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Developmental expression of genes involved in neural estrogen biosynthesis and signaling in the brain of the orange-spotted grouper *Epinephelus coioides* during gonadal sex differentiation☆

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ABSTRACT

In the brain, the synthesis of neurosteroids and receptor activation during gonadal sex differentiation in teleosts are poorly understood. In the present study, the protogynous orange-spotted grouper (*Epinephelus coioides*) was selected as a model fish, and we hypothesized that *de novo* synthesis of neural estrogen may play a role in the female grouper brain during gonadal sex differentiation. We investigated the temporal expression of the genes *StAR*, *cyp19a1b* and *pcna* and the sex steroid nuclear receptors for estrogen (*ERα*, *ERβ1* and *ERβ2*), androgen (*AR*) and the plasma membrane-associated estrogen receptor (*GPR30*) in the brain during early developmental ages from 90 days after hatching (dah) to 180 dah after gonadal sex differentiation. Our results revealed that mRNA for ERs and *GPR30* but not *AR* was significantly increased at 110 dah (a time close to gonadal sex differentiation) in the forebrain and midbrain and for *cyp19a1b* at 110 dah in the forebrain. Brain aromatase activity and estradiol (E2) levels, but not testosterone (T), were increased in the forebrain at 110 and 120 dah, respectively. Furthermore, exogenous E2 stimulated *cyp19a1b* transcripts in the forebrain and hypothalamus and immunoreactive (ir)Cyp19a1b (aromatase enzyme) in the forebrain. irCyp19a1b localized in the glial cells of the forebrain regions. Therefore, we identified a peak of functional aromatase activity and estrogen signaling in the early grouper brain during gonadal sex differentiation. Moreover, *pcna* transcripts (a marker for cell proliferation activity) were higher in the early brain at 110–150 dah. Thus, a peak time of development in the brain is suggested to occur during gonadal sex differentiation in the grouper.

1. Introduction

The brain is considered to be an important sex steroid producing tissue in teleosts. Sex differences in the brain during development are often linked to hormonal changes, and temperature (thermolabile sex determination, TSD) is often capable of modifying sex ratios during early phases of development in teleosts [1]. In the brain, *de novo* production of neurosteroids is involved in a variety of physiological functions in the central nervous system [2,3]. Transfer of cholesterol from the outer to the inner mitochondrial membrane is the rate-limiting step in hormone-induced steroid formation by steroidogenic acute regulatory protein (*StAR*) [4]. In a previous study, we reported that the developmental expression of the mRNAs encoding four key neurosteroidogenic enzymes (*cyp11a1*, *hsd3b1*, *cyp17a1* and *cyp19a1b*) in the brain of protandrous black porgy (*Acanthopagrus schlegeli*) had significant peaks during gonadal sex differentiation. Hence, we suggested that early brain sex steroids enhance male brain development (neurosteroidogenesis) during the period of gonadal sex differentiation [5,6]. Cytochrome P450 aromatase is the key enzyme that catalyzes the conversion of C19 androgens (androstenedione and testosterone, T) into C18 estrogens (estrone and estradiol-17β, E2) in the brain, which regulates many physiological processes such as brain sexual differentiation and activation of female sexual behavior during development [7]. Brain sex steroids, especially E2, may cause brain sex differentiation at an early developmental age and lead to sexually dimorphic adult reproductive behaviors in mammals [8,9]. In addition, aromatase activity is regulated by estrogens and aromatizable androgens, and several studies in teleost fish indicate that *cyp19a1b* transcripts are up-regulated by E2 through estrogen receptor signaling [10–13], indicating the reproductive role and functional relationship of estrogen with brain aromatase. However, another study indicated that the *cyp19a1b* gene is also up-regulated by androgens, especially T, suggesting a potential androgenic regulation of *cyp19a1b* through the estrogen receptor [14].

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Brain aromatization occurs in all classes of vertebrates, but teleost fish are known for exhibiting high aromatase activity mainly due to the expression of the *cyp19a1b* gene [15–17]. Interestingly, high levels of brain aromatase activity, corresponding to the strong expression of *cyp19a1b*, have been reported in the teleost forebrain, particularly in the telencephalon, preoptic area and hypothalamus, where the expression was 100–1000 times higher than in adult mammal brains [18]. This finding was confirmed in several teleost fishes including the Atlantic salmon, *Salmo salar* [19], stickleback (*Gasterosteus aculeatus*) [20], African catfish (*Clarias gariepinus*) [21], European sea bass (*Dicentrarchus labrax*) [16] and peacock blenny (*Salaria pavo*) [22]. This high neural aromatase activity has been attributed to the high levels of neurogenesis present in the teleosts brain compared to mammals [7,13,15]. Moreover, estrogen is an important neurotrophic factor, playing this role throughout life and influencing neuronal differentiation [23,24], participating in the process of embryonic and adult neurogenesis [25,26]. It is important to point out that in teleosts, *cyp19a1b* is expressed in a cell type called radial glial cells. In a recent study, the distribution of *cyp19a1b* in the brain, as determined by *in situ* hybridization and immunohistochemistry, was determined to be in the radial glial cells especially in the forebrain area, suggesting that brain aromatase gene is expressed in the glial cells and not in the neurons [27–29]. Further, it suggested that E2 could, among other functions, modulate some cellular mechanisms involved in neurogenesis [15,27–29]. However, in birds and mammals, expression of *cyp19a1b* was mainly found in the neuronal cells [30,31]. The relationship between aromatase and estrogen receptors and their presence in the developing brain raised questions about their potential roles during gonadal sex differentiation and brain development.

The effects of E2 are typically mediated through two structurally related estrogen receptors, *ERα* and *ERβ*, subtypes that function as ligand-activated transcription factors. During neurosteroidogenesis, E2 is synthesized from T via the P450 enzyme aromatase. The ER and aromatase play important roles in the brain during early development and sex differentiation in the European sea bass [32]. Further, estrogen has been reported to be essential for female gonadal differentiation [33,34] and sex change [35,36] in teleosts. Molecular cloning and identification of differential expression patterns of the different forms of the estrogen receptor (*ERα*, *ERβ1*, and *ERβ2* or *ERγ*) during early development and adult stages have been reported in a number of vertebrate species [37–39] including fish [40–42]. In addition, ERs are highly expressed during ovarian differentiation in tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*) and black porgy [34,43,44], suggesting reproductive roles for ERs in sex differentiation in fish species. Recently, in addition to data on the intracellular nuclear receptors (*ERα* and *ERβ*), some studies have shown that E2 binds with the non-nuclear steroid receptor G-protein coupled receptor 30 (*GPR30*, a plasma membrane protein), which is activated intracellularly by E2 that readily diffuses across cell membranes during development [45,46]. However, how the expression of ERs changes with aging in the brain during gonadal differentiation is still a matter of debate, and most studies on the temporal pattern of ER expression are mainly focused in the liver [47] and gonad [34,43,48]. Additionally, the transcriptional regulation of these ERs during early development in the brain in response to gonadal sex differentiation remains unclear. The functional differences in their roles in brain development and other physiological processes during gonadal differentiation should be studied in fish.

The orange-spotted grouper (*Epinephelus coioides*) is a protogynous hermaphrodite with a mono-female sex development pattern during its early life that undergoes sex change at about 7 years of age. In this framework, we hypothesized that the neural estrogen biosynthesis and receptor actions occurring in the early developing grouper brain play important roles in naturally feminizing the grouper brain during gonadal sex differentiation. In order to understand the neuroendocrine signaling pathway of estrogen synthesis in the developing brain during gonadal sex differentiation, we studied the mRNA expression of *StAR*, *cyp19a1b* and *pcna*, the genomic nuclear receptors (*ERα*, *ERβ1*, *ERβ2*, and *AR*) and the non-nuclear, plasma membrane-associated estrogen receptor (*GPR30*) at different developmental ages from 90 to 180 dah. Here, we used the *StAR* gene as a marker for the rate-limiting step in hormone-induced steroid formation and *cyp19a1b* for the final step of neurosteroidogenesis in which C_{19} androgen is converted to C_{18} estrogen. We also measured brain aromatase activity and brain steroid hormone levels (brain E2 and T) at different developmental ages. E2 effects on the expression of *cyp19a1b* transcripts and immunoreactive Cyp19a1b-positive staining, and a marker gene for cell proliferation (proliferating cell nuclear antigen, *pcna*) in brain tissue were also studied.

