Activation of Brain Steroidogenesis and Neurogenesis during the Gonadal Differentiation in Protandrous Black Porgy, *Acanthopagrus schlegelii*

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ABSTRACT: **The early brain development, at the time of gonadal differentiation was investigated using a protandrous teleost, black porgy. This natural model of monosex juvenile fish avoids the potential complexity of sexual dimorphism. Brain neurogenesis was evaluated by histological analyses of the diencephalon, at the time of testicular differentiation (in fish between 90 and 150 days after hatching). Increases in the number of both Nissl-stained total brain cells, and Pcna-immunostained proliferative brain cells were observed in specific area of the diencephalon, such as ventromedialis thalami and posterior preoptic area, revealing brain cell proliferation. qPCR analyses showed significantly higher expression of the radial glial cell marker *blbp* and neuron marker *bdnf*. Strong immunohistochemical staining of Blbp and extended cellular projections were observed. A peak expression of aromatase (*cyp19a1b*), as well as an increase in estradiol (E_2) content were also detected in the early brain. These data demonstrate that during gonadal differentiation, the early brain exhibits increased E_2 synthesis, cell proliferation, and neurogenesis. To investigate the role of E_2 in early brain, undifferentiated fish were treated with E_2 or aromatase inhibitor (AI). E_2 treatment upregulated brain *cyp19a1b* and *blbp* expression, and enhanced brain cell proliferation. Conversely, AI reduced brain cell proliferation. Castration experiment did not influence the brain gene expression patterns and the brain cell number. Our data clearly support E_2 biosynthesis in the early brain, and that brain E_2 induces neurogenesis. These peak activity patterns in the early brain occur at the time of gonad differentiation but are independent of the gonads.**
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Keywords: **brain development; neurogenenesis; neurosteroidogenesis; aromatase; estradiol**

INTRODUCTION

Neurosteroidogenesis and neurogenesis are occurring during the early brain development in vertebrates (Compagnone and Mellon, 2000; Tsutsui et al., 2000). Steroid hormones, especially estradiol (E_2), play important roles in vertebrate brain development, by promoting neurogenesis, cell migration and survival (McCarthy, 2008). E_2 may regulate brain neurogenesis, cell proliferation, cell fate, dendritic growth, synaptic plasticity, synaptic activity, and may promote neuroprotection (Martinez-Cerdeno et al., 2006; Sasahara et al., 2007; Mouriec et al., 2008). E_2 in the brain may determine the survival or death of pre-existing cells. Brain steroids may be originally synthesized in the gonad and reach the brain via the

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general circulation. The brain may also produce steroids locally including E_2 via neurosteroidogenesis (Diotel et al., 2011).

It has been well documented in rodents (Lenz et al., 2012) and avians (Schlinger, 1998) that the establishment of sexual dimorphism in brain regions is regulated by sex steroids (androgens or estrogens). There are a growing number of researches on brain sex differentiation in fish models such as in killifish and zebrafish (Lauer et al., 2006; Ampatzis and Dermon, 2007; Ampatzis et al., 2012). Sex steroids and cortisol were shown to affect the gonadal sex as well as sex behavior related to sexual function of the brain in fish (Guiguen et al., 2010; Fernandino et al., 2013; Saoshiro et al., 2013).

Radial glial cells (RGCs), a unique cell type in the brain, are involved in cell proliferation, migration and differentiation. RGCs are considered to be the progenitor cells in the brains of vertebrates (Hartfuss et al., 2001; Pinto and Gotz, 2007), including fish (Pellegrini et al., 2007). These cells produce new born glial cells and neurons and provide a track for neurons to migrate to their final destinations and to differentiate into the appropriate functions (Pinto and Gotz, 2007). Brain lipid-binding protein (BLBP) belongs to a family of low molecular weight hydrophobic ligand-binding proteins and is expressed in RGCs. BLBP is involved in the signal transduction and metabolism that initiates the adhesive interaction between glia and neurons. BLBP is required for neuronal differentiation and migration in the developing CNS (Feng et al., 1994; Feng and Heintz, 1995). Brain-derived neurotrophic factor (BDNF) produced in neurons is a member of the neurotrophin family of growth factors that is involved in neurogenesis and neuron development (Lessmann et al., 2003). BDNF is the most active neurotrophin and promotes the survival of existing neurons, the differentiation of new neurons, and the growth of synapses (Neveu and Arenas, 1996).

The cytochrome P450 aromatase, which is the rate-limiting enzyme in the conversion of androgens to estrogens, is encoded by *Cyp19a1*. A single type of aromatase gene is present in mammals and expressed in the gonad and in the brain, both in neurons and RGC (Balthazart and Ball, 1998; Golovine et al., 2003). In contrast, teleosts have two tissue-specific types of aromatase, one that is expressed in the gonad (aromatase A) and the other in the brain (aromatase B). Teleost brain aromatase, encoded by *cyp19a1b*, is expressed only in RGCs and is considered to be critical for E_2 biosynthesis in the fish brain (Lephart, 1996; Forlano et al., 2001). Brain aromatase enzyme activity is much higher in teleost fish than in mammals. The higher *cyp19a1b* expression in fish is related to neurogenic activity and has been suggested to ensure the plasticity of the teleost brain (Godwin, 2010; Le Page et al., 2010).

Black porgy, *Acanthopagrus schlegelii*, is a hermaphrodite protandrous teleost, that spends its first 2 years as a functional male and changes to a female in the third year. This sex pattern provides a unique model of natural mono-sex male fish population to investigate the early brain development. From previous studies, we know that increased germ cell proliferation occurs in the gonads of 4-month-old (120 days after hatching, dah) black porgy fish and that spermatogonia and lobular testicular tissue are present at 150 dah. It is during this period, that the main events of testicular differentiation occur (Wu et al., 2008). These observations were confirmed by the increased gonadal gene expression profiles of the male differentiation gene (*dmrt1*), germ cell development marker (*vasa*), and other gonad-specific genes (Wu et al., 2008, 2012). Our previous studies also showed that during the period of gonad differentiation, there is a peak expression of neurosteroidogenesis-related genes in the brain, including *star* (steroidogenic pathway), *cyp19a1b* (estrogen biosynthesis) and *er* (estrogen receptors) (Tomy et al., 2009). The biological significance of this peak of neurosteroidogenesis is still not known.

In this study, we applied histology and immunohistochemistry (IHC) to investigate the neurogenic activity, cell proliferation and activity of brain cells during the period of gonad differentiation. We first aimed to investigate whether brain neurosteroidogenic activity was associated with the induction of brain neurogenesis. We analyzed the variation in brain cell proliferation and cell projections during gonad differentiation by histology, gene expression analysis, and IHC, using markers such as Blbp, Bdnf, and proliferating cell nuclear antigen (Pcna). We further investigated the role of E_2 in brain neurogenesis by treating the fish with E_2 or with an aromatase inhibitor (AI). Finally, as these peaks of brain activity, neurosteroidogenesis, and neurogenesis, appeared to occur at the time of gonad differentiation, we determined whether gonad factors affect neurogenic activity in the early brain, by performing castration experiment.

MATERIAL AND METHODS

Animals, Biometry, and Tissue Sampling

Black porgy (60 dah) were obtained from an aquaculture farm and acclimated to the ponds at the National Taiwan

Ocean University culture station (NTOU; Keelung, Taiwan) in seawater with a natural light system (salinity 33 ppt; temperature 20–24°C). The fish were fed with commercial feed (Fwa Sou Feed, Taichung, Taiwan) twice a day (8% of their body weight in total feed per day). The study was performed on juvenile fish, which were all mono-sex males, because black porgy is a protandrous fish. We took advantage of natural mono-sex male for the experiment to avoid the complexity of sex dimorphism. The gonad developmental stages were defined according to previous reports (Tomy et al., 2007; Wu et al., 2008).

Fish were anesthetized in a solution of 0.1% 2-phenoxyethanol and were killed by decapitation. Body weight (BW) and body length (BL) were measured. The encephalon length (EL) as indicator of brain size, was measured from the rostral olfactory bulbs to the caudal metencephalon on a dorsal view of the brain using a Vernier caliper [dashed line in Fig. 1(A)]. The biometric parameters are described in Supporting Information Table 1. The BW, BL, and EL increased with age ($P < 0.05$).

