



E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2016; 4(5): 532-536
© 2016 JEZS
Received: 17-07-2016
Accepted: 18-08-2016

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Comparative expression of androgen receptor in gonads and brain during different stages of gonadal development in *Cirrhinus mrigala*

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Abstract

Androgens are necessary for normal male phenotype expression, including the initiation and maintenance of spermatogenesis. Many physiological actions of androgens are mediated by the androgen receptor (AR), a member of the nuclear receptor super family. In the present study focused to identify the expression of AR in the gonads and brain of *Cirrhinus mrigala*. The gene specific primers were used to find out the genes in the brain and gonads of an Indian major carp, *C. mrigala*. Present finding shows the variations of expression of AR receptor mRNA levels in gonads and brain of this fishes. In the present results suggest the gene expression is sex specifically varied in different developmental stages of gonads in the teleosts fish *C. mrigala*.

Keywords: Androgen, receptor, *Cirrhinus mrigala*, expression

Introduction

Steroid hormones are known to regulate cellular processes through two distinct receptor mechanisms. The classically defined, genomic pathway is initiated after the steroid molecule diffuses through the plasma membrane and binds to an intracellular transcription factor belonging to the nuclear steroid receptor superfamily. Subsequently, the steroid/receptor complex translocates to the nucleus and activates or represses the transcription of target genes [1-5]. Androgens are necessary for normal male phenotype expression, including the initiation and maintenance of spermatogenesis. Many physiological actions of androgens are mediated by the androgen receptor (AR), a member of the nuclear receptor super family. AR functions as a ligand-dependent transcription factor, regulating expression of an array of target genes that are important in male development and fertility [6].

A nuclear androgen receptor, distinctly different from the mammalian AR, has been identified in the brain of the Atlantic croaker, *Micropogonias undulatus* [7, 3, 4, 5]. The two receptors have different binding affinities for certain androgens, AR1 binding being more specific for testosterone [7-8]. ARs of goldfish resembled those of mammals in all important physicochemical characteristics. They were unusually abundant compared to levels in rat brain, but comparable to levels in prostate and other male sex hormone target organs. Moreover, there were seasonal variations in total receptors, with a peak at spawning 4- to 5-fold higher than values in reproductively inactive fish. This indicates that brain ARs have a long evolutionary history and have been highly conserved through the vertebrate series [9, 10]. They have shown that AR α levels in the Atlantic croaker brain are regulated by gonadal factors in both sexes. In females, physiological levels of testosterone or estradiol can upregulate the receptor. The regulation of AR α by testosterone and estradiol represents a novel action of these steroids in fish.

Upregulation of AR α by gonadal steroids ensures high levels of AR α and, thus, presumably maximal responses to androgens during gonadal recrudescence. AR α is involved in behavioural responses linked to reproduction and/or feedback by androgens on the hypothalamus-pituitary-gonadal axis [10]. AR α and AR β mRNA were found in distinct nuclei throughout the telencephalon and diencephalons of *A. burtoni*, with AR β expressed more widely and at higher levels than AR α [11]. Many of the brain areas which shows AR expression, including central nucleus of the dorsal telencephalon, ventral nucleus of the ventral Telencephalon, dorsal nucleus of the ventral telencephalon and preoptic area (POA) maintain strong reciprocal interactions in a variety of fish species [12-14], suggesting multiple levels of

potential androgen influence within the teleost brain. The ovarian follicle is not only the major site of synthesis of estrogens, androgens, and progestins in female vertebrates, but is also an important target for sex steroid hormones. Both classic (genomic) and rapid, nongenomic actions of estrogens, androgens, and progestins have been described in the ovaries of a variety of vertebrate species, as well as the nuclear and membrane receptors thought to mediate these effects [15].