2. Materials and methods

2.1. Experimental fish

Orange-spotted groupers (*E. coioides*), were collected from the southern part of Taiwan and acclimated to the pond environment at the University culture station in a seawater and natural light system (salinity of 33 ppt; water temperatures ranged from 20 to 24 °C). The fish were fed *ad libitum* with a commercial food (Fwu Sou Feed Co., Taichung, Taiwan). All procedures and investigations were approved by the National Taiwan Ocean University Institutional Animal Care and Use Committee and were performed in accordance with standard guiding principles.

2.2. Sample collection

Gonadal differentiation occurs around 120 dah in the grouper, as determined by histological gonadal observation. Therefore, we chose two time points before and three points after gonadal sex differentiation to investigate the changes in steroidogenic enzymes and sex steroid receptors in response to early brain development during gonadal differentiation. Prior to sampling, the fish were anesthetized in ethylene glycol monophenyl ether (0.05%) and sacrificed by decapitation. Brain tissues were dissected into three parts: forebrain (prosencephalon including olfactory bulb, telencephalon and part of the preoptic area; located between the anterior commissure and the optic chiasm), midbrain (mesencephalon, mainly optic tectum, tegmentum and cerebral crura) and hypothalamus (mainly diencephalon including thalamus, epithalamus, subthalamus and hypothalamus) as previously described in the black porgy [5]. Three batches of brain samples were collected: (1) 90, 110, 130, 150 and 180 dah for the gene expression studies and aromatase activity measurements, (2) 90, 120, 150 and 180 dah for the measurement of sex steroids (E2 and T) and (3) one^{+}-year-old fish in the E2-treatment for gene expression and immunohistochemical staining. Tissues were sampled and immediately frozen in liquid nitrogen and stored at −80 °C for gene cloning, RT-PCR, Q-PCR ($n = 8$ fish per value), aromatase activity ($n = 6$ fish per value) and enzyme immunoassays for brain E2 and T ($n = 6$ fish per value).

2.3. Experimental design

2.3.1. Experiment 1: gene expression profiles during gonadal sex differentiation in the grouper brain

In order to clarify the steroidogenic enzyme gene profile in the grouper brain during gonadal sex differentiation, brain samples were collected from 90 (body weight, BW = 11.6 ± 0.5 g; body

length, BL = 9.1 ± 0.2 cm), 110 (BW = 24.5 ± 0.8 g, BL = 11.7 ± 0.1 cm), 130 (BW = 28.1 ± 1.1 g, BL = 12.3 ± 0.2 cm), 150 (BW = 31.2 ± 1.2 g, BL = 12.9 ± 0.2 cm) and 180 dah fish (BW = 48.1 ± 2.8 g, BL = 15.3 ± 0.3 cm) (n = 8 fish in each value). mRNA transcripts of the genes were quantified by quantitative real-time PCR (Q-PCR) analysis.

2.3.2. Experiment 2: aromatase activity and sex steroids in the grouper brain during gonadal sex differentiation

In order to measure the aromatase activity (n = 5 fish in each value) and sex steroids (E2 and T) (n = 6 fish in each value) in the grouper brain at different developmental ages, we collected two sets of different brain samples (forebrain, midbrain and hypothalamus). For aromatase activity, samples were collected 90 (BW = 12.2 ± 0.8 g, BL = 9.3 ± 0.2 cm), 110 (BW = 23.4 ± 1.1 g, BL = 11.5 ± 0.3 cm), 130 (BW = 29.1 ± 1.3 g, BL = 12.5 ± 0.2 cm), 150 (BW = 30.4 ± 1.5 g, BL = 13.1 ± 0.3 cm) and 180 dah (BW = 51.3 ± 3.8 g, BL = 15.5 ± 0.3 cm). For sex steroids quantification, samples were collected at 90 (BW = 11.4 ± 0.6 g, BL = 8.9 ± 0.2 cm), 120 (BW = 26.2 ± 1.0 g, BL = 12.1 ± 0.2 cm), 150 (BW = 31.8 ± 1.7 g, BL = 12.7 ± 0.23 cm) and 180 dah (BW = 45.1 ± 4.1 g, BL = 15.1 ± 0.5 cm). Aromatase activity was quantified by the radiometric method and sex steroids were measured by enzyme immunoassay.

2.3.3. Experiment 3: effects of E2 on gene expression profiles of the brain of one-year-old grouper fish

In order to further investigate the effects of E2 on *cyp19a1b* and *pcna* expression, one-year-old female groupers (BW = 81.5 ± 4.9 g, BL = 17.1 ± 0.45 cm) were divided into two groups, with n = 10 fish in each of the following experimental groups: control (vehicle alone, coconut oil; Sigma, St. Louis, MO) and E2 treatment (1 μg/g BW; Sigma). Fish were given intramuscular injections on day 1 and 5 (two injections in total) similar to our previous study published in black porgy [49]. Fish forebrain, midbrain and hypothalamus were collected 24 h (day 6) after the 2nd injection (day 5) and stored at −80 °C until further use. The changes in the mRNA transcripts of *cyp19a1b* and *pcna* in control and E2 treatment groups were analyzed by Q-PCR.

2.3.4. Brain histology and immunohistochemical staining with Cyp19a1b antisera

Brain tissues of one-year-old groupers were fixed in a 4% paraformaldehyde solution up to 16 h. Further, fixed brain was dehydrated, embedded in paraffin, and sectioned at 5 μm. Standard histological sections were prepared using hematoxylin and eosin (H&E) or immunohistochemical staining. Immunoreactive (ir) cells were localized in the brain.

For immunohistochemistry (IHC) analysis of Cyp19a1b immunoreactivity, slides were allowed to come to room temperature (RT), immersed in xylene three times for 5 min each, hydrated in a series of ethanol washes, air dried and sections were outlined in PAP pen (Fisher Scientific, St. Louis, MO). The slides were then rinsed in sodium phosphate buffer with saline (PBS) and incubated with 3% H_2O_2 in PBS, blocked in 1.5% normal goat serum (NGS, Vector Laboratories, Burlingame, CA) in PBS (30 min, RT), and with primary antibody diluted in PBS containing 1.5% NGS (16–20 h, 4 °C). The Cyp19a1b polyclonal antibody used in the study was induced in guinea pig (*Cavia porcellus*) against orange-spotted grouper Cyp19a1b peptide fragment (NH2-CAMRFIPRTTQPQHSQQNHH-COOH) based on a conserved amino acid sequence alignment of known teleost brain. Following the 16–20 h incubations, sections were rinsed in PBS (3 × 10 min) and placed in biotinylated anti-guinea pig IgG secondary antibody (1:1000 dilutions) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Color formation was visualized with an ABC kit (avidin–biotin; Vector Laboratories) and DAB (3,3′-Diaminobenzidine; Sigma). Elimination and serial dilutions of the guinea pig pre-sera instead of the primary antibody was used for the negative control. The neuroanatomical nomenclature employed for the grouper brain nuclei in this paper is based on the brain atlas of zebrafish [50].

2.3.5. Key factors related to brain development and/or neurogenesis

In the present study, we cloned the steroidogenic enzyme pathway genes involved in neural estrogen biosynthesis in the grouper brain including *StAR*, *cyp19a1b*, *ERα*, *ERβ1*, and *ERβ2* and *GPR30* during early developmental ages, and the temporal expressions of the genes were observed during gonadal sex differentiation.

PCNA was initially identified as an auxiliary protein for mammalian DNA polymerase δ and it is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle [51]. PCNA can be used as a marker for cell proliferation [52] and for detection of dynamic changes during morphogenesis [53]. Thus, in the present study, we also studied the *pcna* gene expression profile during early development of the grouper brain in response to gonadal differentiation.