For the quantitative PCR analyses (qPCR), the brains were dissected into three parts (telencephalon [TE] including olfactory bulbs; diencephalon [DE] including hypothalamus, preoptic area, and thalamus; and mesencephalon [ME] including optic tectum, and valvula cerebelli; Fig 1[A,B]) using a stereomicroscope (SZX 16; Olympus, Tokyo, Japan), and were stored at −80°C until analysis. For the steroid enzyme immunoassays (EIA), the brains were similarly divided into 3 parts that were weighed before being stored at −80°C. For the histological examination, the brains were quickly isolated and fixed overnight with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate-buffered saline (PBS, pH 7.4). Gonad samples were also collected and fixed with 4% PFA for histological examination.

All the procedures and experiments were approved by the NTOU Institutional Animal Care and Use Committee and were conducted in an appropriate way in accordance with the animal experimentation procedure guidelines.

Experimental Design

Experiment 1: Gene Expression and Sex Steroids in the Brain during Testicular Differentiation. Brain tissues were collected from 90 dah (early differentiated gonad), 120 dah (middle differentiating gonad), and 150 dah (late differentiated gonad) fish for gene expression qPCR

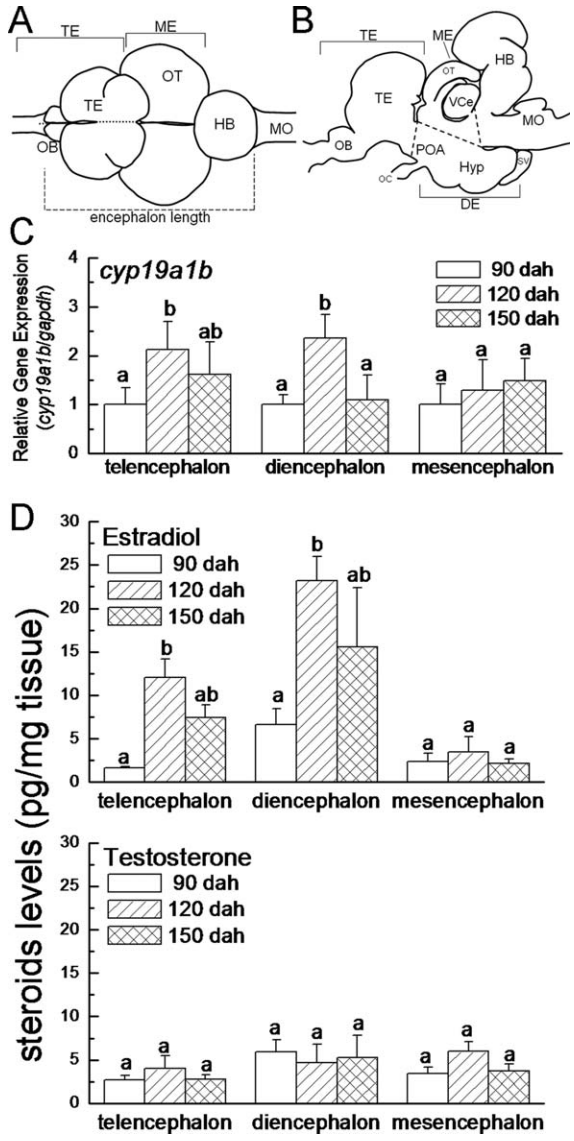


Figure 1 Variation in neurosteroidogenesis during black porgy early development. (A, B) Drawings of the black porgy brain, dorsal (A) and lateral (B) views. Brain tissues were collected and dissected into three parts as telencephalon, including olfactory bulbs (TE), diencephalon (DE) and mesencephalon (ME) for qPCR analysis and enzyme immunoassays (EIA). (C) Relative gene expression of brain aromatase *cyp19a1b*, as measured by qPCR in brain parts of black porgy at 90, 120, and 150 days after hatching (dah). The transcript values of *cyp19a1b* were calibrated with an internal control *gapdh*, and then normalized with the value at 90 dah, defined as 1. No differences in *gapdh* expression were observed between groups (Supporting Information Fig 2). The results are expressed as the mean ± SD ($n = 8$ fish/group). Different lowercase letters indicate significant differences ($P < 0.05$) in *cyp19a1b* expression between ages for each brain part (one-way ANOVA followed by Tukey's test). (D) Brain contents of estradiol (E_2) and testosterone (T), as measured by EIA in brain parts of 90, 120, and 150 dah fish. The results are expressed as the mean ± SD ($n = 6$ fish/group). Different lowercase letters indicate significant differences ($P < 0.05$) in steroid content between ages for each brain part (one-way ANOVA followed by Tukey's test). DE, diencephalon; HB, hindbrain; Hyp, hypothalamus; ME, mesencephalon; MO, medulla oblongata; OB, olfactory bulb; OC, optic chiasm; OT, optic tectum; POA, preoptic area; SV, saccus vasculosus; TE, telencephalon; VCe, valvula cerebelli.

Table 1 The number of Pcna-immuno- staining cells in posterior preoptic area (PP) in the Exp. 1 (fish at 90–150 days after hatching, dah) and Exp. 3 (fish fed with estradiol [E_2] or aromatase inhibitor [AI]).

	Pcna-positive cells ($\times 10^3$)/mm^2
Exp. 1: Different ages	
90 dah	0.16 ± 0.02^a
120 dah	0.37 ± 0.04^b
150 dah	0.15 ± 0.02^a
Exp. 3: Fish fed with E_2 or AI	
Control	0.39 ± 0.04^b
E2-fed	1.71 ± 0.15^c
AI-fed	0.19 ± 0.03^a

The results are expressed as the mean ± SD ($n = 3$ fish).
Different lowercase letters indicate the significant difference ($P < 0.05$).

analysis ($n = 8$ per age group), measurement of brain steroid levels ($n = 6$ per age group), and histology ($n = 3$ per age group). Gonad samples were also collected for histological examination as previously published (Wu et al., 2008). The characteristics of gonadal histology with H&E staining and Pcna-staining were shown in the Supporting Information Fig. 1.

Experiment 2: Fish Treatment with E_2. To investigate the effects of E_2 on gene expression and cellular changes in the brain, fish were treated *in vivo* with E_2. Fish at 90 dah were randomly divided into two groups (control and E_2); the E_2-treated group received 6 mg E_2 (Sigma-Aldrich, St. Louis, MO)/kg feed, according to the dosage previously established in black porgy (Lee et al., 2000). We previously reported that this dose of E_2 stimulated brain aromatase enzyme activity by 1.5-fold in black porgy (Lee et al., 2000). The control group was fed the same diet but without E_2. Brains were collected after one month of feeding, and the brain changes between the control and E_2 treated group were analyzed by gene expression ($n = 8$ per group) and histology ($n = 3$ per group). The gonad histology for the fish in this experiment has been previously reported (Wu et al., 2008).

Experiment 3: Fish Treatment with E_2 or an AI. To further investigate the role of E_2 in the induction of brain cell proliferation, E_2 (6 mg/kg feed, as in Experiment 2) or an AI (1,4,6-androstatrien-3,17-dione; Steraloids, Newport, RI) (20 mg/kg feed) were administered *in vivo* to black porgy. We previously reported that this dose of AI reduced brain aromatase enzyme activity by 70–80% (Lee et al., 2002), and *cyp19a1b* mRNA levels by 60–90% (Tomy et al., 2009). Fish at 90 dah were randomly divided into three groups: E_2, AI, and control groups. The control group was fed the same diet but without E_2 or AI. After one month of feeding, the brains were collected for histology studies. The gonad histology of the fish in this experiment has been previously reported (Wu et al., 2008). Both E_2 and AI treatments resulted in the regression of testicular tissue, development of oogonia/primary oocytes and growth of ovarian tissue (Wu et al., 2008). Treatments with E_2 or AI had no side effect on the growth performance (Supporting Information Table 1).

