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Research paper

Characterization of CYP11A1 and its potential role in sex asynchronous gonadal development of viviparous black rockfish *Sebastes schlegelii* (Sebastidae)

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ABSTRACT

Mitochondrial cytochrome P450 side-chain cleavage (P450scc), encoded by the *cyp11a1* gene, initiates the first step of steroid biosynthesis. In this study, a 1554-bp open reading frame (ORF) of black rockfish (*Sebastes schlegelii*) *cyp11a1* was cloned. The *cyp11a1* gene is located on chromosome 5 and has 9 exons. The ORF encodes a putative precursor protein of 517 amino acids, and the predicted cleavable mitochondrial targeting peptide is located at amino acids 1–39. P450scc shares homology with other teleosts and tetrapods, which have relatively conserved binding regions with heme, cholesterol and adrenodoxin. Tissue distribution analysis revealed that the highest expression levels of *cyp11a1* were detected in mature gonads and head kidney but that low levels were detected in gestational/regressed ovaries, regressed testes and other tissues. Immunostaining of P450scc was observed in testicular Leydig cells, ovarian theca cells, interrenal glands of head kidney, pituitary and multiple regions of brain. Particularly, two kinds of fish-specific P450scc-positive cells, including coronet cells of brain saccus vasculosus and hypophyseal somatolactin cells, were identified in black rockfish. Our results provide novel evidence for the potential role played by P450scc in reproduction behavior by mediating steroidogenesis in viviparous teleost.

1. Introduction

In vertebrates, steroids play crucial roles in regulating sex determination, sex differentiation, secondary sexual character formation, reproductive behavior activation and gametogenesis, and steroids participate in sexual maturation in vertebrates (Tokarz et al., 2015). Steroids also transmit information between organisms as pheromones in vertebrates (Doyle and Meeks, 2018), especially in teleosts (Li et al., 2018). In teleosts, the biosynthesis of steroid hormones in animal tissues involves the participation of at least five distinct forms of cytochrome P450 and two hydroxysteroid dehydrogenases (Rajakumar and Senthilkumaran, 2020). The initial and rate-limiting step in steroidogenesis is the cleavage of the side chain from cholesterol to yield pregnenolone, which is catalyzed by cholesterol side chain cleavage cytochrome P450 (P450scc) (Nelson et al., 1993; Omura and Morohashi, 1995). P450scc, also called cholesterol side-chain cleavage enzyme, is a mitochondrial monooxygenase encoded by *cyp11a1* and catalyzes the conversion of cholesterol to pregnenolone (Hu et al., 2004). P450scc is part of an electron transport system located on the matrix side of the inner mitochondrial membrane (Hu et al., 2004).

Pregnenolone, the product generated by P450scc from gonads and adrenals, is the metabolic precursor of all steroid hormones in most vertebrates (Hanukoglu, 1992; Miller, 1988). However, P450scc is also detected in other tissues that are not generally considered steroidogenic. Several studies have reported that P450scc expression is present in the following different cell types: skin (Keeney et al., 1995; Slominski et al., 1996); peripheral nervous system and white matter cells of the brain (Iwahashi et al., 1990; Le Goascogne et al., 1987; Strömstedt and Waterman, 1995; Zhang et al., 1995); hindgut (Keeney et al., 1995); and pancreas (Morales-Montor et al., 1999). In teleosts, such as olive flounder (*Paralichthys olivaceus*) and Japanese eel (*Anguilla japonica*), *cyp11a1* is expressed in head kidney (homologous to adrenal tissue) and gonads (Kazeto et al., 2006; Liang et al., 2018).

Black rockfish (*Sebastes schlegelii*), which belongs to the *Sebastes*

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genus in the Sebastidae family, has a viviparous reproductive pattern and long-term sperm storage (Gao et al., 2018). The reproductive strategy of asynchronized gonadal development may have developed from adaptive evolution. Spermatogenesis starts in July, and the sperm matures from December to the following January. After mating, sperm are dispersed in the ovary during the early vitellogenesis period until the late period when sperm are detected under the ovigerous lamellae epithelium (Mori et al., 2003). Oocytes start vitellogenesis in November and mature around late March. After oocyte fertilization, females become pregnant, and parturition occurs in May (Kawaguchi et al., 2008). It is well-known that black rockfish has an asynchronous gonadal development process, but steroid synthesis during this process is less known.