2.4. RNA extraction, cDNA library construction and molecular cloning of neural estrogen biosynthetic genes

Total RNA from forebrain, midbrain and hypothalamus at 90–180 dah (n = 8 in each age) was extracted using TRIzol® Reagent (Gibco BRL; Grand Island, NY) according to the manufacturer's instruction. The quality and concentration of RNA were assessed by spectrophotometry and checked by running an aliquot (1 μg) on a 1.8% agarose–formaldehyde gel. The cDNAs were synthesized from 1 μg of total RNA using Superscript II (Invitrogen; Carlsbad, CA) and oligo $(dT)_{12-18}$ primers in a 20 μl reaction volume with incubation at 42 °C for 60 min, 37 °C for 15 min, and 70 °C for 15 min and cDNAs were then stored at −80 °C until use.

Grouper *StAR* (GenBank accession number GU929702), *pcna* (HM637102), *ERα* (GU929705), *ERβ1* (JF304611), *ERβ2* (GU929706), *GPR30* (HM637103) and *AR* (GU929707) were partially cloned using respective degenerate primers based on the conserved regions of genes belonging to other teleosts, whereas for grouper *cyp19a1b* (AY510712), a 941 base pair product was amplified by RT-PCR using the specific primers designed from an already published complete cds in the NCBI database (Table 1). The PCR reaction was performed in a final volume of 25 μl reaction containing 2.5 μl of 10× reaction buffer (200 mM Tris–HCl, pH 8.4, 500 mM KCl), 1 μl of 10 mM dNTP, 1 μl of 2 mM $MgCl_2$, 0.5 μl each of 10 μM forward and reverse primer (degenerate primer), respectively, 0.2 μl superscript enzyme (Invitrogen I) and 1 μl cDNA. The reaction conditions for degenerate PCR were as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s and 72 °C for 7 min. Each PCR product was electrophoresed on 1.5% agarose gel and the fragment showing the predicted molecular weight was then excised using Gel-M™ Gel Extraction system kit (VIOGENE, Bio 101, La Jolla, CA). Extracted cDNAs were ligated into pGEM-T Easy vector (Promega, Madison, WI) and transformed into *Escherichia coli* competent cells following the manufacturer's instruction. Plasmid containing the insert was sequenced and compared with the NCBI database using BLAST.

2.5. Tissue distribution mRNA expression

In order to further analyze the tissue specific expression, we selected only *StAR* and *cyp19a1b*, because these genes play initial and terminal roles during steroidogenesis, respectively. Specifically, *StAR* is essential for transporting cholesterol from the outer to the inner mitochondrial membrane where the rate limiting step

Table 1
Oligonucleotide primers used for degenerate PCR, RT-PCR and Q-PCR analyses.

Gene	Orientation	Sequence	Usage
StAR	S	5′-CCTGCAACYTTYAARCTG-3′	Degenerate PCR
	AS	5′-CCACCTGYGTCTGAGAGAG-3′	Degenerate PCR
	S	5′-TCAGCACAGGGCTTCATCACTAT-3′	Q-PCR
	AS	5′-TGCAAAAATGCCTGAGCAAAG-3′	Q-PCR
cyp19a1b	S	5′-TGAGGAGGATGCAGCTGC-3′	RT-PCR
	AS	5′-GTGTCCTGCAGTTCCTGGGC-3′	RT-PCR
	S	5′-GCAAAATAGGGCTGGCATGTA-3′	Q-PCR
	AS	5′-TTTTTTCCAGAGCTCGACATCAC-3′	Q-PCR
pcna	S	5′-GGTBCTKGARGCKCTGAARG-3′	Degenerate PCR
	AS	5′-CDGGYTCRTTCATCTCAATBG-3′	Degenerate PCR
	S	5′-TTCCTGCACACTTCAGGATTTTT-3′	Q-PCR
	AS	5′-CTCATACCGGTGCGACAGAA-3′	Q-PCR
ERα	S	5′-GGGCTGC AAGGCCTT CTTTAAG-3′	Degenerate PCR
	AS	5′-TGGCCCAKGCGATCATGT-3′	Degenerate PCR
	S	5′-GGGCTGCAAGGCCTTCTTTAAG-3′	RT-PCR
	AS	5′-GGCCCAGGCGATCATGTG-3′	RT-PCR
	S	5′-CCTCAGGACGGGAGGAAAC-3′	Q-PCR
	AS	5′-TGCCAAGCAGCGGTGATT-3′	Q-PCR
ERβ1	S	5′-CTGGGCCAAGAAGATTCCAG-3′	Degenerate PCR
	AS	5′-GGCACYATGTTCTTCATTTTC-3′	Degenerate PCR
	S	5′-CTGGGCCAAGAAGATTCCAG-3′	RT-PCR
	AS	5′-GGCACTATGTTCTTCATTTTC-3′	RT-PCR
	S	5′-CCATCCAGGGAAACTTATCTTCTC-3′	Q-PCR
	AS	5′-CAGCTATCAGCATATCAAATATCTCAGA-3′	Q-PCR
ERβ2	S	5′-GAGGGSTGYAAGGCYTTCTTC-3′	Degenerate PCR
	AS	5′-CTCCTCCYTCTGHAGCTT-3′	Degenerate PCR
	S	5′-GCCCCGCCACTAATCAATG-3′	RT-PCR
	AS	5′-CGAAGCCAGGGATCTTTTTC-3′	RT-PCR
	S	5′-ATATCCTGATGTTGGGCCTGAT-3′	Q-PCR
	AS	5′-GAGTTTGAAGTCTGGAGAGAAGATGA-3′	Q-PCR
ARβ	S	5′-GCSMGRGAGYTSTGCAAAG-3′	Degenerate PCR
	AS	5′-CAGGAYGGRCARTTCTTYC-3′	Degenerate PCR
	S	5′-GGGTCTGGGAGCTCGTAAAC-3′	RT-PCR
	AS	5′-GGGGCGAAGTAAAGCATC-3′	RT-PCR
	S	5′-TCTCCTAAATTAGGTCTGAACACCAA-3′	Q-PCR
	AS	5′-ACCACCTCAGGCTCAATGGA-3′	Q-PCR
GPR30	S	5′-GGCAGATTCCCTCATTGAG-3′	RT-PCR
	AS	5′-GGGGTTGAGGCAGCTGTTG-3′	RT-PCR
	S	5′-GCTCAGAAACACCGTGGATTG-3′	Q-PCR
	AS	5′-ACCAGAACCACCACCACGAT-3′	Q-PCR
β-actin	S	5′-CCCCAAAGCCAACAGGGAGAAGATG-3′	RT-PCR
	AS	5′-GGGCAACGGAACCTCTCATTG-3′	RT-PCR
	S	5′-AGGGAGAAGATGACCCAGATCA-3′	Q-PCR
	AS	5′-GGGACAGCACAGCCTGCAT-3′	Q-PCR

S, sense strand; AS, antisense strand.

occurs, and aromatase (*cyp19a1b* gene) is the key enzyme that catalyzes the transformation of androgens into estrogens in neurosteroidogenesis. Thus, it is of interest to study their expression in different tissues during the period of brain development and gonadal sex differentiation in the grouper. Total RNA (1 μg) was isolated from the brain, pituitary, gill, head kidney, muscle, heart, liver, gonad, spleen and intestine. RT-PCR was performed using gene specific primers (Table 1). *β-actin* (AY510710) was used as housekeeping gene for RT-PCR. The PCR conditions were as follows: 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s and 72 °C for 7 min.

2.6. Quantification of neural estrogen related genes

Quantitative real-time PCR was employed to measure the transcript abundance of *StAR*, *cyp19a1b*, *ERα*, *ERβ2*, *GPR30*, *AR* and *pcna* in the forebrain, midbrain and hypothalamus at different developmental ages (90–180 dah) ($n = 8$ fish for each value). Q-PCR primers were designed for the respective genes using Primer Express software (Applied Biosystems) (Table 1). We used β-actin as housekeeping gene in the Q-PCR, because the transcript of β-actin did not change significantly in different parts of the brain in different developmental ages. Gene quantification of standards, samples and controls was conducted simultaneously in a Q-PCR machine (iQ™ Multicolor Real-Time PCR Detection System; Bio-Rad Co., Hercules, CA) with iQ™ SYBR green (Bio-Rad) as a dsDNA minor-groove binding agent, forward and reverse primers and water. Determination of transcript abundance for *StAR*, *cyp19a1b*, *pcna*, *ERα*, *ERβ2*, *GPR30* and *AR* was conducted in duplicate, normalized and calculated relative to the average of β-actin. Calculation of PCR efficiency was based on the slope of the relationship between log input cDNA (transcript concentrations) *vs* Ct (the calculated fractional cycle number at which the PCR-fluorescence product is detectable above a threshold) was obtained. We used an efficiency-corrected method for the calculation of relative expression level of grouper steroidogenic genes and sex steroid nuclear receptors in different developmental ages. The correlation of the standard curve for *StAR*, *cyp19a1b*, *pcna* and *GPR30* was −0.999 and −0.990 for the rest of genes.

