml. Plasma LH levels were measured by a homologous competitive ELISA according to (Mateos et al., 2006). The sensitivity of the assay was 0.65 ng/ml.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) and polymerase chain reaction

Sea bass total RNA was isolated from head kidney using the TRI Reagent (Molecular Research Center, Inc. Cincinnati, OH) according to the manufacturer's instructions. For cDNA synthesis, 4 μg of total RNA were denatured at 65 °C for 5 min in the presence of 100 ng of random hexamers and 1 μl of dNTPs (10 mM each dNTP), and then chilled on ice. RT was performed at 42 °C for 50 min using Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA). Protection of mRNA from ribonucleases during the cDNA synthesis was assured by using 40 U of RNasin (Promega Corp.). The reaction was stopped by heating at 70 °C for 15 min.

In order to obtain a fragment of sea bass *StAR* cDNA, a PCR was performed using 2 μl of cDNA and the degenerate primers star1 (5′-CC(T/A)CCTGCTTC(C/T)TGGC(G/T)GG(A/G)-3′) and star2 (5′-GC ATCTTGTGTCAGCAGGC(A/G)TG-3′) designed to conserved regions of *StAR* from the largemouth bass (*Micropterus salmoides*, GenBank:DQ166820). Thermal cycling was performed using a touchdown PCR program (Don et al., 1991). The following conditions were used: an initial denaturation step at 94 °C for 2 min followed by 20 cycles of 94 °C for 30 s, the highest annealing temperature (70 °C) for 30 s, and an extension temperature of 72 °C for 30 s. The annealing temperature was then decreased 0.5 °C per cycle