In this study, we analyzed the protein domains, evolutionary relationship and mRNA expression patterns of *cyp11a1* related to the reproductive cycle in adult black rockfish. We also detected the immunolocalization of the Cyp11a1 (P450scc) protein in mature gonad, head kidney and different brain regions. Our study provides novel insights into the distribution and morphology of steroidogenesis cells, as well as the molecular mechanisms of sex steroid biosynthesis in viviparous black rockfish.

2. Materials and methods

2.1. Ethics statement

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Ocean University of China. The protocol for animal care and handling used in this study was approved by the Committee on the Ethics of Animal Experiments of Ocean University of China (Permit Number: 20141201). Before sacrificing and handling, experimental fish were anesthetized with 100 ng/ml ethyl 3-aminobenzoate methanesulfonic acid (MS222), and all efforts were made to minimize suffering of the animals. The field studies did not involve endangered or protected species.

2.2. Fish and sampling

In total, 89 adult black rockfish (over 3-year-old; body length: 31.7 ± 1.6 cm; body weight: 799.1 ± 150.2 g) were obtained monthly from Nanshan Fisheries Market (from November 2018 to July 2020). Gonads at different stages were collected based on the reproductive cycle of adult black rockfish. Testes were sampled in September 2019, November 2019, January 2020 and May 2020, which correspond to early spermatogenesis stage (ES), mature stage (M), regressed stage (Rgs) and regenerated stage (Rgn), respectively. Similarly, ovaries were collected in October 2019, December 2019, March 2020, April 2020, May 2020 and August 2020, which correspond to previtellogenesis stage (Pv), vitellogenesis stage (V), mature stage (M), gestation stage (G), regressed stage (Rgs) and regenerated stage (Rgn), respectively. Brains and pituitaries were collected from male fish in November 2020. Other tissues were mainly taken from female fish in August 2019. All samples originated from marine cages located offshore of Penglai, Shandong, China (37.80°N, 120.75°E).

2.3. Total RNA extraction and reverse transcription

Total RNA was extracted from gonads at different stages and other tissues using the TRIzol reagent (Vazyme, China) according to the manufacturer's instructions. Gonad samples were fixed in 10% neutral buffered formalin (10% NBF) for histological identification. RNA quantity and purity were assessed by a Biodropsis BD-1000 nucleic acid analyzer (OSTC, China) and electrophoresis using a 1% agarose gel. cDNA was prepared using the Hiscript® III SuperMix (+gDNA wiper) kit (Vazyme, China) according to manufacturer's instructions.

Table 1
Primers used in this study

Primer	Sequence (5′-3′)	Length of products (bp)	Purpose
ORF-*cyp11a1*-F	CCAAGCCTACTGGAGAACCT	1963	ORF cloning
ORF-*cyp11a1*-R	CACACAGACTACACATTCCCATC		
ORF-*fdx1*-F	CTGTGGGTTTCTCTCAGTGG	630	ORF cloning
ORF-*fdx1*-R	CTGTCCAAGTCCACGTTTCC		
qPCR-*cyp11a1*-F	CTGGAGATCGAACCGTGTGA	165	qPCR
qPCR-*cyp11a1*-R	TCTTGAGAGAGGTCCGTGGT		
qPCR-*18s*-F	CCTGAGAAACGGCTACCACAT	119	qPCR
qPCR-*18s*-R	CCAATTACAGGGCCTCGAAAG		

2.4. Molecular cloning and sequencing of cyp11a1 and fdx1

Based on the gonadal transcriptome (PRJNA573572) data and genome (unpublished) data of black rockfish, the *cyp11a1* and *fdx1* sequences were cloned and verified because Cyp11a1 (P450scc) forms a heterodimer with adrenodoxin (Adx, also named ferredoxin-1/Fdx1) (Mast et al., 2011; Miller, 2013). All primers used in the present study were designed with Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Table 1. Head kidney cDNA was used as the template for cloning. The PCR product was purified, cloned into the pCE2 TA/Blunt-Zero vector (Vazyme, China) and sequenced.