2.7. Measurement of brain aromatase activity

Aromatase activity in the brain during different developmental ages from 90 to 180 dah was quantified by the radiometric method in which the stereospecific loss of hydrogen from the C-1β position of 1β-[^{3}H] androstenedione (^{3}H-A) during aromatization and the formation of H_2O was measured according to our previous study, with minor modification [35,54]. Briefly, brain tissues

such as the forebrain, midbrain and hypothalamus ($n = 5$ fish for each value) were homogenized in a potassium phosphate buffer (1 mM EDTA, 100 mM KCl, 10 mM KH_2PO_4 and 10 mM dithiothreitol, pH 7.4). The protein concentrations of the crude supernatant fraction were measured by a Bradford assay kit (Bio-Rad Co., Hercules, CA). Absorbance was measured in samples at 595 nm in an ELISA reader using bovine serum albumin as a protein standard. Aromatase activity was expressed as fmol of ^3H_2O/h mg protein (femtomole/h mg protein).

2.8. Enzyme immunoassay for brain E2 and T

Brain E2 and T concentrations were measured in the forebrain and hypothalamus ($n = 6$ fish per value) at 90, 120, 150 and 180 dah using an enzyme immunoassay kit (EIA) (Cayman Chemical Company, Ann Arbor, MI). It is well known that estrogen and aromatase are expressed highly in the forebrain and hypothalamic area of the teleost brain [15,18]. Therefore, in the present study we used only the forebrain and hypothalamus tissues to measure the concentrations of brain E2 and T during gonadal sex differentiation. Briefly, the assays were validated prior to use by adding different concentrations of steroid hormones (spikes) to the brain samples. The values detected from the spiked samples were parallel to the respective standard curve and the recovery was satisfactory. The brain samples were homogenized in 10× volume (mg/ml) of phosphate buffered saline and centrifuged at 13,000 rpm at 4 °C for 15 min. The supernatants were purified by extraction using organic solvent to prevent the interference of lipids and proteins in the analysis. The supernatant was mixed with 3 ml diethyl ether using a vortex mixer. After phase separation, the aqueous portion was frozen in a freezer. The lipophilic phase was decanted into a clean tube, and the ether phase was evaporated by heating to 40 °C. The dry extract was reconstituted in 500 ml EIA buffer by vortexing and stored at −20 °C until further use. Steroid levels of brain samples were tested from different dilutions (1:10, 1:20, 1:40, 1:60 and 1:100) and high levels of E2 and T were detected between the dilution ranges of 1:60 to 1:100. We thus used 1:80 dilutions of brain samples for the present study. No lipid interference was observed for the brain samples because of small size of brain tissues were used. Enzyme immunoassays were run according to the manufacturer's instructions with a development time of 60–90 min for both E2 and T. Sample plates were read at a wavelength of 410 nm for E2 and 415 nm for T, respectively using an ELISA reader. Data were quantified against a standard curve that was linearized using a logit transformation of B/B_0 (% Sample bound/maximum bound).

2.9. Data analysis

Data are presented as the mean ± SEM of the values obtained from the forebrain, midbrain and hypothalamus at different developmental ages (90–180 dah). All data were analyzed using the SPSS (version10.0; SPSS Inc., Chicago, IL) statistical package. Statistical significance of the differences between different developmental ages was assessed by one-way ANOVA followed by S–N–K (Student–Newman–Keuls) multiple comparison test with a significance level of $P < 0.05$. Student t-test was also conducted to compare the significant difference ($P < 0.05$) between control and E2 injected fish.

3. Results

3.1. Tissue distribution

The spatial distributions of *StAR* and *cyp19a1b* were investigated in different tissues of the orange-spotted grouper (Fig. 1). Expression of *StAR* was detectable in the forebrain and midbrain, but the strongest band was found in the head kidney and gonad. *cyp19a1b* expression was very strong only in the forebrain, but a weak band was also observed in the midbrain, hypothalamus and spleen. No expression of *cyp19a1b* was found in the gonad.

3.2. Developmental expression profiles of neural estrogen related genes by quantitative real-time PCR analysis

3.2.1. StAR

Relative transcripts of *StAR* significantly ($P < 0.05$) increased at 110 dah in the forebrain, midbrain and hypothalamus compared to 90 dah (Fig. 2A). *StAR* mRNA levels at 110 dah in the forebrain, midbrain and hypothalamus were 3.2-, 3.5-, 2.8-fold and 1.5-, 1.7-, 1.3-fold higher when compared to 90 and 180 dah, respectively.

3.2.2. cyp19a1b

mRNA expression of *cyp19a1b* was more abundant ($P < 0.05$) at 110 dah in the forebrain compared with other brain regions, with 2.6- and 6.2-fold higher expression when compared to the midbrain and hypothalamus, respectively (Fig. 2B). In the hypothalamus, *cy19a1b* expression gradually and significantly increased from 90 to 130 dah, but was not different from 130 to 180 dah. In the midbrain, no significant differences were found among the different developmental ages (Fig. 2B).

3.2.3. pcna

Transcripts of *pcna* were highest at 110 dah in the midbrain and 110–150 dah in the hypothalamus ($P < 0.05$) (Fig. 2C). In the forebrain, the *pcna* level was highest at 110 dah, but was only significantly different ($P < 0.05$) when compared to 130 dah. There was no significant difference among 90, 150 or 180 dah in the forebrain and midbrain. The transcripts showed a 1.3-fold and 1.7-fold increase ($P < 0.05$) in the forebrain and midbrain, respectively, at 110 dah compared to 90 dah (Fig. 2C). Moreover, significantly lower expression was found at 180 dah in the hypothalamus compared to the forebrain and midbrain.

3.2.4. AR

AR transcripts gradually decreased from 110 dah to 150 dah, showed a significant ($P < 0.05$) increase at 90 dah and had a high expression level at 180 dah in the forebrain (Fig. 2D). The level of the transcripts in the midbrain increased at 110 dah, and showing highest expression at 180 dah. Expression of *AR* at 180 dah was 2.5- and 4-fold higher compared to that at 150 dah in the forebrain and midbrain, respectively (Fig. 2D). The transcript levels in the hypothalamus were not significantly different among the different developmental ages.

3.2.5. ERα

ERα mRNA expression was relatively high at 110 dah in the forebrain and midbrain, whereas contrasting results were found in the hypothalamus; the expression level of *ERα* in the hypothalamus significantly increased from 90 to 150 dah and showed very low expression at 180 dah (Fig. 3A). Expression levels in the forebrain, midbrain and hypothalamus at 110 dah were 2.4-, 2.3- and 5.2-fold higher compared to that at 90 dah in the respective brain regions (Fig. 3A). A statistically significant ($P < 0.05$) peak in *ERα* expression was also found at 180 dah in the midbrain, and this expression was 146-fold higher than the level in the forebrain.

3.2.6. ERβ1

ERβ1 had highest expression at 110 dah in the forebrain and midbrain whereas high expression in the hypothalamus was found from 130 to 180 dah (Fig. 3B). *ERβ1* exhibited 6.7- and 1.8-fold higher expression in the forebrain and midbrain, respectively, at 110 dah compared to 90 dah. In addition, *ERβ1* expression was also

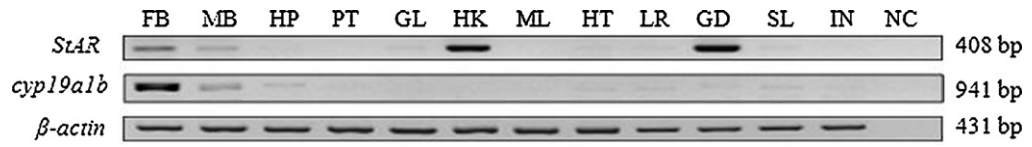

Fig. 1. Tissue mRNA expression of the gene encoding *StAR* and *cyp19a1b* in grouper. Tissues were collected from forebrain (FB), midbrain (MB), hypothalamus (HYP), pituitary (PT), gill (GL), head kidney (HK), muscle (ML), heart (HR), liver (LR), gonad (GD), spleen (SL), intestine (IN) and negative control (NC) in order to analyze tissue distribution. *β-actin* was used as an internal control for tissue distribution analysis.