The teleost ovary has proven to be a particularly useful model for investigating nongenomic steroid actions and the receptors on the plasma membrane through which they act. However, a comprehensive understanding of the expression of ARs in the brain, ovaries and testis of Indian major carps is currently lacking. Hence, an attempt has been made in the present investigation to study the androgen receptor gene expression in both gonads and brain of male and female fish during different stages of gonadal development.

Materials and Methods

Male and female *Cirrhinus mrigala* every month samples (January 2013 to August 2015) were collected from Sathanur Dam, TNFDC. Testis, ovary and brain samples were collected during different stages of reproductive period. Oocytes were collected during previtellogenic (stage I), vitellogenic (stage II) and matured stages (stage III) and testicular fragments were collected during stasis (stage I), pre-spawning (stage II) and spawning stage (stage III). The fish were sacrificed and the gonadal and brain samples were immediately preserved in RNA later (Sigma Aldrich Chemicals Pvt. Ltd, India) for further analysis.

Total RNA isolation

The tissue was homogenized (100 mg) with 500 µl of Trizol (Sigma Aldrich Chemicals Pvt. Ltd., India) and 200 µl of DEPC water. The homogenized mixture was incubated at -20 °C for 5 min. 200 µl of chloroform was added to the mixture and was shaken vigorously for 15 sec. The mixture was incubated for 5 min at -20 °C and centrifuged at 12,000 rpm for 15 min. The supernatant was collected.

Equal volume of isopropanol was added to the supernatant and was incubated at -20 °C for 45 min. The sample was centrifuged at 12,000 rpm for 15 min to obtain the RNA pellet. 1 ml of 75% ethanol was added to the RNA pellet and centrifuged at 12,000 rpm for 5 min. The supernatant was discarded and the pellet was air dried. The RNA pellet was dissolved in 40µl of DEPC water and stored at -20 °C for 30 min. It was then subjected to 1.2% agarose gel.

Synthesis of first strand cDNA

The RT-PCR kit was obtained from Medox Biotech India Pvt. Ltd. The procedure given in the kit was followed.

The first strand cDNA was synthesized by adding 1 µl of total RNA, 1 µl of oligo (dT) [18] primer and 10.5µl of de-ionized water was added in a tube. The mixture was micro centrifuged for few seconds and was incubated for 5 min at 70 °C. It was chilled on ice and was briefly centrifuged before adding 4µl of 5x reaction buffer, 0.5 µl of ribonuclease inhibitor and 2µl of 10mM dNTP mix. It was again centrifuged and incubated at 42 °C for 5 min. After incubation 1 µl of M-MULV reverse transcriptase was added and incubated at 42 °C for 60 min. The reaction was stopped by heating for 10 min at 70 °C and chilled on ice. The cDNA synthesized was loaded in the gel electrophoresis. The first strand cDNA synthesized were used directly for PCR reactions.

PCR amplification

The PCR amplification was performed using PCR Master Mix Kit (Bangalore Genei, India). PCR Master Mix consisted of 2x concentration (Taq DNA polymerase, dNTPs and reaction buffer with 1.5 mM magnesium chloride at optimum concentration). The primer was obtained from the Sigma Aldrich Chemicals Pvt. Ltd, India. Specific androgen receptor gene primers used for the amplification:

Sense 5' CACTCGCAGTTGATCTTCTGAA 3'

Antisense 5' GTTACGGAAACCTGGAAGTCCTT 3'

The reaction mixture consisted of 1 µl of the forward and the reverse primers each, 2µl of cDNA and 25µl of PCR master mix. PCR was carried out for 35 cycles in a PCR thermocycler (BIO-RAD) with a pre-heating for 2 min at 95 °C and each cycle consisting of a denaturation for 30 sec at 95 °C, annealing for 30 sec at 54 °C and an extension for 60 sec at 72 °C. After the amplification, the product was allowed to run in 1.2% agarose gel with 2 µg/ml of ethidium bromide and 1× TAE buffer. It was observed in a gel documentation unit (JH BIO geldoc system) for positive bands.