2.5. Multiple alignments, phylogenetic tree construction and homology analysis

Multiple alignments of amino acid sequences were performed based on Cyp11a1/2 amino acid sequences of several species using ClustalX2.1. Based on the deduced amino acid sequences of *S. schlegelii cyp11a1* and *fdx1*, the tertiary structure of P450scc and the quaternary structure of P450scc-Adx heterodimer were modeled by homology modeling methods, and modeling quality (QMEAN Z-scores (Benkert et al., 2010)) was assessed using SWISS-Model (https://swissmodel.expasy.org/). The four-digit Protein Data Bank-formatted file (*.pdb) of Cyp11a1 exported from SWISS-Model and multiple sequence alignments were comprehensively analyzed by ESPrit 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi).

The mitochondrial transit peptides (MTPs) and cleavage site were predicted by TargetP-2.0 Server (http://www.cbs.dtu.dk/services/TargetP/). The protein molecular weight and theoretical isoelectric point (pI) were configured by ProtParam (https://web.expasy.org/protparam/).

A phylogenetic tree was reconstructed by multiple alignments of deduced amino acid sequences with the neighbor-joining method using MEGA-X. Values on the trees represent bootstrap scores of 1000 iterations, indicating the credibility of each branch.

2.6. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed to determine the expression levels of *cyp11a1* in black rockfish gonads at different stages and other tissues. qPCR was conducted using the ChamQ™ SYBR® Color qPCR Master Mix (High Rox Premixed) kit (Vazyme, China) following the manufacturer's instructions. The qPCR program was as follows: 95 °C for 30 s; 40 cycles of 95℃ for 10 s and 60 °C for 30 s; and followed by melting curves: 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. The threshold cycle (C_T) values were measured for each sample, and *18s* (KF430619.1) was selected as the reference gene (Liman et al., 2013). The relative mRNA expression levels of the genes were calculated using the $2^{-\Delta\Delta C_T}$ method and triplicate samples. qPCR was run in triplicates to confirm the results.