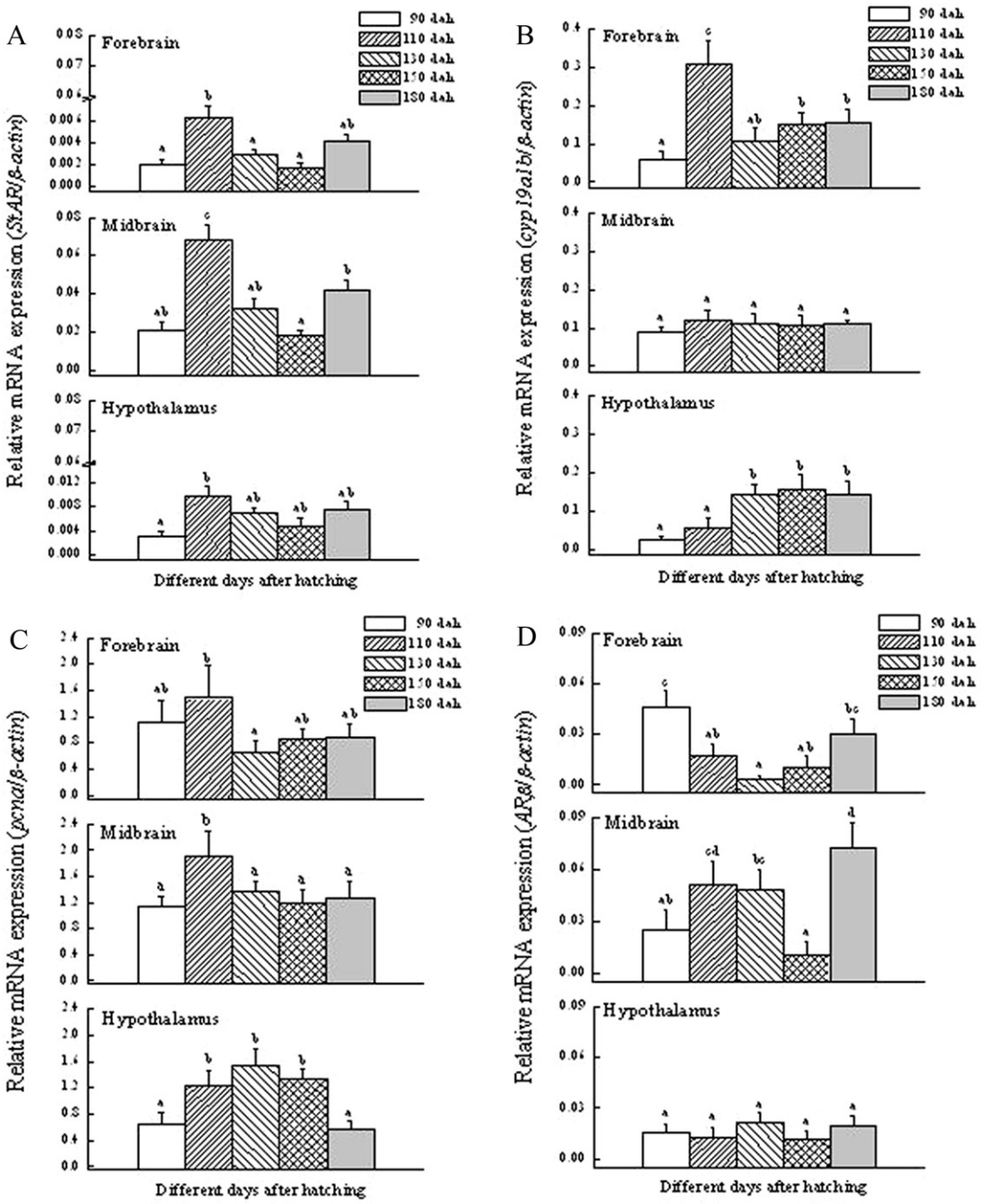

Fig. 2. Relative transcripts of *StAR*, *cyp19a1b*, *pcna* and *AR* in the forebrain, midbrain and hypothalamus from 90 to 180 dah. Gene expression levels are expressed as mean normalized expression (±SEM) of eight samples as determined by Q-PCR. Different letters indicate statistically significant differences ($P < 0.05$) in gene expression at different developmental ages.

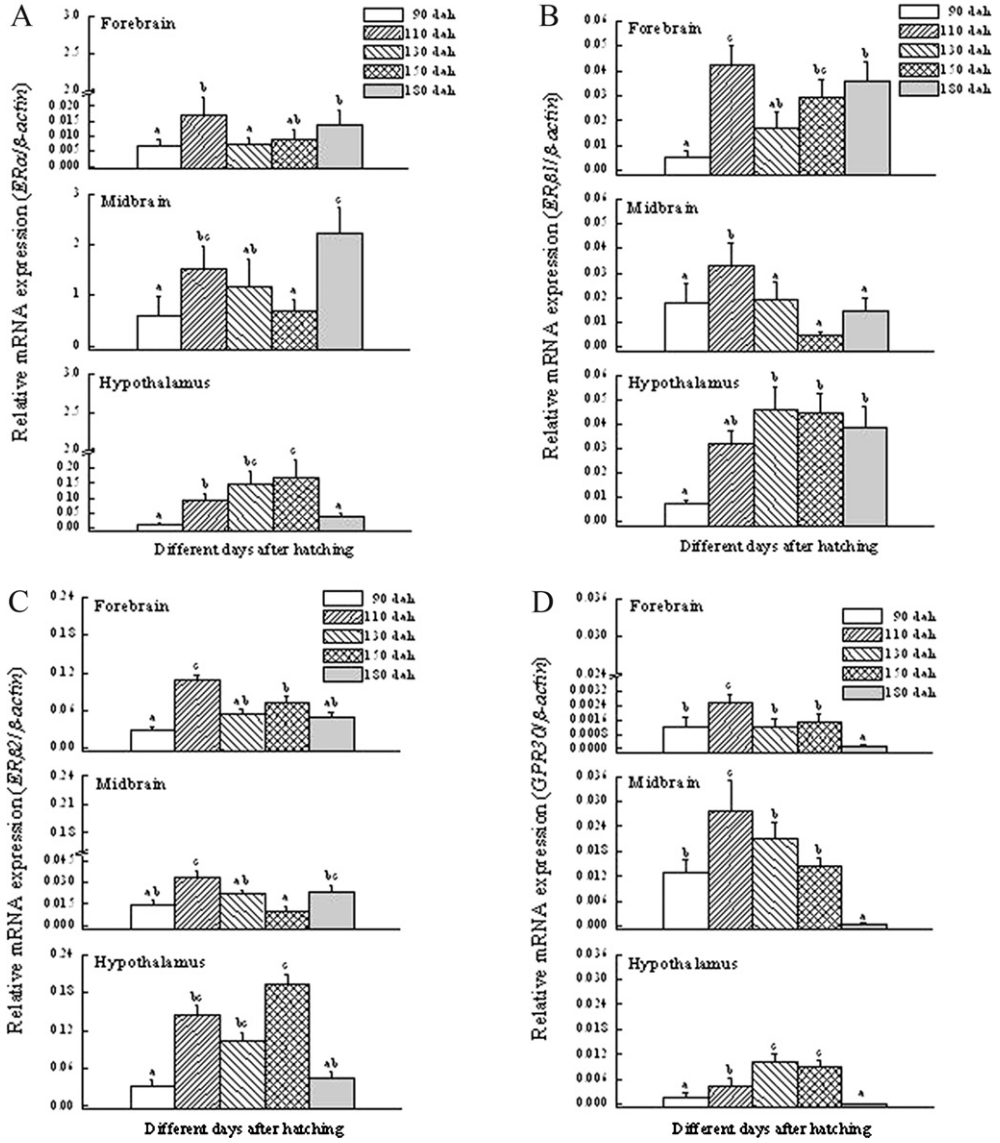

Fig. 3. Relative transcripts of sex steroid receptors of *ERα*, *ERβ1*, *ERβ2*, and *GPR30* in the forebrain, midbrain and hypothalamus from 90 to 180 dah. Gene expression levels are expressed as mean normalized expression (±SEM) of eight samples as determined by Q-PCR. Different letters indicate statistically significant differences ($P < 0.05$) in gene expression at different developmental ages.

significantly ($P < 0.05$) higher in the forebrain at 180 dah. Expression of *ERβ1* in the hypothalamus was not significantly different in 110 dah compared to 90, 130, 150 or 180 dah (Fig. 3B).