Preparation of TAE buffer

TAE buffer (10×) was prepared by dissolving 9.6 g of Tris, 0.74 g of EDTA and 2 ml of acetic acid in 200 ml of distilled water at pH 8.

Quantification

Total RNA, cDNA and PCR amplified products were quantified using UV-visual Smart Spec Plus Spectrophotometer (BIO-RAD).

Results

Comparative study of AR gene expression was carried out in brain of male and female fish during breeding period (June, July and August). Expression was observed in all the samples and intense band was obtained in the brain of matured male and female fish (Fig. 1). Comparative analysis of AR gene expression was observed in different stages of testicular and ovarian samples. AR expression was high in stage-III oocytes when compared with stage -I and stage-II oocytes. Similarly dark band was obtained in stage III testicular fragments when compared to stage-I and stage-II (Fig. 2). Month wise expression study was also carried out in gonadal and brain samples. Figure (3-5) shows the comparative analysis of AR in the gonadal and brain samples of male and female fish during the month of June, July and August respectively. AR expression was noticed in all the samples. Expression was less in the female brain when compared with male brain and gonadal samples.

Discussion

Testosterone exerts its actions in brain directly via androgen receptors or, after aromatization to estradiol, via estrogen receptors. Brain aromatase activity in teleost fish is 100–1000 times greater than in mammals and would be expected to significantly reduce the quantity of androgen available for receptor binding [9]. Tissue levels of androgen receptor in vertebrates appear to be under complex transcriptional and post-transcriptional control [10, 17, 18]. Moreover, androgen receptor may be regulated differently in various regions of certain androgen responsive organs, such as the brain and the prostate [17, 18].

Brain AR α levels in both male and female Atlantic croaker are greatly influenced by the gonadal status and circulating levels of sex steroids [10]. Studies in the Atlantic croaker [5, 7] and the goldfish (*Carassius auratus*) [9] have demonstrated

seasonal variations in brain AR levels, with the highest concentrations during gonadal recrudescence. This supports our present finding wherein intense AR bands were observed in the brain samples of matured male and female fish (Fig. 1). In the Japanese eel, AR α and AR β are co-expressed in cells in the testes [19]. AR was highest in tissues characterized by spermatocytes mainly in spermatogonial stages, decreasing in successive developmental stages of testes in the spiny dogfish *Squalus acanthias* [20]. Testosterone binding activity varies 2- to 4-fold with the pattern premeiotic greater than meiotic and postmeiotic, indicating that changes are due to total receptor abundance and not to intracellular location [20]. In the present finding, the AR expression was more in the matured (stage III) testis when compared to stage I and stage II testicular samples (Fig. 2).

Physiological studies in numerous non-mammalian species, including teleost fish, indicate that the androgen dependence of spermatogenesis is a general vertebrate phenomenon [21]. In the fish brain aromatase is the steroidogenic enzyme that has received most attention. Indeed, a wealth of information is available regarding aromatase activity, regulation, and expression in the brain [4, 22-25]. A rapid, nongenomic action of androgens initiated at the cell surface to down-regulate ovarian estradiol production in croaker has been described by Braun and Thomas [26]. It is possible that the membrane AR (mAR) is responsible for mediating this nongenomic effect of androgens. Studies by Braun and Thomas [27] support a role for the mAR in the reproductive cycle of female *Atlantic croaker*.

Elevated expression of AR-mRNA in the brain during late vitellogenesis is correlated with higher circulating concentrations of androgens and greater propensity for

receptive behaviour in female leopard geckos, *Eublepharis macularius* [28]. AR levels increased 10-fold during ovarian recrudescence, reaching maximum levels in fully mature ovaries, which suggests a likely physiological role for this receptor during the reproductive cycle of female Atlantic croaker, *Micropogonias undulatus* [27]. This is in agreement with the present finding wherein intense bands were observed in the matured oocytes (stage III) when compared to stage I and stage II oocytes (Fig. 2).

