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The expression of nuclear and membrane estrogen receptors in the European eel throughout spermatogenesis

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ABSTRACT

Estradiol (E_2) can bind to nuclear estrogen receptors (ESR) or membrane estrogen receptors (GPER). While mammals possess two nuclear ESRs and one membrane GPER, the European eel, like most other teleosts, has three nuclear ESRs and two membrane GPERs, as the result of a teleost specific genome duplication. In the current study, the expression of the three nuclear ESRs (ESR1, ESR2a and ESR2b) and the two membrane GPERs (GPERa and GPERb) in the brain-pituitary-gonad (BPG) axis of the European eel was measured, throughout spermatogenesis.

The eels were first transferred from freshwater (FW) to seawater (SW), inducing parallel increases in E_2 plasma levels and the expression of ESRs. This indicates that salinity has a stimulatory effect on the E_2 signalling pathway along the BPG axis.

Stimulation of sexual maturation by weekly injections of human chorionic gonadotropin (hCG) induced a progressive decrease in E_2 plasma levels, and different patterns of expression of ESRs and GPERs in the BPG axis. The expression of nuclear ESRs increased in some parts of the brain, suggesting a possible upregulation due to a local production of E_2. In the testis, the highest expression levels of the nuclear ESRs were observed at the beginning of spermatogenesis, possibly mediating the role of E_2 as spermatogonia renewal factor, followed by a sharply decrease in the expression of ESRs. Conversely, there was a marked increase observed in the expression of both membrane GPERs throughout spermatogenesis, suggesting they play a major role in the final stages of spermatogenesis.

1. Introduction

In male vertebrates, sex steroids, androgens, estrogens, and progestins, play significant roles in the control of spermatogenesis (Schulz and Miura, 2002), process in which diploid spermatogonia differentiate to mature haploid spermatozoa. Estrogens have been shown to be indispensable for the early spermatogenic cycle, controlling the spermatogonial stem cell renewal through its receptor (Miura et al., 1999; Miura and Miura, 2011). Estradiol (E_2), as all sex steroids is a small lipophilic hormone, which can diffuse through the cell membrane (Oren et al., 2004). E_2 can bind to intracellular nuclear estrogen receptors (ESRs) and modulates gene transcription (Mangelsdorf et al., 1995), which corresponds to the classic genomic mechanism of steroid action. Two nuclear ESRs, ESR1 and ESR2 (also named ERα or NR3A1, and ERβ or NR3A2, respectively), are present in mammals. They belong to the nuclear steroid receptor superfamily, as well as androgen, progestin, gluco- and mineralocorticoid receptors (Carson-Jurica et al., 1990; Laudet et al., 1992). Teleost species have at least three distinct ESR subtypes, including ESR1, ESR2a and ESR2b (Hawkins et al., 2000; Ma et al., 2000; Menuet et al., 2002), with ESR2a (also named ERβ2) and ESR2b (also named ERβ1) resulting from the third whole genome duplication (3R) event that occurred in teleost lineage (Hawkins et al., 2000; Lafont et al., 2015).

In addition to the classic genomic functions, E_2 can bind itself to membrane receptors, which activates intracellular signalling pathways through a fast, non-genomic action (for review see: Thomas, 2012, or Nelson and Habibi, 2013). In mammals, the former orphan receptor GPR30 was characterized as an E_2 membrane receptor, and is also called G-protein coupled estrogen receptor GPER (Filardo and Thomas, 2005; Filardo et al., 2007; for review see Prossnitz and Maggiolini, 2009). Two membrane GPERs have recently been observed in most teleosts including the eel, likely resulting from teleost 3R (Lafont et al., 2015).

The European eel (*Anguilla anguilla*) has a complex catadromous life cycle which includes a 5000–6000 km oceanic reproductive migration to reach its spawning site in an unknown area of the Sargasso sea. Eels

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are euryhaline fish which are subjected to high variations in salinity during their life cycle (Daverat et al., 2006). After their juvenile growth period in continental waters, eels change from yellow eels to prepubertal silver eels, future genitors that will undergo the transoceanic reproductive migration. In captivity, the reproductive cycle is still not closed, and long-term hormonal treatments (fish pituitary extracts for females, and human chorionic gonadotropin, hCG, for males) are required to induce sexual maturation in silver eels (Boëtius and Boëtius, 1967; Pérez et al., 2000; Asturiano et al., 2006; Gallego et al., 2012). This, together with the dramatic reduction in the wild European eel population (ICES, 2012) has increased the interest in deciphering the basic mechanisms controlling the reproduction of this species. Furthermore, the phylogenetical position of the European eel, branching at the base of teleosts, may provide insights into ancestral regulatory functions in teleosts, the largest group of vertebrates (Henkel et al., 2012a,b). As far as we know, this is the first study on male teleosts to look at the expression of the three nuclear (ESR1, ESR2a and ESR2b) and two membrane (GPERa, GPERb) estrogen receptors in the BPG axis throughout the spermatogenetic process.

2. Material and methods

2.1. Fish maintenance, hormonal treatments and sampling

Eighty male European eels (mean body weight 100 ± 6 g) were purchased from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia, Spain) and transferred to the Aquaculture Laboratory in the Polytechnic University of Valencia. The 80 males were randomly distributed and kept at 20 °C in two freshwater 200-L aquaria equipped with separated recirculation systems, thermostats/coolers, and covered to maintain constant darkness.