3.2.7. ERβ2

At 110 dah, the expression of *ERβ2* was significantly ($P < 0.05$) higher in the forebrain, midbrain and hypothalamus as compared to that at 90 dah, whereas in the hypothalamus highest expression levels were found at 150 dah (Fig. 3C). The expression levels of *ERβ2* were similar in the forebrain, hypothalamus and midbrain at 90 dah. The increased expression of *ERβ2* at 110 dah was 2.8-, 2.5- and 3.5-fold higher than that at 90 dah in the forebrain, midbrain and hypothalamus, respectively (Fig. 3C).

3.2.8. GPR30

GPR30 transcripts exhibited a significant ($P < 0.05$) peak at 110 dah in the forebrain and midbrain and gradually increased from 90 to 150 dah in the hypothalamus (Fig. 3D). The transcript levels of *GPR30* showed 2.1- and 2.2-fold higher expression at 110 dah in the forebrain and midbrain, respectively, and 5.5-fold at 130 dah in the hypothalamus compared to 90 dah (Fig. 3D). The expression pattern of *GPR30* followed a similar trend to that of *ERα* and *ERβ2*, with a significantly higher expression level at 110 dah in the forebrain and midbrain. However, the mRNA expression level of *GPR30* was low compared to the mRNA levels of *ERα* and *ERβ*, and it was low transcript in all parts of the brain at 180 dah.

3.3. Aromatase activity

Aromatase activity was measured in the forebrain, midbrain and hypothalamus at different developmental ages (90, 110, 130, 150 and 180 dah) (Fig. 4). Aromatase activity was significantly ($P < 0.05$) higher in the forebrain at 110 dah compared to other ages (Fig. 4). However in the hypothalamus, highest levels of aromatase activity were detected at 130, 150 and 180 dah compared to 90 and 110 dah (Fig. 4). In contrast to this, in the midbrain, no significant differences were found among the different ages (Fig. 4).

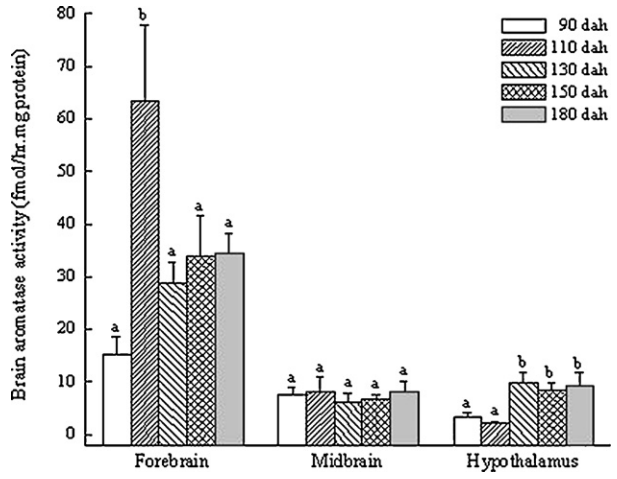

Fig. 4. Changes in brain aromatase activity in the forebrain, midbrain and hypothalamus during different developmental ages (90–180 dah). Aromatase activity was expressed as femtomole of ^3H_2O/h mg protein. Values are expressed as mean ± SEM of five samples. Different letters indicate significant ($P < 0.05$) differences among the different developmental ages.

3.4. Brain E2 and T

The levels of E2 and T in brain tissues were quantified by an EIA method (Fig. 5). Brain E2 levels increased at 120 dah in the forebrain, with significantly higher concentrations ($P < 0.05$) when compared to 90 dah but not 150 or 180 dah (Fig. 5A). The concentrations of E2 in the forebrain at 120 dah were 4-fold higher when compared to 90 dah. In the hypothalamus, E2 levels were significantly ($P < 0.05$) higher at 150 dah than that at 90, 120, and 180 dah (3.1-fold higher as compared to that the value at 90 dah). The levels of brain T were significantly ($P < 0.05$) higher in the forebrain at 90 dah, whereas brain T levels did not show any significant differences between the age group of 120, 150 and 180 dah in the forebrain. In the hypothalamus, significantly ($P < 0.05$) higher levels of T were observed at 90 dah when compared with 120–180 dah (Fig. 5B).

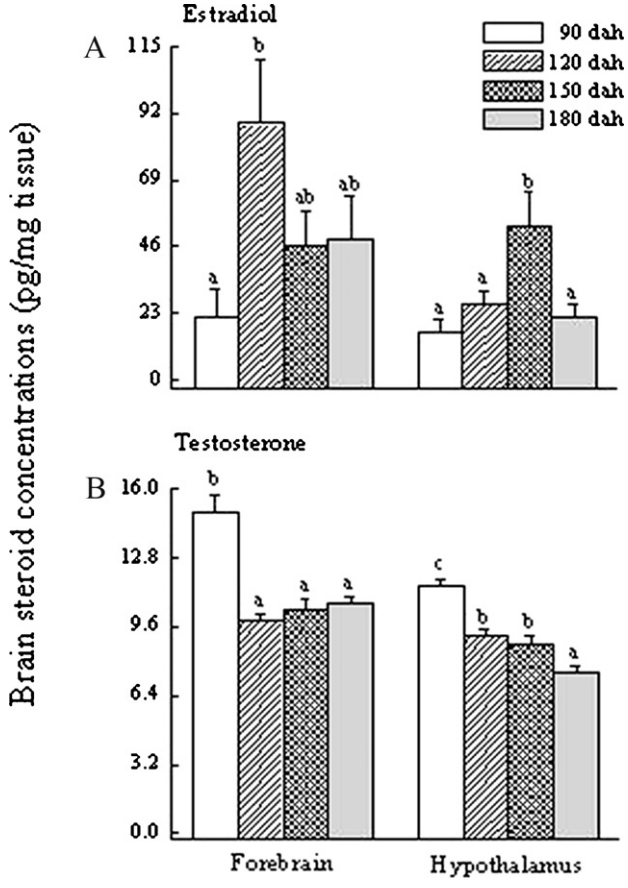

Fig. 5. Steroid concentrations (pg/mg tissue) in orange-spotted grouper brain during early developmental ages. A, estradiol and B, testosterone in the forebrain and hypothalamus at 90, 120, 150 and 180 dah. Data are given as mean ± SEM of six samples. Different letters indicate statistically significant differences ($P < 0.05$) between the different developmental ages.