Experiment 4: Fish Castration. To investigate whether gonadal factors are responsible for brain changes, the gonad removal surgery was performed in 80 dah fish. Fish were anesthetized by immersion in a solution of 0.01% 2-phenoxyethanol until spontaneous motor activity and gill movement ceased. The fish were quickly removed from the container and placed on a sponge to stabilize the body laterally. After a ventral incision, the gonads were located using a stereomicroscope (SZX 16; Olympus) and excised. The surgical incision was sutured with a nylon suture (Ethilon 697; Ethicon Inc., Somerville, NJ), and the fish were transferred into an aquarium with aeration. The survival rate was 82.0%. Control and castrated fish were sacrificed at 90, 120, and 150 dah. To confirm the success of castration, the peritonea was collected and examined for the lack of *vasa* expression (a germ cell marker; Wu et al., 2012), by qPCR (n= 13 per group). Brain diencephalon samples were collected for the analyses of *vasa* gene expression ($n = 10$ per group) and histology examination ($n = 3$ per group).

Measurement of Gene Transcripts by qPCR Analysis

Total RNA was extracted from dissected brain tissues (Exp. 1, 2, and 4) or peritonea (Exp. 4) using TRIzol reagent (Invitrogen, Carlsbad, CA) and was reversely transcribed to the first-strand cDNA using Superscript III (Invitrogen) with oligo(dT)$_{12–18}$ primers (Promega, Madison, WI) according to the manufacturer's protocol. The first-strand cDNA was used for qPCR analysis.qPCR was conducted using a 7500 Real-Time PCR System (Applied Biosystems, CA) and SYBR Green as described in our previous study (He et al., 2003). All transcript values were calibrated with an internal control gene, *gapdh*. No significant changes in the expression profiles of *gapdh* were found among groups ($P > 0.05$) including age, E_2 or AI treatment, and castration (Supporting Information Fig. 2). The gene-specific primers and annealing temperatures are described in Supporting Information Table 2.

Measurement of Brain Steroids by EIA

Each brain part sample from Exp. 1 was homogenized in PBS (0.1 M, pH 7.4). Steroids were extracted with ethyl ether, and aliquots were assayed using EIA kits and following the manufacturer's protocol for testosterone (T), E_2 and 11-ketotestosterone (11-KT) (Cayman Chem., Ann Arbor, MI). The standard curve for E_2 ranged from 0.33 to 200 pg, for T from 0.2 to 25 pg, and for 11-KT from 0.78 to 100 pg. E_2 and T contents in each individual brain part were within the range of EIA detection, while brain 11-KT levels were undetectable.

Counting Brain Cells Using the Nissl Staining Method

Brains (Exp. 1 and 4) were fixed in 4% PFA and dehydrated through a graded ethanol series, embedded in paraffin, and cut into 4 μm transverse serial sections. Nissl staining (0.5% cresyl violet; Sigma-Aldrich) was utilized, and brain areas were located by identifying neuroanatomical structures by using the brain atlas of gilthead seabream as a reference (Muñoz-Cueto et al., 2001). Black porgy and gilthead seabream are closely related species, among the Sparidae, and the brain anatomy is basical the same between the two species according to our preliminary studies. Nissl-labeled brain cells were quantified using photographs imaged at 20X on a microscope (BX51; Olympus) with ImageJ (National Institutes of Health) using the following steps: (i) a digital photograph was imaged by a CCD (DP71; Olympus); (ii) spatial calibration was set by ImageJ "Set Scale" function (the same magnification and resolution for all pictures); (iii) ImageJ "Measure" function was used to determine the estimated area (μm^2) of each brain region; (iv) the Nissl-stained nuclei, including glial cells or neurons, were counted as positive cells. The result of cell density (number of Nissl-stained cells/μm^2) was statistically compared among groups. The transverse sections of fish brain were positioned at the level of the preoptic area (POA) and thalamus. The target regions were: dorsalis posterior thalami (DP), ventromedialis thalami (VM), and posterior POA (PP). The average cell density of 3 serial sections in each fish brain was calculated from 3 fish for each group.

Immunohistochemistry

Primary Antibodies. The mouse monoclonal anti-PCNA (M0879; DakoCytomation, Glostrup, Denmark) and rabbit polyclonal anti-BLBP (ab32423; Abcam, Cambridge, UK) antibodies were used to detect immunoreactivity in black porgy brains. The BLBP antibody has been used as RGC marker in zebrafish (Kim et al., 2013) and in our previous study in grouper (Nagarajan et al., 2013). The PCNA antibody has been used to assess cell proliferation, in our previous study in black porgy (Wu and Chang, 2009), and in other fishes such as zebrafish (Diotel et al., 2010; Kishimoto et al., 2011), killifish (Terzibasi Tozzini et al., 2014), medaka (Kong et al., 2008), and goldfish (Vigliano et al., 2011).

A guinea pig antipeptide antibody was generated in the present study, which against a synthetic peptide of black porgy Cyp19a1b: DEHSLAMRFIPRTTQTPHSH (Kelowna International Scientific, Taiwan).

Western Blotting. The specificity of the Cyp19a1b antibody was assessed by Western blotting. Black porgy (120 dah) brain crude proteins were extracted by TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The BCA protein assay kit (Pierce Biotechnology, Rockford, IL) was used to determine the protein concentration according to the manufacturer's protocol. Protein samples (10 μg) were electrophorised on a NuPAGE Novex 10% Bis-Tris Mini Gel (Invitrogen), and the gel was subsequently transferred to a nitrocellulose membrane (Hybond-C Extra 0.45 μm; GE Healthcare, Buckinghamshire, UK). After blocking, the membranes were incubated with the anti-Cyp19a1b antibody (diluted at 1:5,000 in Tris-buffered saline [TBS] containing 0.1% Tween-20 [TBST] and 1% skim milk) for 1 h at room temperature. After washing with TBST, the membranes were incubated with 0.25 μg/ml alkaline phosphatase (AP)-conjugated goat anti-guinea pig IgG (Yao-Hong Biotechnology Inc., New Taipei, Taiwan) for 1 h at room temperature. NBT/BCIP solution (Sigma-Aldrich) was used to visualize the immunoreactive band. A single and specific Cyp19a1b immunoreactive band of approximately 55 kDa was detected in the brain extracts by Western blot analysis, which corresponds to the molecular weight of black porgy Cyp19a1b [Supporting Information Fig. 3(A), anti-AromB]. This immunoreactive band could be eliminated by the pre-adsorbing antibody with 200 ng/ml of the peptide antigen [Supporting Information Fig. 3(A), Preadsorbed]. Positive (with Cyp19a1b antibody) and negative (with preadsorbed Cyp19a1b antibody) immunohistochemical stainings were shown in the Supporting Information Fig. 3(B).

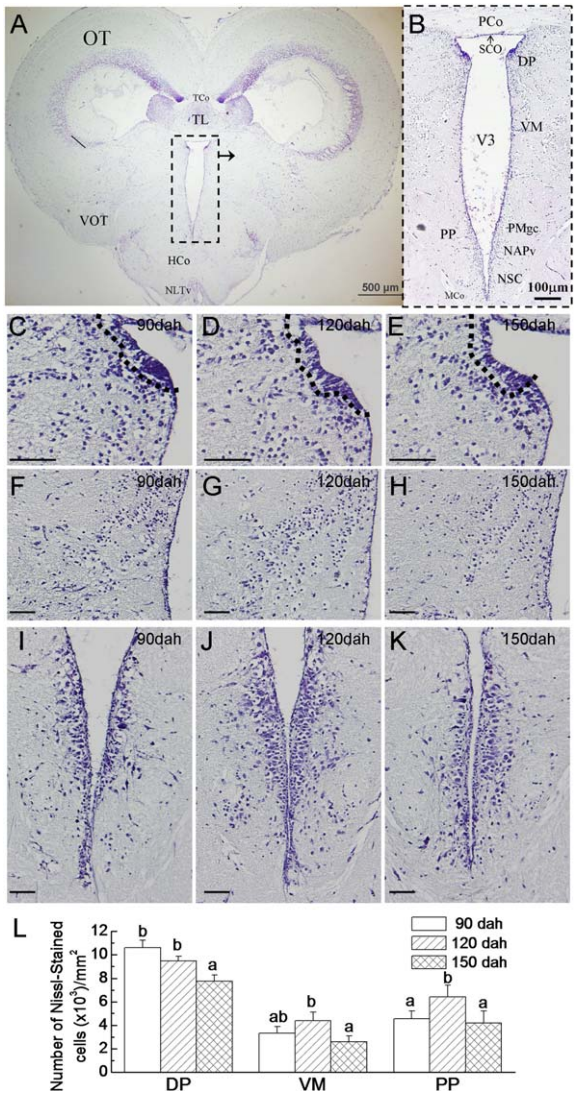
Brain Immunostaining. Brain sections were immunostained using a standard avidin-biotinylated-peroxidase complex (ABC) kit (Vector Laboratories, Burlingame, CA). The hydrated sections were incubated for 10 min in 3% H_2O_2 and for 1 h in 5% skim milk. The sections were incubated overnight at 4°C with primary antibodies against PCNA (1:200; Exp. 1 and 3), BLBP (1:200; Exp. 1 and 2), or Cyp19a1b (1:1000; Exp. 2). After washing in PBS with 0.1% Tween-20, the sections were incubated with biotinylated goat IgG antibodies (1:2000; anti-mouse [BA-9200], anti-rabbit [BA-1000], or anti-guinea pig [BA-7000]; Vector Laboratories), washed again and incubated in ABC solution. The peroxidase reaction was visualized with a solution of 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) and counterstained with hematoxylin. For immunofluorescent staining, the sections were immersed in Alexa Fluor goat IgG antibodies (1:200; anti-mouse [488, A11029], anti-rabbit [488, A11034 and 546, A11035], or anti-guinea pig [546, A11074]; Invitrogen). The tissue sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) to visualize the cell nuclei.