One group of 8 eels was anaesthetized with benzocaine (60 ppm) and sacrificed by decapitation in freshwater (FW). The rest of the fish were gradually acclimatized over the course of one week to seawater (37 ± 0.3‰ of salinity). Groups of 8 eels were anaesthetized and sacrificed by decapitation in seawater conditions (SW). Once a week for 8 weeks the rest of the fish were anaesthetized, weighed and injected with hCG (1.5 IU g^{-1} fish; Profasi, Serono, Italy), to induce the spermatogenesis as previously described by Pérez et al. (2000). Groups of 8 eels were anaesthetized and sacrificed by decapitation each week (W1–8) through the hormonal treatment. For the analysis of ESR expression through the spermatogenesis, the 8 latter groups have been redistributed to 4 groups based on their spermatogenic stage.

Total body weight and testis weight were recorded to calculate the gonadosomatic index [GSI = (gonad weight/total body weight)*100]. Blood samples were collected, centrifuged and stored at −20 °C until E_2 plasma level analysis. Testicular tissue samples were fixed in 10% formalin buffered at pH 7.4 for histological analysis.

Samples of anterior brain (dissected into three parts: olfactory bulbs, telencephalon, mes-/di-encephalon), pituitary and testis were stored in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at −20 °C until extraction of total RNA.

Because eels stop feeding at the silver stage and throughout sexual maturation thee fish were not fed throughout the experiment. They were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.2. Gonadal histology

The formalin-fixed mid-part testis samples were dehydrated in ethanol, embedded in paraffin, sectioned to 5–10 μm thickness with a Shandom Hypercut manual microtome (Shandon, Southern Products Ltd., England), and stained using the haematoxylin and eosin method of National Diagnostic (www.nationaldiagnostics.com/histology/article/staining-procedures). Five slides per fish were observed with a Nikon Eclipse E-400 microscope, and pictures were taken with a Nikon DS-5M camera attached to the microscope (Nikon, Tokyo, Japan). The stages of spermatogenesis were determined according to the germ cell types present in the testis (Miura and Miura, 2011; Leal et al., 2009) their relative abundance, the degree of development of the seminal tubules and the sperm production of the male at the time of sacrifice (Morini et al., submitted for publication). The stages considered were: Stage SPGA: dominance of A spermatogonia, B spermatogonia present in low numbers; Stage SPGB/SPC: dominance of B spermatogonia and spermatocytes, in some cases low numbers of spermatids; Stage SD: dominance of spermatids, in some cases a small number of spermatozoa; Stage SZ: dominance of spermatozoa (Fig. 1).

2.3. Extraction and reverse-transcription

Total RNA of the testis, anterior brain parts and pituitary were isolated using a Trizol reagent (Life Technologies, Inc., Carlsbad, CA) as described by Peñaranda et al. (2013). RNA concentration was evaluated using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain). The testis RNA was treated using a DNase I of NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany). Twenty μl cDNA were synthesized from 500 ng of testis total RNA, using a qScript cDNA Synthesis Kit (Quanta Bioscience, MD, USA). The brain parts and pituitary RNAs were treated using a DNase (gDNA Wipeout Buffer, Qiagen, Hilden, Germany). Using a Quantiscript Reverse Transcriptase (Qiagen, Hilden, Germany), 20 μl cDNA was synthesized from 500 ng of total RNA in the case of the olfactory bulb and pituitary, and from 1 μg in the case of the telencephalon and the mes-/diencephalon.

2.4. Gene expression analyses by quantitative real-time PCR

The quantitative real-time Polymerase Chain Reactions (qPCR) were carried out using specific qPCR primers for each European eel estrogen nuclear and membrane receptor (Lafont et al., 2015) and the Acidic ribosomal phosphoprotein P0 (ARP) (Weltzien et al., 2005) was used as the reference gene (Table 1).

2.4.1. Reference gene

The stability of the reference gene was determined using the BestKeeper program (Pfaffl et al., 2004), reporting a standard deviation (SD[±Cq]) lower than 1. The BestKeeper calculated that variations in the reference gene are based on the arithmetic mean of the Cq values. Genes with a SD value higher than 1 are defined as unstable. In the testis: SD = 0.83; $p < 0.05$ with a Cq geometric mean of 24.21 ± 1.77; in the brain and pituitary, olfactory bulb: SD = 0.81; telencephalon: SD = 0.48; mes-/diencephalon: SD = 0.58, pituitary: SD = 0.63; $p < 0.05$ and the Cq geometric mean of the olfactory bulb: 23.39 ± 1.76; telencephalon: 21.76 ± 1.40; mes-/diencephalon: 21.89 ± 1.49; pituitary: 22.34 ± 1.55.

2.4.2. SYBR Green assay

To determine the expression of each ESR and GPER gene, qPCR assays were performed using a model 7500 unit (Applied Biosystems; Foster City, CA, USA) with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas Corp. Glen Burnie, MD, USA). The qPCR program used for all was an initial step of 50 °C for 2 min, followed by 95 °C for 10 min, and 40 cycles of 95 °C for 1 s and 60 °C for 10 s and 72 °C for 7 s. To evaluate assay specificity, the machine performed a melting curve analysis directly after PCR by slowly (0.1 °C/s) increasing the temperature from 68 to 95 °C, with a continuous registration of any changes in fluorescent emission intensity.

The total volume for each qPCR reaction was 20 μl, with 5 μl of diluted cDNA template, forward and reverse primers (250 nM each), and SYBR Green/ROX Master Mix (12 μl). The transcript levels were determined by the efficiency-adjusted relative quantification method described by Weltzien et al. (2005). Serial dilutions of the cDNA pool of the gonad tissues were run in duplicate and used for the standard