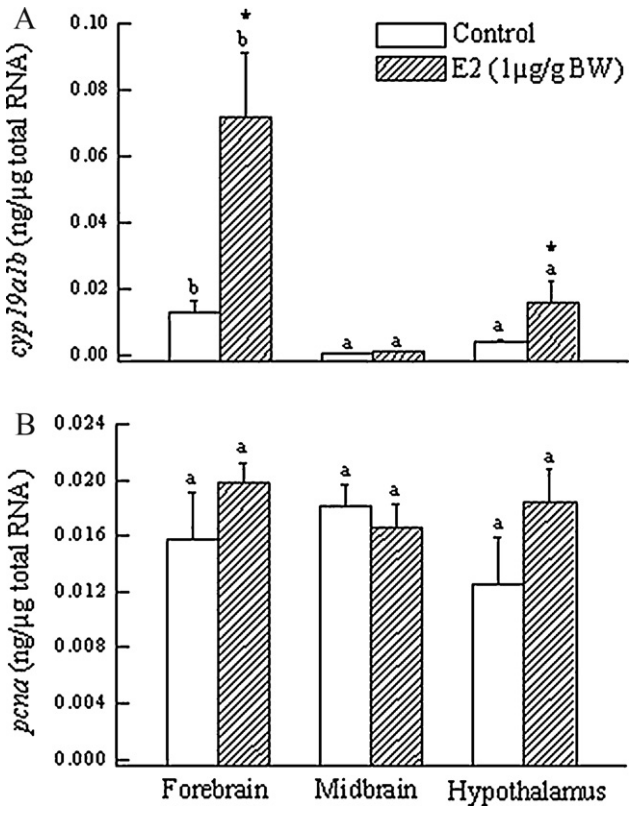

Fig. 6. *In vivo* effects of estradiol (E2, 1 μg/g BW) on *cyp19a1b* and *pcna* expression in the forebrain, midbrain and hypothalamus. The results are expressed as mean ± SEM ($n = 10$ in each value). Different letters indicate statistically significant differences ($P < 0.05$) between the different parts of the brain in the same group. Asterisk (*) represent significant ($P < 0.05$) differences between the control and E2 injected group.

3.5. *In vivo* sex steroid effects on grouper *cyp19a1b* and *pcna* expression

3.5.1. *cyp19a1b*

E2 (1 μg/g BW) significantly induced *cyp19a1b* expression in the forebrain and hypothalamus but not in the midbrain. E2 injection caused an increase in *cyp19a1b* transcripts of 5.6-fold ($P < 0.05$) in the forebrain and 4.2-fold ($P < 0.05$) in the hypothalamus (Fig. 6A). However, the E2 effect on *cyp19a1b* expression in the midbrain was not significantly different from the control group (Fig. 6A).

3.5.2. *pcna*

Injection with E2 (1 μg/g BW) had no significant effect on *pcna* expression in the forebrain, midbrain or hypothalamus. However, non-significant increases in expression were detected in the forebrain and hypothalamus as compared to the control (Fig. 6B).

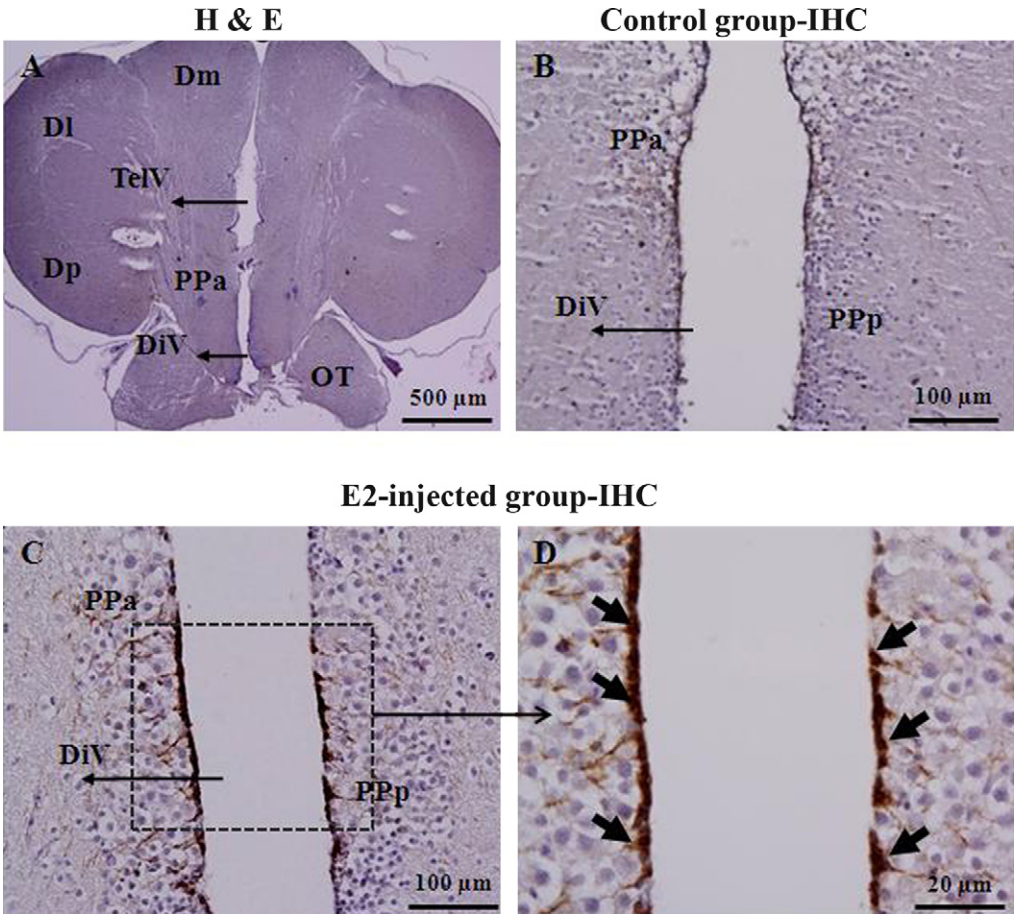

Fig. 7. Transverse sections of brain showing distribution of immunoreactive Cyp19a1b-possitive cells in the control and E2 injected (1 μg/g BW) groupers. A–D: A, control brain stained with hematoxylin and eosin; B, control brain showing expression of Cyp19a1b on the diencephalic ventricle; C, E2-injected brain showing expression of Cyp19a1b on the diencephalic ventricle and preoptic area, and D, higher magnification *f* the radial glial cells along the ventricles of the forebrain. Stronger Cyp19a1b expression was observed in the radial glial cells (indicated by arrows) along the ventricle of the preoptic area in E2 injected fish as compared to the control fish. Dl, lateral zone of dorsal telecephalic area; Dm, medial zone of dorsal telecephalic area; Dp, posterior zone of dorsal telecephalic area; DiV, diencephalic ventricle; OT, optic tract; PPa, parvocellular preoptic nucleus-anterior part; PPp, parvocellular preoptic nucleus-posterior part; TelV, telecephalic ventricle.

3.6. Cyp19a1b immunoreactivity in the radial glial cells of the grouper forebrain

The histological section of the forebrain stained with H&E is shown in Fig. 7A. Stronger staining of irCyp19a1b cells was found in the forebrain of E2 injected fish (Fig. 7C and D) compared to the preoptic area and diencephalic ventricle of the control group (Fig. 7B). The expression of Cyp19a1b was clearly found in the radial glial cells of the forebrain (Fig. 7C). Expressions of irCyp19a1b were abundantly found along to the radial glial cells of the diencephalic ventricle and preoptic area (Fig. 7C and D) of the E2 injected fish. However, no staining was observed corresponding to the other cell types, such as neurons, in the E2 injected fish. A large majority of the radial cells had a small round nucleus located close to the ventricle (Fig. 7D). No staining was observed when the sections were incubated with normal guinea pig pre-sera instead of the primary antibody (data not shown).

4. Discussion

In the present study, we characterized the developmental expression patterns of genes that are involved in neural estrogen biosynthesis including *StAR*, *cyp19a1b*, and ERs during the early developmental ages from 90 to 180 dah in the forebrain, midbrain and hypothalamus by quantitative real-time PCR analyses. We also measured brain aromatase activity and brain sex steroid levels (brain E2 and T) at the same ages. The changes of temporal expression of these genes in brain were demonstrated for the first time in accordance with neurosteroidogenesis and gonadal sex differentiation in the orange-spotted grouper.

Quantitative real-time PCR analysis demonstrated that the mRNA expression levels of genes in the neural estrogen biosynthetic pathway in the grouper brain were expressed from 90 dah onward. The mRNA level of *StAR*, a key gene in the steroidogenic enzyme pathway for transferring of cholesterol from the outer to the inner mitochondrial membrane, was significantly higher at 110 dah in all brain regions when the gonad was differentiating. This significant increase in the mRNA of *StAR*, considering its rate-limiting function and its role in the regulation of neurosteroidogenesis, also suggested a functional significance in the brain. Developmental expression changes of *StAR* have been reported in the protandrous black porgy brain, which showed high expression from 75 dah with significant increases at 120 dah in the forebrain and hypothalamus [6]. Moreover, Kim et al. [55] reported that *StAR* mRNA was expressed at very low levels in various brain regions of the rat after adrenalectomy and gonadectomy. The continued expression of steroid compounds several weeks after the surgical removal of the adrenal glands and gonads was due to their *de novo* synthesis in the brain [56]. Thus, the above findings suggest that *StAR* expressed in the early brain is independent of expression in adrenal or gonadal tissue. However, in freshwater stingrays (*Potamotrygon* spp.), *StAR* mRNA is expressed in several tissues such as

the atria, ventricle, gill, interrenal gland and muscle, suggesting that *StAR* may be critical to processes other than steroidogenesis [57].