TUNEL Assay. TUNEL assay is a specific labeling of nuclear fragmentation and using the recombinant terminal deoxynucleotidyl transferase (rTdT) enzyme-mediated dUTP-biotin nick end-labeling method to demonstrate apoptosis. TUNEL staining was performed on brain sections according to the manufacturer's protocol (Promega) and apoptotic nuclei were stained dark brown by DAB. The hydrated sections were incubated for 30 min in 20 μg/ml proteinase K, for 5 min in 4% PFA, and for 10 min in equilibration buffer. The sections were incubated for 60 min at

37°C with the TdT reaction mix solution, including rTdT enzyme, biotinylated nucleotide mix and equilibration buffer. The reactions were stopped in 2X SSC for 15 min. After washing in PBS, the sections were incubated with streptavidin HRP and visualized with a DAB solution. TUNEL-positive cells were counted in the PP, and the results were statistically analyzed ($n = 5$ fish per age group).

Statistical Analysis

The data are presented as the mean ± SD (standard deviation). The comparisons were performed in the Exp. 1–3 (Table 1; Figs. 1, 2, 4, 5) by one-way analysis of variance (ANOVA) followed by Tukey's test using Statistical Package for the Social Sciences (SPSS). Two-way ANOVA was performed to determine the effect of age, surgery, and the interaction in Exp. 4 (Fig. 7). A value of $P < 0.05$ was considered statistically significant.



RESULTS

Brain Aromatase Transcript Levels and Steroid Contents at the Time of Gonadal Differentiation

qPCR was performed on brain parts from 90, 120, and 150 dah fish to determine the gene expression profile of *cyp19a1b*. The relative gene expression of *cyp19a1b* increased significantly at 120 dah in the telencephalon and diencephalon but exhibited no significant changes in the mesencephalon at 120 dah [Fig 1(C); 2.1-fold, $P < 0.05$, TE; 2.4-fold, $P < 0.01$, DE; 1.3-fold, $P > 0.05$, ME; compared with 90 dah].

Brain steroid hormone (E_2 and T) levels were analyzed by EIA [Fig 1(D)]. Peak E_2 levels were observed in the telencephalon and diencephalon with no significant changes in the mesencephalon at 120 dah (7.4-fold, $P < 0.01$, TE; 3.5-fold, $P < 0.05$, DE; 1.4-fold, $P > 0.05$, ME; compared with 90 dah). T was detected but exhibited no significant changes at that period (1.5-fold, TE; 0.8-fold, DE; 1.6-fold, ME; all $P > 0.05$; compared with 90 dah).

Brain Cell Number at the Time of Gonadal Differentiation

Transverse serial sections of POA were examined. The brain cell numbers in the POA appeared

Figure 2 Variation in brain cell numbers in diencephalic area during black porgy early development. (A and B) Transverse section of the black porgy brain at the level of the third ventricle, stained by Nissl method, with higher-magnification view as shown in B. (C–K) Nissl-stained brain cells distributed in DP (C–E), VM (F–H) and PP (I-K) area of 90 dah (C, F, I), 120 dah (D, G, J) and 150 dah (E, H, K) fish. (L) Statistical analysis of Nissl-stained brain cell numbers in DP, VM and PP of 90, 120, and 150 dah fish. The results are expressed as the mean ± SD ($n = 3$ fish/group). Different lowercase letters indicate significant differences ($P < 0.05$) between ages for each area (one-way ANOVA followed by Tukey's test). In DP, the high density of cell clusters in the vicinity of the ventricle made it impossible to accurately count cell numbers in this area. The values of DP were calculated from the rest of DP (excluding the dash lines of high density cell clusters in figures C–E). DP, nucleus dorsalis posterior thalami; HCo, horizontal commissure; MCo, minor commissure; NAPv, nucleus anterioris periventricularis; NLTv, nucleus lateralis tuberis, pars ventralis; NSC, nucleus suprachiasmaticus; OT, optic tectum; PCo, posterior commissure; PMgc, nucleus preopticus magnocellularis, pars gigantocellularis; PP, posterior POA (posterior preoptic area); SCO, subcommissural organ; TCo, tectal commissure; TL, torus longitudinalis; V3, third ventricle; VM, nucleus ventromedialis thalami; VOT, ventral optic tract. Untext bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

highest at 120 dah as compared to other periods (Supporting Information Fig. 4), but in some area, cells were too dense to allow us to accurately count the cell numbers (Supporting Information Fig. 4). We therefore chose to focus on the posterior part of the POA (PP) as a representative area to count the cell numbers. PP is ventral to the thalamus and dorsal to the anterior part of hypothalamus. The cell populations of the PMgc (nucleus preopticus magnocellularis pars gigantocellularis), NAPv (nucleus anterioris periventricularis), and NSC (nucleus suprachiasmaticus) are included in this area. On the dorsal side of the PP, brain cells in the thalamic DP (nucleus dorsalis posterior thalami) and VM (nucleus ventromedialis thalami) were also counted (Fig. 2). Nissl-stained cells, including glial cells and neurons, were counted in the DP [Fig. 2(C–E)], VM [Fig. 2(F–H)], and PP [Fig. 2(I–K)], and the results were statistically analyzed [Fig. 2(L)]. The Nissl-stained cells distributed in these areas revealed that the cell number varied during the period from 90 to 150 dah. In the DP, the high density of cell clusters in the vicinity of the ventricle made it impossible to accurately count the cell number. The DP cell numbers were calculated based on the rest of the DP [excluding these high density cell clusters, see dashed lines in Fig. 2(C–E)]. Even excluding these cell clusters, higher cell numbers were observed at 90 dah (1.4-fold, $P < 0.05$) and 120 dah (1.2-fold, $P < 0.05$), compared with 150 dah [Fig. 2(C–E, L)]. In the VM, a slight, but not significant, increase in cell number was observed at 120 dah (1.3-fold, $P > 0.05$; compared with 90 dah), and the cell number significantly decreased at 150 dah [0.6-fold, $P < 0.05$; compared with 120 dah; Fig. 2(F–H,L)]. In the PP, the cell number was significantly higher at 120 dah (1.4-fold, $P < 0.05$; compared with 90 dah) than at 90 or 150 dah [Fig. 2(I–K,L)].

Brain Cell Proliferation at the Time of Gonadal Differentiation

The immunohistochemical detection of Pcna was used as a nuclear marker of brain cell proliferation. Figure 3 illustrates the Pcna-immunostained cells in the DP and PP. In the DP, stronger Pcna staining was observed at 90 dah compared with the older ages [Fig. 3(A–C)]. However, the high cell density clusters in this region did not allow for an accurate cell count, as mentioned above. In the PP, Pcna-positive cells [Fig. 3(D–F)] were counted, and the results were statistically analyzed (Table 1). The number of Pcna-positive brain cells was significantly higher at 120 dah [Fig. 3(E) and Table 1; 2.3-fold, $P < 0.01$; compared with 90 dah] than at the other sampling times [Fig. 3(D,F)].