AR transcript levels are increased in maturing testis in rat [29] and also in the developing ovary in pig [30]. Relative abundance of AR transcripts was reported in bisexual testicular tissue, functional testis and ovarian tissue of black porgy, *Acanthopagrus schlegelii* [31]. In the cichlid *Astatotilapia burtoni*, two AR subtypes (AR α and AR β) are differentially located throughout the adult brain in nuclei known to function in the control of reproduction. AR α was expressed in the ventral part of the ventral telencephalon, the POA of the hypothalamus and the ventral hypothalamus, whereas AR β was more widely expressed in the dorsal and ventral telencephalon, the POA, and the ventral and dorsal hypothalamus [5, 11, 32-33].

This present finds provide information on the AR expression in the brain and gonads of *C. mrigala* during different stages of reproductive cycle give us a rationale and focus for further studies, in which quantification of ARs in different regions of the brain and gonadal tissue, and determination of DNA sequence can be done to understand their evolutionary relatedness and to investigate their cellular and tissue distributions. However, the present study reveals the relationship of AR expression between gonads and brain.

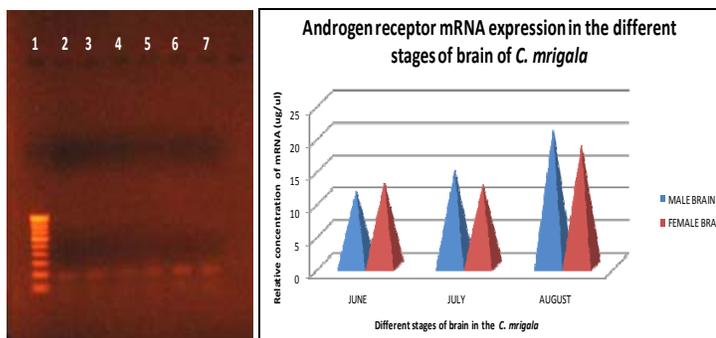


Fig 1: The Gel image shows the Androgen receptor gene expression in the male and female brain of *C. mrigala* during breeding period. Lane 1, DNA marker; Lane 2, PCR product of male brain (June); Lane 3, PCR product of female brain (June); Lane 4, PCR product male brain (July); Lane 5, PCR product of female brain (July); Lane 6, PCR product of male brain (August); Lane 7, PCR product of female brain (August) and the column bar diagram shows the concentration AR mRNA levels measured by UV-Spectrophotometry.

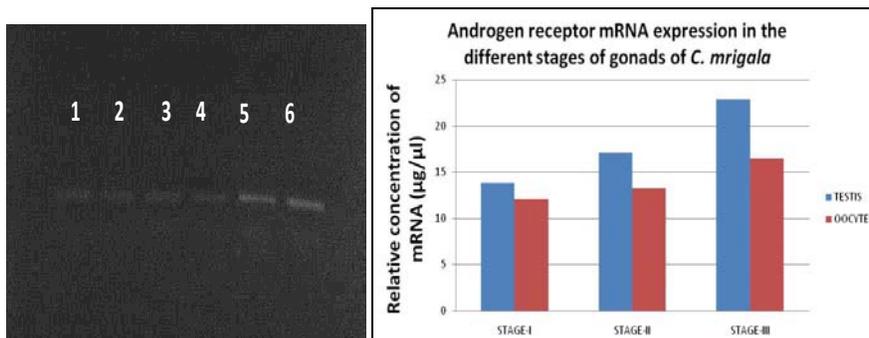


Fig 2: Gel image shows the Androgen receptor gene expression in the oocyte and testis during different stages of gonadal development in *C. mrigala*. Lane 1, testis (Stage I); Lane 2, oocyte (Stage I); Lane 3, testis (Stage II); Lane 4, PCR product of oocyte (Stage II); Lane 5, testis (Stage III); Lane 6, PCR product of oocyte (Stage III) and the bar diagram shows the concentration of AR mRNA levels measured by UV-Spectrophotometry.