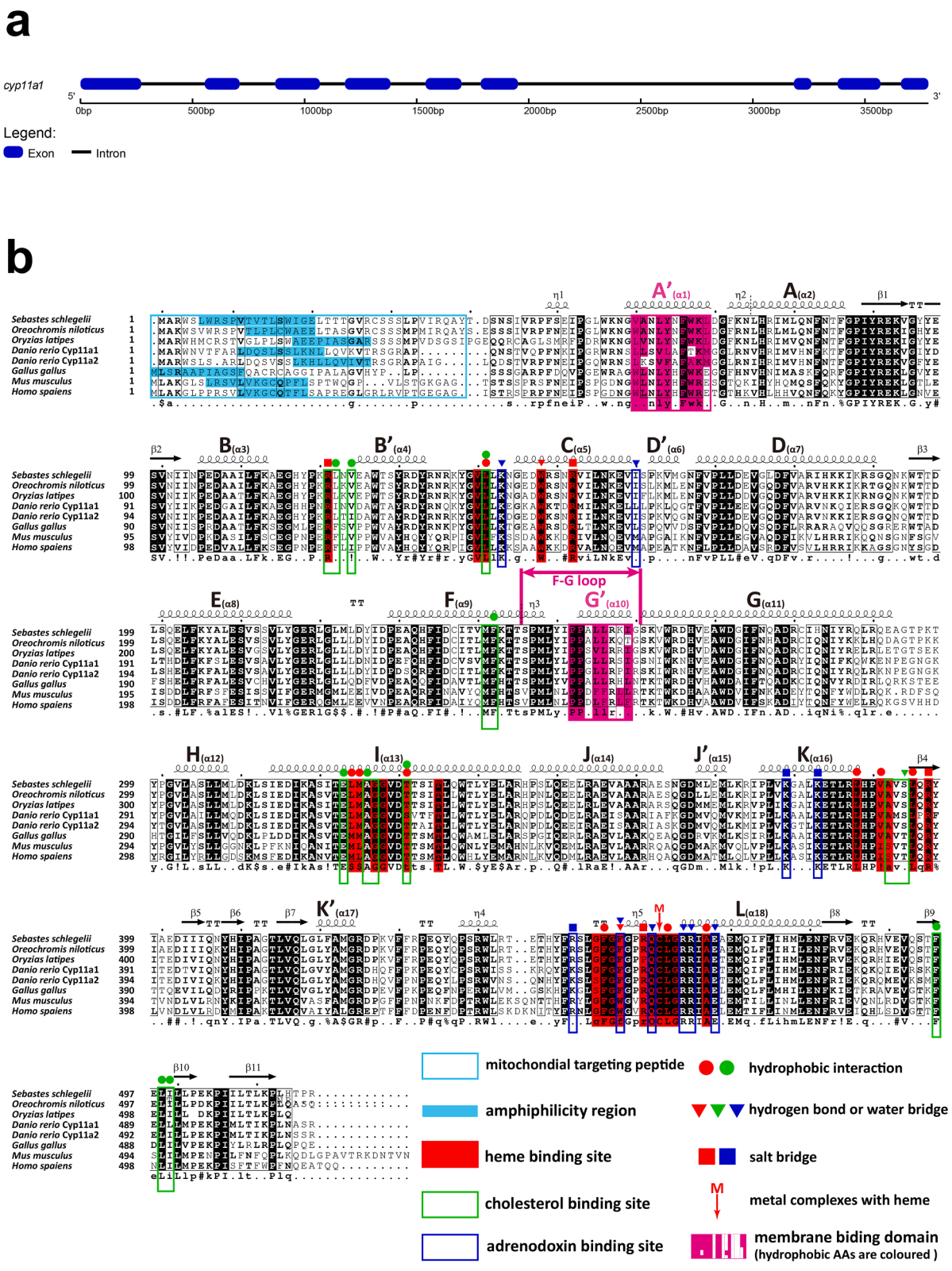

Fig. 1. Genetic structure (a) and multiple sequence alignments of Cyp11a1/2 (b). The first illustration shows the predicted secondary structure elements of *S. schlegelii* (α- or η-helices represented with squiggles, β-strands represented with arrows and turns represented with TT letters). Black background letters indicate the conserved amino acid residues. Each α-helix of black rockfish P450scc is marked alphabetically (A'-K) according to human P450scc. The GenBank accession no. of Cyp11a1 sequences used in this study are as follows: *Homo sapiens* (NP_000772.2), *Mus musculus* (NP_062753.3), *Gallus gallus* (NP_001001756.1), *Xenopus laevis* (XP_018111072.1), *Anguilla japonica* (AAV67332.1), *Oncorhynchus mykiss* (AAB25804.1), *Oryzias latipes* (NP_001156558.1), *Oreochromis niloticus* (XP_003440489.1), *Takifugu rubripes* (XP_003967495.2), and *Danio rerio* [two copies identified: Cyp11a1 (NP_694485.2) and Cyp11a2 (XP_691817.1)].