An antibody against Blbp was used to characterize brain RGCs. Double immunofluorescent staining was performed on 120 dah brain sections [DP, Fig. 3(G1–G4); PP, Fig. 3(H1–H4)] using antibodies against Pcna and Blbp. Many Blbp-immunostained cells were observed in the DP [Fig. 3(G1)] and PP [Fig. 3(H1)]. Pcna immunofluorescent staining produced similar labeling [Fig. 3(G2,H2)]. The results of the double staining showed colocalization of Pcna and Blbp, suggesting that the glial cells were proliferative [Fig. 3(G4,H4)].

Brain Cell Apoptosis at the Time of Gonadal Differentiation

Our recent Tunel-staining data in the posterior part of POA demonstrate the increase of programmed cell death ($P < 0.05$) at 150 dah ($0.51 \pm 0.02 \times 10^3$ cells/mm^2, $n = 5$ fish) as compared to 90 dah ($0.16 \pm 0.02 \times 10^3$ cells/mm^2, $n = 5$ fish) and 120 dah ($0.15 \pm 0.02 \times 10^3$ cells/mm^2, $n = 5$ fish).

Expression Profiles of Brain Cell Markers at the Time of Gonadal Differentiation

qPCR was performed on brain parts from 90, 120, and 150 dah fish to determine the gene expression profiles of a RGC marker, *blbp*, and a neurotrophic growth factor, *bdnf*. The relative expression of *blbp* was significantly increased at 120 dah in each brain part [Fig. 4(A); 2.1-fold, $P < 0.01$, TE; 2.2-fold, $P < 0.01$, DE; 1.8-fold, $P < 0.01$, ME; compared with 90 dah]. The transcript levels of *bdnf* also peaked at 120 dah in all the analyzed parts of the brain [Fig. 4(A); 2.5-fold, $P < 0.01$, TE; 2.7-fold, $P < 0.01$, DE; 2.4-fold, $P < 0.01$, ME; compared with 90 dah].

The antibody against Blbp was used to further characterize Blbp protein expression in the brain. As shown in Figure 4(B–D), stronger staining was observed in the VM at 120 dah compared with the other time points. Blbp immunostaining revealed extended RGC projections at 120 dah [Fig. 4(C)] compared with the other time points [Fig. 4(B, D)].

Effect of E_2 Treatment on the RGC Markers

To investigate the effects of E_2 on gene, protein expression, cell number and cellular projections of RGCs, an E_2-containing diet was provided to 90 dah fish for one month. Brain samples were collected at

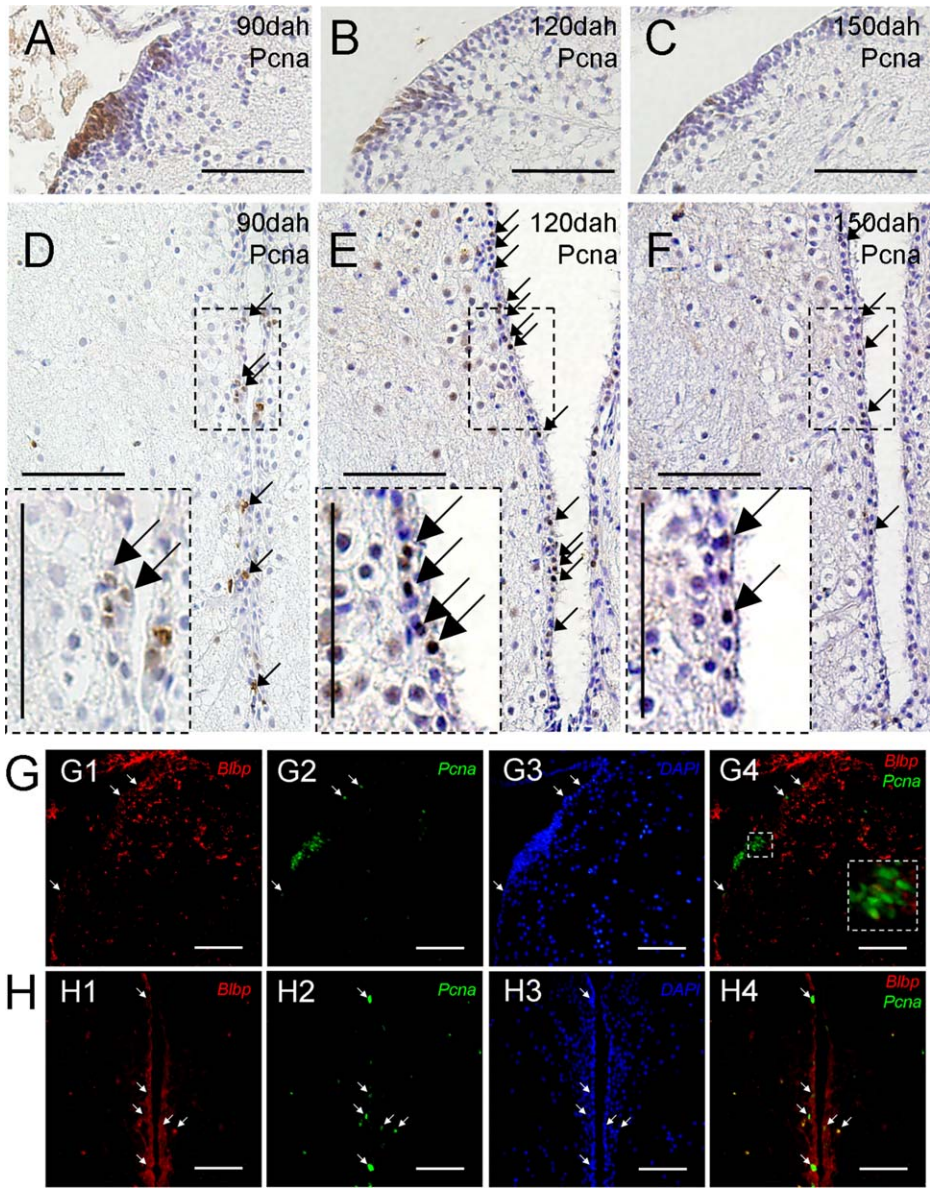


Figure 3 Variation in brain cell proliferation, as shown by Pcna immunostaining, in diencephalic area during black porgy early development. (A–C) Pcna immunostaining in DP of 90, 120, and 150 dah fish. (D–F) Pcna immunostaining in PP of 90, 120, and 150 dah fish. Higher-magnification views of the insets were shown in D-F. Arrows indicate the Pcna signals. The detection showed that stronger signals of Pcna-staining are found in DP at 90 dah than elder ages. However, the high cell density clusters in this region did not allow to accurately count cell number. Pcna-stained brain cell number in PP was significantly higher at 120 dah than at other sampling times. (G and H) Double immunofluorescent staining with Pcna and radial glial cell (RGC) marker, Blbp, on DP (G1–G4) and PP (H1–H4). G1 and H1 for Blbp, G2 and H2 for Pcna, G3 and H3 for DAPI, G4 and H4 for costaining with Blbp and Pcna. Higher-magnification views of the inset are shown in G4. Arrows indicate coexpression of Pcna and Blbp signals by RGC. DP, nucleus dorsalis posterior thalami; PP, posterior preoptic area. Bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

120 dah and examined by qPCR and IHC, and the expressions of *cyp19a1b*/Cyp19a1b and *blbp*/Blbp were analyzed. E_2 significantly increased *cyp19a1b* expression in all three brain parts compared with control fish [Fig. 5(A); 36.7-fold, $P < 0.01$, TE; 20.2-fold, $P < 0.05$, DE; 8.5-fold, $P < 0.01$, ME]. The