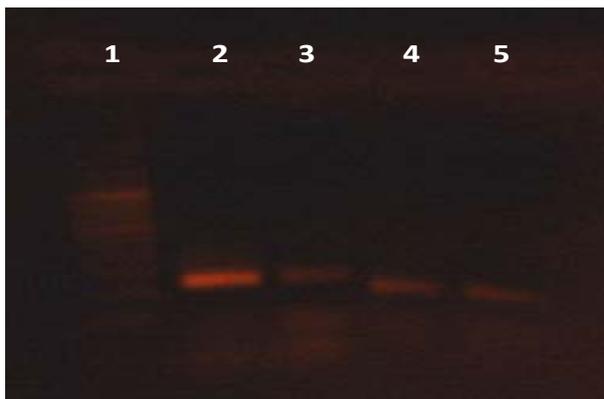


Fig 3: Gel image shows the Androgen receptor gene expression in the male and female brain, oocyte and testis during the month of June. Lane 1, DNA marker; Lane 2, PCR product of male brain; Lane 3, PCR product of female brain; Lane 4, PCR product of testis; Lane 5, PCR product of oocyte.

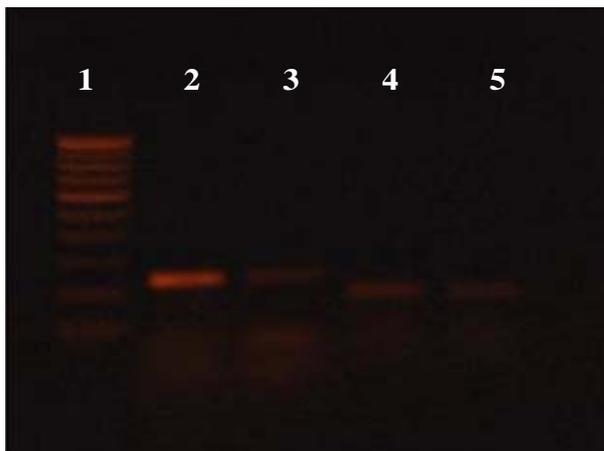


Fig 4: The Gel image shows the Androgen receptor gene expression in the male and female brain, oocyte and testis of *C. mrigala* during the month of July. Lane 1, DNA marker; Lane 2, PCR product of male brain; Lane 3, PCR product of female brain; Lane 4, PCR product of testis; Lane 5, PCR product of oocyte.

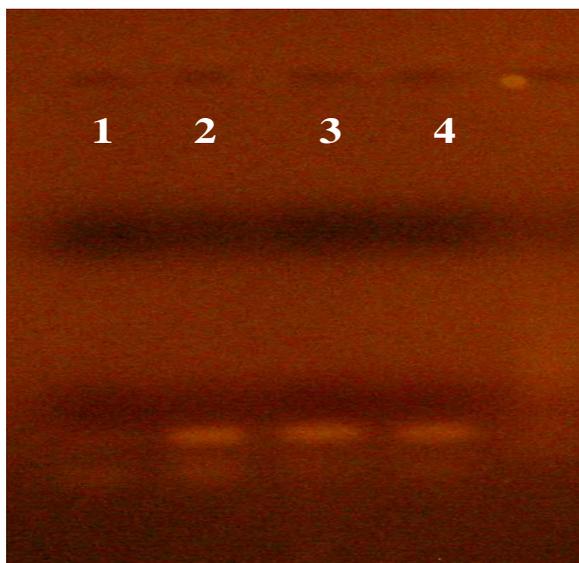


Fig 5: The Gel image shows the Androgen receptor expression in the brain, oocyte and testis of matured male and female *C. mrigala* (August). Lane 1, PCR product of female brain; Lane 2, PCR product of male brain; Lane 3, PCR product of testis; Lane 4, PCR product of oocyte.

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