Estrogens exert robust and wide-ranging effects on the developing brain through their receptors in vertebrates [58]. In zebrafish, early expression of brain ER can regulate *cyp19a1b* expression during early development [13]. In the present study, we revealed that the expression of *ERα*, *ERβ1* and *ERβ2* exhibited similar but not identical expression patterns together with peak expression of *cyp19a1b* at 110 dah in the forebrain, compared to other brain regions. These results suggest that the ERs are involved in the specificity of estrogen signaling and, more specifically, in the proper development of steroid-sensitive brain areas and thus neurosteroidogenesis and female brain development during gonadal sex differentiation. In contrast, the expression of *ERα* and *ERβ2* increased from 110 to 150 dah in the hypothalamus. In rats, double-label *in situ* hybridization/immunohistochemistry experiments established that the two ER subtypes (*ERα* and *ERβ*) were expressed in different cells of the forebrain (neurons in the bed nucleus of the stria terminalis, medial amygdale and preoptic area contain both ERs), suggesting that estrogens may regulate some neuronal populations depending on the expression of *ERα*, *ERβ* or both ERs [59]. Differential expression of *ERα*, *ERβ1* and *ERβ2* was identified in different tissues of the fathead minnow (*Pimephales promelas*) during early development and in adults, indicating different mechanisms of regulation for the different ERs [41]. Tomy et al. [6] reported that these two ER subtypes were expressed in the brain of male black porgy from 75 dah with a later peak during gonadal sex differentiation. The above observations suggest functional roles of each ER in adult and during early developmental ages, although the exact physiological roles of each ER subtype in neurosteroidogenesis with respect to brain development require further research.

The membrane estrogen receptor *GPR30* was also significantly increased at 110 dah in the forebrain and midbrain, suggesting a role for *GPR30* as a plasma membrane-associated GPCR mediating rapid estrogen effects on the developing grouper brain [46]. Distribution of *GPR30* in the adult rat and mouse brain, as revealed by *in situ* hybridization/immunohistochemistry, was observed with the intense signals in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus, anterior and intermediate lobe of the pituitary and the medulla oblongata. These data revealed the expression of *GPR30* in many different types of neurons [60,61], whereas another study in zebrafish suggested that *GPR30* regulates oocyte maturation [62]. However, data on the early expression of the membrane estrogen receptor (*GPR30*) associated with nuclear estrogen receptors (*ERα*, *ERβ1* and *ERβ2*) in the brain during gonadal sex differentiation of teleosts in response to neurosteroidogenesis remain poor. In the present study, we reported for the first time that the developmental expression patterns of the nuclear and non-nuclear membrane estrogen receptors in the developing brain changed in response to gonadal sex differentiation. However, the precise location of transcripts and their relative abundance in the developing grouper brain remain to be determined. Furthermore, *AR* was expressed from 90 dah and significantly decreased from 110 to 150 dah in the forebrain, whereas expression of the ERs increased. However, higher expression of *AR* was also observed at 110 and 180 dah in the midbrain, but no significant changes were observed in the hypothalamus among the ages. Thus, this finding suggests that *AR* expression in the brain did not have a close association with the gonadal differentiation when compared with the expression of the ERs. Further studies defining the precise localization of ERs transcripts in the brain in response to physiological responses of neural development are needed.

Expression of *cyp19a1b* was significantly ($P < 0.05$) elevated in the forebrain at 110 dah (a period close to gonadal sex differentiation), and aromatase activity was also significantly increased in the forebrain at the same time point. These data indicate that the forebrain is more sensitive to expression of sex steroids than other regions in the brain [7,15]. In the blue-headed wrasse (*Thalassoma bifasciatum*), aromatase-labeled cell bodies were visualized throughout the brain, but the greatest abundance was observed in the forebrain [63]. Thus, we suggest the involvement of the estrogen system in early brain development of the female grouper. However, the exact relationship between brain and gonadal sex differentiation is of great interest but is still not clear and needs further investigation. Brain E2 levels were significantly ($P < 0.05$) high at 120 dah when compared to 90 dah in the forebrain. These data were consistent with the findings of brain *cyp19a1b* transcripts and aromatase activity in the grouper at 110 dah and brain E2 results in the male black porgy at 120 dah, in which the expression of brain E2 was high at 120 dah compared to 90 and 150 dah [5]. The slight difference in the timing for the peak expression of *cyp119a1b* transcripts/aromatase activity (110 dah) and sex steroids (120 dah) may be due to the different batches of samples used for the experiments. The level of E2 in the hypothalamus as compared to the forebrain was low, but a significant increase was observed at 150 dah compared to 90, 120 and 180 dah in the hypothalamus. Since aromatase expression and enzyme activity levels are correlated [64,65], aromatase mRNA levels have been reported to be an indicator of estrogen production and sexual differentiation in fish [28,29,66,67]. In our results, *cyp19a1b* mRNA, aromatase enzyme activity, brain E2 levels and mRNA expression of the ERs were consistently and significantly higher during early development in the forebrain. Therefore, it is suggested that the peak of neurosteroidogenesis and *de novo* synthesis of E2 in the early brain may play a physiological role at an early developmental age. This E2 peak locally produced in the brain may be responsible for the early brain neurogenesis and initiation of brain development. High *pcna* mRNA expression (a marker of cell proliferation activity) was also found in the early grouper brain.

Further, E2 treatment resulted in significantly increased *cyp19a1b* transcripts in the forebrain and hypothalamus but not in the midbrain. *pcna* had a very slight but non-significant increase in E2 injected fish. Increased expression of *cyp19a1b* transcripts in E2 injected fish implied a potential regulatory effect of E2 on *cyp19a1b* expression. IrCyp19a1b-positive cells were more abundant in the radial glial cells of the preoptic area and diencephalic ventricle of E2 injected fish. However, no staining was observed in neurons or other cell types in the forebrain area, suggesting brain aromatase expression is restricted to the radial glial cells and brain aromatase expression could probably be regulated by estrogenic activity in the developing and adult grouper fish brain due to the high levels of neurogenesis. Thus, locally produced E2 in the brain could be responsible for brain development. In agreement with the E2 treatment studies, *cyp19a1b* has been shown to be up-regulated by E2 in a dose-dependent manner in the early development of teleost fish [10,11,67] and the expression of brain aromatase enzyme was mainly found in the radial glial cell population, but not in the neurons of the adult zebrafish [27,68,69] and plainfin midshipman (*Porichthys notatus*) [15]. Consequently, the present results supported the hypothesis that E2 may play an important role for the development in the early brain.

In summary, our present study measured for the first time the relative expression of *StAR*, *cyp19a1b*, *pcna* and steroid receptors (*ERα*, *ERβ1*, *ERβ2*, *GPR30* and *AR*) during early development in the brain of the grouper. The present results showed significantly higher expression of *StAR*, *cyp19a1b*, ERs and *GPR30* but not *AR* at 110 dah in the brain of the grouper. Moreover, aromatase activity and brain E2 levels were dramatically increased in the forebrain early at 110 and 120 dah, whereas brain T levels were significantly decreased from 110 to 180 dah demonstrating that the synthesis of neural E2 but not T are responsible for early brain development in the female grouper. Further, exogenous E2 upregulated

cyp19a1b transcripts and irCyp19a1b-positive cells in the brain. irCyp19a1b expression was localized specifically in the radial glial cells of the forebrain regions. Cell proliferation activity (as indicated by *pcna* expression) was also increased between 110 and 150 dah in the brain. Therefore, our data illustrated that the development of the female brain during early development could be mediated by sex steroids and estrogen receptor-dependent events. Finally, peak development of the brain is suggested to be associated with gonadal sex differentiation.

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