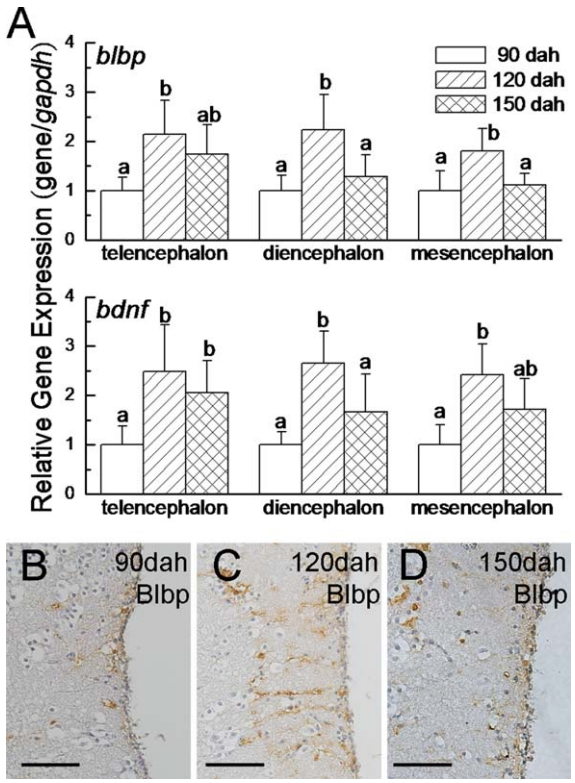


Figure 4 Variation in brain expression of radial glial cell marker blbp and neurotrophic factor bdnf during black porgy early development. (A) Relative gene expression of *blbp* and *bdnf* in brain parts of black porgy at 90, 120 and 150 dah, as determined by qPCR. The transcripts of *blbp* and *bdnf* were calibrated with an internal control *gapdh*, and then normalized with the value at 90 dah, defined as 1. No differences in *gapdh* expression were observed between groups (Supporting Information Fig. 2). The results are expressed as the mean ± SD ($n = 8$ fish/group). Different lowercase letters indicate significant differences ($P < 0.05$) in gene expression between ages for each brain part (one-way ANOVA followed by Tukey's test). (B–D) Blbp immunostaining in ventromedialis thalami (VM) at 90, 120, and 150 dah. A stronger staining was observed at 120 dah. Blbp immunostaining revealed the extended projections of radial glial cells at 120 dah as compared to the other sampling times. Bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

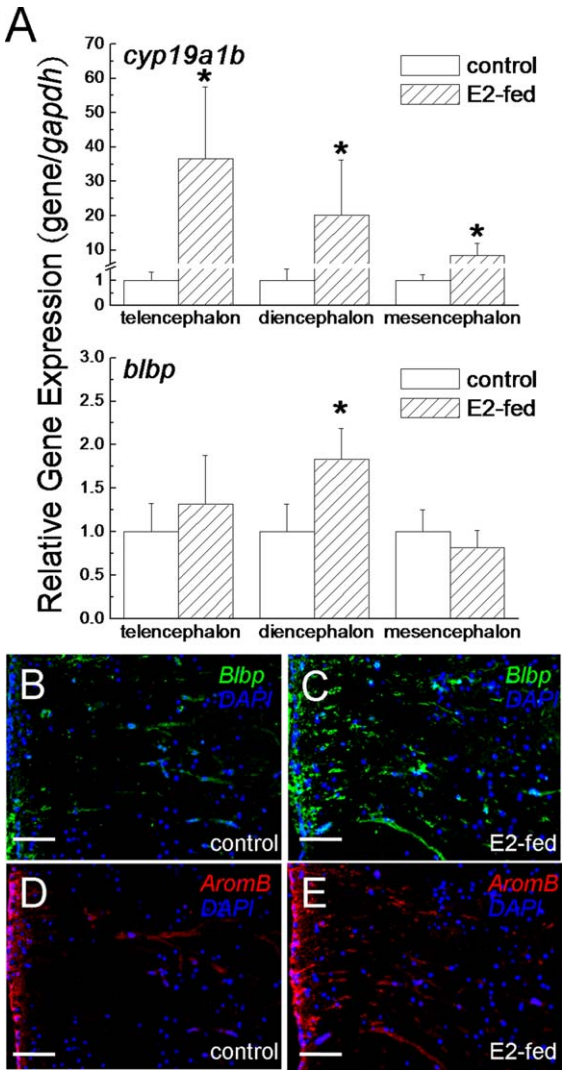


Figure 5 Effect of estradiol (E_2)-treatment on brain expression of aromatase and blbp during black porgy early development. E_2-treated fish were fed with E_2 diet (6 mg/kg feed) for one month and sacrificed at 120 dah. (A) Relative gene expression of aromatase B (*cyp19a1b*) and *blbp* in brain parts of control or E_2-treated fish, as measured by qPCR. The transcript values of *cyp19a1b* and *blbp* were calibrated with an internal control *gapdh*, and then normalized with the value from control fish, defined as 1. No differences in *gapdh* expression were observed between groups (Supporting Information Fig. 2). The results are expressed as the mean ± SD ($n = 8$ fish). Asterisks indicate significant differences ($P < 0.05$) between control and E_2-treatedt groups (one-way ANOVA followed by Tukey's test). (B–E) Immunofluorescence staining of Blbp and Cyp19a1b (AromB) in ventromedialis thalami (VM) in control or E_2-treated fish. Stronger Blbp- and Cyp19a1b-immunostaining were observed in VM after E_2-treatment. Immunostaining reveled extensive processes of radial glial cells in VM area in E_2-treated fish as compared to control fish. Bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

gene expression *blbp* only increased significantly in the diencephalon [Fig. 5(A); 1.3-fold, $P > 0.05$, TE; 1.8-fold, $P < 0.01$, DE; 0.8-fold, $P > 0.05$, ME].

The antibodies against Cyp19a1b (AromB) and Blbp were used to further characterize protein expression and cellular changes after E_2 treatment. A stronger Blbp immunostaining was observed after E_2 treatment in VM as compared to the control [Fig. 5(B,C]. After IHC of Cyp19a1b, extended

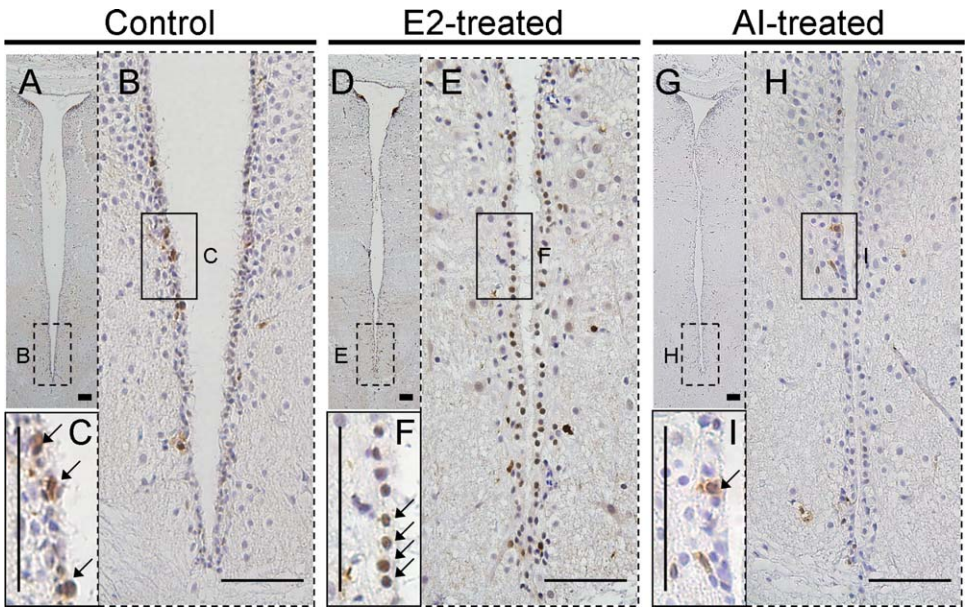


Figure 6 Effects of estradiol (E_2) or aromatase inhibitor (AI) treatments on brain cell proliferation, as shown by Pcna immunostaining in the posterior preoptic area (PP). Fish were fed for one month with control feed or E_2-(6 mg/kg feed) or AI-(20 mg/kg feed) containing feeds, and were sacrificed at 120 dah. (A–C) Pcna immunostaining in PP of control fish. (D–F) Pcna immunostaining in PP of E_2-treated fish. (G–I) Pcna immunostaining in PP of AI-treated fish. Arrows indicate the Pcna signals. Higher-magnification views of the insets were shown in the figure. The data showed that Pcna-stained brain cell number in PP was significantly higher in E_2-treated fish and lower in AI-treated fish, as compared to control fish. Bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RGC projections were labeled. Cyp19a1b immunostaining revealed increased signals and cell extended projections in the VM of E_2-treated fish compared with control fish [Fig. 5(D,E)]. These data indicated that E_2 treatment consistently increased Cyp19a1b and Blbp gene and protein expressions.

Effect of E_2 and AI on Brain Cell Proliferation

To investigate the effect of E_2 on brain cell proliferation, an E_2- or an AI-containing diet was given to 90 dah fish for one month. Brain samples were collected at 120 dah and examined for proliferative activity via the immunohistochemical detection of Pcna. Immunohistochemistry against Pcna was performed on the PP from control [Fig. 6(A–C)] and fish treated with E_2 [Fig. 6(D–F)] or AI [Fig. 6(G–I)]. The number of Pcna-positive brain cells was significantly higher in E_2-treated fish (4.4-fold, $P < 0.01$) and significantly lower in AI-treated fish (0.5-fold, $P < 0.01$) compared with control fish (Table 1).

Effect of Castration on Brain Gene Expression and Cell Numbers

As we found that E_2 induced neurogenic activity, castration was performed to further investigate whether the gonads are the key factor responsible for the changes in the brain. The transcript levels of germ cell marker *vasa* showed an increasing pattern in peritonea of control fish during the gonad differentiation, but no *vasa* transcripts were detected by qPCR in castrated fish [Fig. 7(A)]. The *vasa* expression thus confirmed that the castration operation were successful [Fig. 7(A)].

The transcript levels of the brain cell markers *blbp* (1.5-fold, $P < 0.05$, control; 1.4-fold, $P < 0.05$, castrated) and *bdnf* (2.0-fold, $P < 0.05$, control; 1.6-fold, $P < 0.05$, castrated) significantly increased at 120 dah both in nonsurgical control fish and in castrated fish. There were no significant differences between the control and castrated groups for the same ages [Fig. 7(B)]. Nissl-stained cells were counted in the PP, and the results were statistically analyzed, indicating that the number of brain cells varied both in control and castrated fish during this period. The cell number was significantly higher in both groups at 120 dah (1.5-

fold, $P < 0.05$, control; 1.3-fold, $P < 0.05$, castrated), but there were no significant differences between the control and castrated groups for the same ages [Fig. 7(C)].

DISCUSSION

In the present study, we demonstrated a significant peak of neurogenesis in the early brain in mono-sex male black porgy. It is not clear whether this neurogenesis peak in POA at 120 dah is related to the brain sex dimorphism in black porgy.

Peak Neurogenesis in the Early Brain

The POA, which is organized by E_2, is important for sex-specific behavirors and neuroendocrine control during reproduction. Focusing on the diencephalon, we showed more Nissl-stained brain cells at 120 dah, as compared to 90 and 150 dah in the posterior POA (PP) including PMgc, NAPv, and NSC. Cell proliferation was further assessed by Pcna immunostaining, and qPCR analyses showed increased expression of the RGC marker *blbp* and neuron marker *bdnf*. After Blbp immunostaining, extended cellular projections of RGCs were also observed at 120 dah in the VM (a thalamic area in the dorsal vicinity of the PP).

RGCs serve as neuronal precursors in the brain (Hartfuss et al., 2001; Pinto and Gotz, 2007), and their extended cell projections also allow neurons to migrate to their final location. During the migration, neurons become functional and adopt a mature morphology. The formation and maintenance of a radial glial scaffold is fundamental in the developing vertebrate brain. We report here for the first time in fish the integrative increase of neurosteroidogenesis (E_2 levels) and neurogenesis activity, brain cell projections and cell numbers during the period of gonadal differentiation. The peak aromatase activity or *cyp19a1b* expression was detected in the brain of

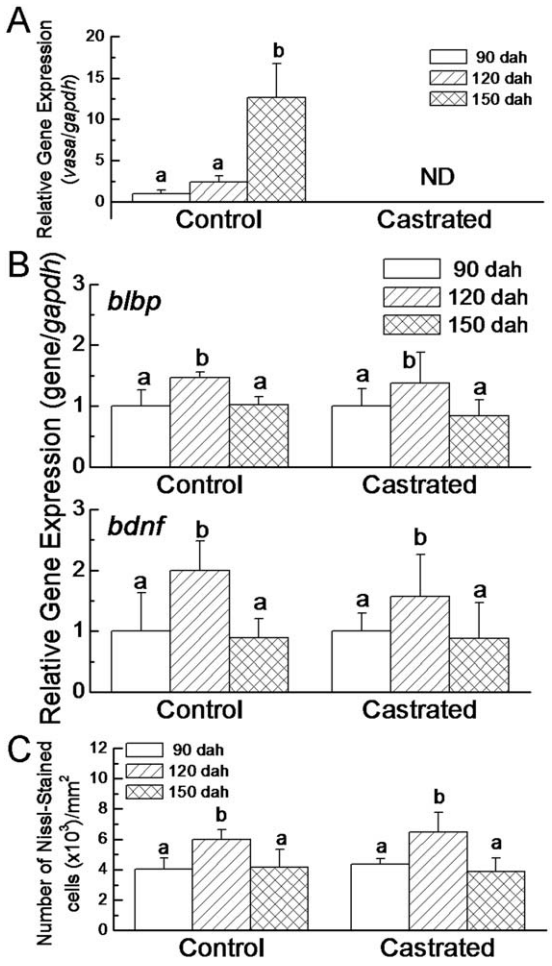


Figure 7 Effect of castration on gene expression profiles and brain cell number during black porgy early development. The surgery of gonad removal was operated at 80 dah (day after hatching), and fish were sacrificed at 90, 120, and 150 dah. (A) Relative gene expression of germ cell marker *vasa* in the peritonea of control fish or castrated fish, at different ages, as measured by qPCR. The *vasa* transcripts were undetectable in castrated fish, assessing that the surgery was successful. The *vasa* transcript values in control fish were calibrated with an internal control *gapdh*, and then normalized with the value at 90 dah fish, defined as 1. No differences in *gapdh* expression were observed between groups (Supporting Information Fig. 2). The results are expressed as the mean ± SD ($n = 13$ fish/group). Different lowercase letters indicate significant differences ($P < 0.05$) in *vasa* expression between ages in control fish (one-way ANOVA followed by Tukey's test). (B) Relative gene expression of brain cell markers *blbp* and *bdnf* in the diencephalon of control fish or castrated fish, at different ages, as measured by qPCR. The transcript values of *blbp* and *bdnf* were calibrated with an internal control *gapdh*, and then normalized with the value of diencephalon at 90 dah fish, defined as 1. No differences in *gapdh* expression were observed between groups (Supporting Information Fig. 2). The results are expressed as the mean ± SD ($n = 10$ fish/group). Different lowercase letters indicate significant differences ($P < 0.05$) in gene expression (two-way ANOVA followed by Tukey's test). Castration had no significant effect on brain gene expression, at any age. (C) Nissl-stained cell number in the posterior preoptic area (PP) of control and castrated fish at different ages. The results are expressed as the mean ± SD ($n = 3$ fish/group). Different lowercase letters indicate significant differences ($P < 0.05$) in cell numbers between groups (two-way ANOVA followed by Tukey's test). Castration had no significant effect on brain cell number, at any age.

specific gender of fish species such as female European sea bass (Blazquez et al., 2008) and male rainbow trout (Vizziano-Cantonnet et al., 2011) during gonad differentiation. The peak increases of aromatase mRNAs and enzymatic activity were also found in the brain during the prenatal period in rat (Lephart et al., 1992; Lephart, 1996).

The increase in brain cell numbers at 120 dah could be contributed by two pathways: increased proliferation (based on Pcna assay) or decreased apoptosis (based on the TUNEL assay) of the brain cells. The present data showed an increase in Pcna-positive cells in the brain at 120 dah (Table 1), as compared to 90 and 150 dah. This demonstrated that an increase in brain cell proliferation could be involved in the increase in brain cell number at 120 dah. Our TUNEL-staining studies showed no variation in programmed cell death between 90 and 120 dah, but an increase at 150 dah. This suggests that the increase in brain cell number between 90 and 120 dah would not be due to reduced apoptosis. In contrast, increased apoptosis, together with reduced cell proliferation would contribute to the decrease in brain cell number at 150 dah.

Brain Neurogenesis is E_2-Dependent

The peak activities of neurogenesis in the early brain might result from sex steroid regulation. To test this hypothesis, E_2 and AI were orally administered to black porgy. We demonstrate that E_2 stimulates gene and protein expression of aromatase and of Blbp (a radial glial cell marker) in the brain, and also upregulates proliferative activity (Pcna-positive cells). These data indicate that E_2 induces neurosteroid- and neuron-related gene expressions, and cell proliferation in specific brain region of black porgy. In contrast to the E_2 stimulation, AI treatment significantly decreased cell proliferation activity and cell numbers as shown by Pcna immunohistochemical staining. This further supported the role of E_2 in neurogenesis.

Our results of stimulatory effects of E_2 on neurogenesis are in agreement with previous data in other vertebrates, such as rodents (Orikasa et al., 2007). E_2 induced *Bdnf* gene expression in the cerebellum, and BDNF mediated estrogen action in promoting spinogenesis and synaptogenesis in developing neurons in mice (Haraguchi et al., 2012). RGCs serve as neuronal precursors in the brain (Hartfuss et al., 2001; Pinto and Gotz, 2007), and E_2 stimulates progenitor cell proliferation and inhibits apoptosis in fish (Tsai et al., 2001) and mammals (Perillo et al., 2000; Martinez-Cerdeno et al., 2006; Wang et al., 2008). E_2 accelerates and enhances dendritic growth in the POA and other specific brain regions in mammals (Toran-Allerand et al., 1983; Kuppers et al., 2001; Sakamoto et al., 2003). E_2 is indicated as a major factor for neurogenesis (Toran-Allerand, 1984) and migration (Henderson et al., 1999) in the mammalian brain.

In contrast, previous studies also reported that E_2 induced apoptosis or inhibited brain cell proliferation (Davis et al., 1996). In the anteroventral periventricular nucleus (AVPN) of the rat brain, a structure associated with the ovulatory surge of gonadotrophin, the cells die in males in response to E_2, which results in fewer cells than in females (Tsukahara, 2009). In zebrafish, estrogens inhibit the activity of aromatase and Pcna during repair of brain lesion in adult (Diotel et al., 2013). The inhibitory E_2 effects were found on neurogenesis in the optic tectume of sexually mature female medaka (Takeuchi and Okubo, 2013) and on the proliferation in brain cells in adult female zebrafish (Makantasi and Dermon, 2014). These data suggest a complex role for E_2 in the brain neurogenesis and development which may vary according to developmental stage, age, physiological status, treatment, and species. Our data in the black porgy further highlight a major stimulatory role of E_2 in the induction of neurogenesis in the early brain in vertebrates.

Peak of Steroidogenesis in the Early Brain

We demonstrate in the present study that neuroestrogen E_2 is synthesized in the early brain of mono-male black porgy at the time of gonadal differentiation. We showed by qPCR a peak expression of brain aromatase transcripts *cyp19a1b*. Furthermore, the development of an antibody to black porgy Cyp19a1b allowed us to reveal the specific and enhanced expression of aromatase protein in the RGCs. Our previous study also demonstrated that the peak aromatase enzyme activity was detected in the early brain of black porgy (Tomy et al., 2007). These findings further support our previous hypothesis of a peak of brain neurosteroidogenesis activity at the time of gonadal differentiation in black porgy (Tomy et al., 2007, 2009; Wu et al., 2010) and grouper (Nagarajan et al., 2013). Black porgy and grouper are protandrous (male gonad develops first) and protogynous (female gonad develops first), respectively. Our results in these two species suggest that the occurrence of peak neurostroidogenesis and neurogenesis in the early brain may be not related to the gender of fish gonad.

In some other teleost species, higher levels of E_2, aromatase enzymatic activity or *cyp19a1b*

transcript expression in brain were detected in male as compared to female during gonad differentiation in pejerrey (Strobl-Mazzulla et al., 2008) and rainbow trout (Vizziano-Cantonnet et al., 2011). Sex steroids (T or E_2) could regulate the cell numbers in specific regions of the brain in the rodents (Lenz et al., 2012) or avians (Schlinger, 1998) to establish sexual dimorphisms in brain. For example, in rats, the sexually dimorphic nucleus of the preoptic area (SDN-POA) is larger in males than in females and the sex difference is due to cell death in female. This cell death could be rescued by treating females with T or E_2 (Arai et al., 1996; Davis et al., 1996). The high vocal center (HVC) nucleus in the brain of song birds is crucial for a uniquely male behavior (the acquisition and retention of song). This HVC area is more developed in males than in females, treating females with E_2 enlarges the HVC nucleus and produces a masculine neural song system (Schlinger, 1998). We still do not know whether the peaks of steroidogenesis and neurogenesis of the early brain of protandrous male may also play a role in the control of brain sex dimorphisms and sexual behavior in black porgy.

Peak of E_2 Levels in the Early Brain

In the present study, we showed an increase in early brain E_2 concentrations. In contrast, there were no peak T levels in black porgy brain. In our studies, 11-KT levels were also measured in the brain but 11-KT levels were too low to be detected in the early brain. Therefore, only T and E_2 data were presented. In present data, brain E_2 levels were much higher (4-fold) than T in black porgy. Our unpublished data further showed that T levels were much higher than 11-KT levels in the telencephalon (>69-fold), diencephalon (>59-fold), and mesencephalon (>111-fold) of early brain in black porgy. The concentrations of T were much higher than 11-KT (100-fold) or E_2 (30-fold) in bluebanded goby (Lorenzi et al., 2012). The decreased T and aromatase activity and increased 11-KT were found in the adult brain during the sex change from a female to male in bluebanded goby (Black et al., 2005; Lorenzi et al., 2012). In the future, the concentrations and roles of 11-KT should be further examined in the early brain of black porgy by a more sensitive method than EIA.

Neurogenesis is Regulated by Local E_2 in the Brain

We showed that the peaks of steroidogenesis (aromatase and E_2 levels) and neurogenesis occurred in the early brain at the same time, and that AI also significantly decreased brain cell numbers. Together with the castration experiment, all these data support the possibility that the neurogenesis peak in the early brain of the black porgy is induced by locally biosynthesized brain E_2.

As we showed that peaks in brain neurosteroidogenesis, E_2 and neurogenesis occurred during the period of gonadal differentiations, we addressed the question of the potential role of the gonads in early brain activity. For answering these questions, we developed a protocol to surgically remove the gonads in small fish at 80 dah. After castrating the fish and analyzing the brains, we found that castration did not have any effect on the peak pattern of brain cell numbers (Nissl positive staining-brain cells), nor on *blbp* and *bdnf* gene expression. The peak of brain neurogenesis similarly occurred in both the control and castrated fish. We demonstrate for the first time that the peak neurogenesis in the early fish brain of black porgy is independent of the gonad and positively regulated by brain local E_2.

Our present studies of the neurosteroidogenesis and neurogenesis peak in the early brain during gonad differentiation also brought up interesting aspects for the interaction between brain and gonad in fish. In bluehead wrasse, the arginine vasotocin (Avt) system is androgen dependent and is involved in the reproductive behavior and physiology (Semsar and Godwin, 2003). Avt-positive cell numbers and soma size in POA of bluehead wrasse were independent from gonad (Semsar and Godwin, 2003). A sexually bipotential brain allows hermaphroditic wrasse to sense social signals and change behavior and gonad sexuality in a short time (Munakata and Kobayashi, 2010). The data support the effects of brain factors on the gonad development.

Concerning the gonadal effects on the brain, it has been shown that gonadal factors (i.e., sex steroids) administered to manipulate the gonadal sex may also affect brain sex in fish (Guiguen et al., 2010; Saoshiro et al., 2013). It has been well documented that exogenous androgens (T or 11-KT) induced male gonad and E_2 induced female gonad in black porgy (Wu and Chang, 2013) and other fish species (Guiguen et al., 2010), and also that androgens stimulated male-type sexual behavior in goldfish (Saoshiro et al., 2013). In pejerrey, higher temperatures induced gonad masculinization in accordance with the increased circulatory cortisol and 11-KT levels; further, exogenous cortisol induced the increased 11-KT levels and produced gonad masculinization (Fernandino et al., 2013).

CONCLUSION

In this study, we revealed that peaks in brain neurosteroidogenesis and neurogenesis activities occur at the time of gonad differentiation in the black porgy. Exogenous E_2 stimulated neurosteroidogenesis and neurogenesis, while AI decreased brain cell proliferation. These findings support the hypothesis that E_2 plays important roles in the induction of early brain development in protandrous black porgy. No significant differences in gene expression and brain cell numbers were found between control and castrated fish. These data further support that the peak in early brain activity is controlled by locally produced brain E_2, and is independent of the gonads.

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