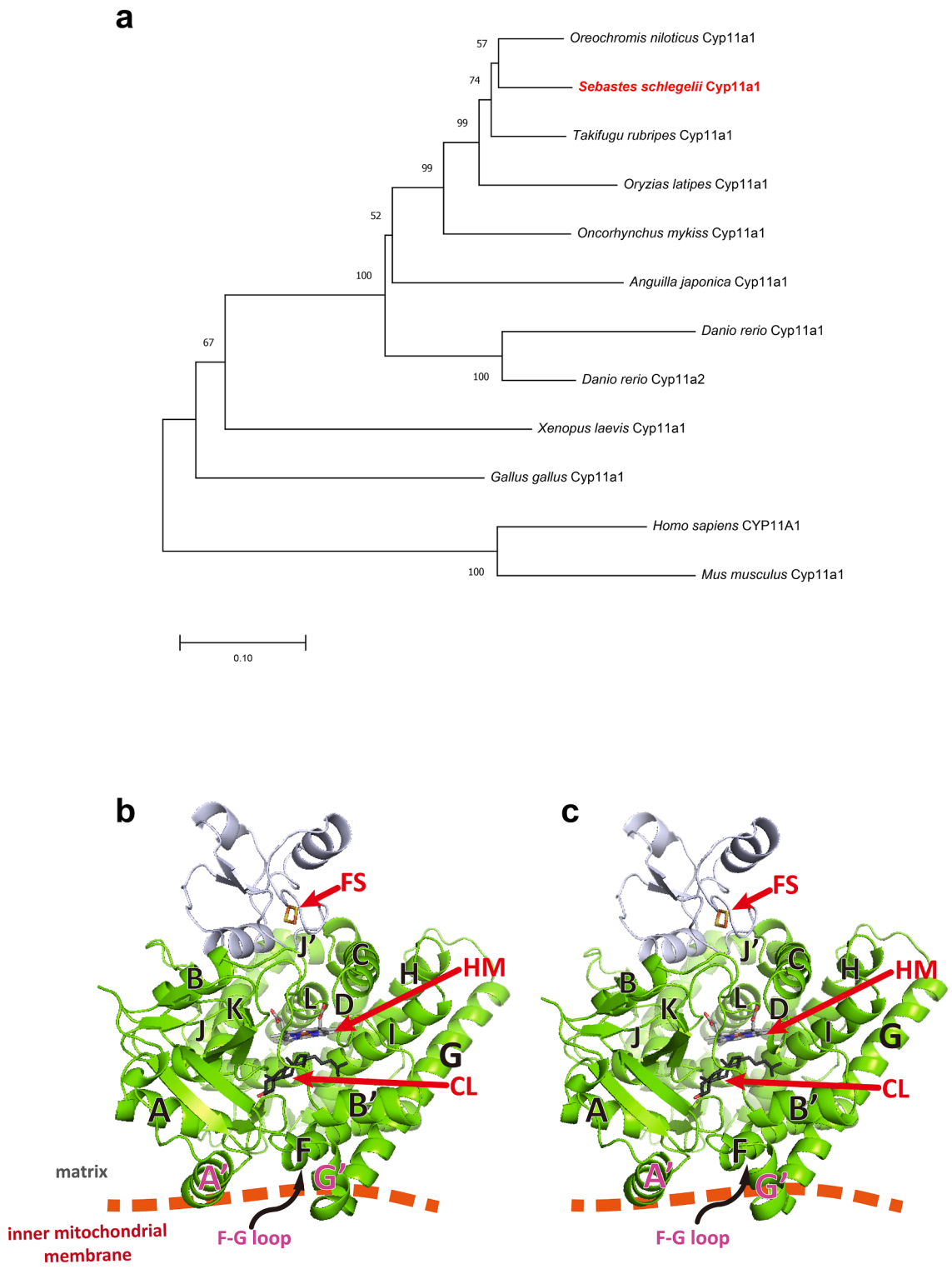

Fig. 2. Phylogenetic tree (a) and predicted 3D structure of the black rockfish P450scc-Adx heterodimer (b) using human templates (c, Protein Data Bank ID code: 3N9Y.1). a: Values at the branch points indicate the percentage of 1000 bootstrap replicates supporting the division. Red words represent Cyp11a1 of black rockfish. b-c: Green peptide: P450scc; white peptide: adrenodoxin (Adx). FS: Fe_2S_2; HM: heme; CL: cholesterol. Each α-helix of black rockfish P450scc is marked alphabetically (A'-K) according to human P450scc.

2.7. Rabbit polyclonal antibody preparation and immunohistochemistry

The following specific amino acid sequence was selected from Cyp11a1: aa 478–492 (MLENFRVEKQRHVEV). A polypeptide was artificially synthesized, and the rabbit anti-Cyp11a1 polyclonal antibody was generated by Sangon Biotech Co., Ltd. (Shanghai, China).

Mature testis, ovary (at vitellogenesis stage), head kidney and brain were collected from black rockfish. Tissues were then embedded in paraffin, fixed in 10% NBF for 6–24 h at 4 °C and sectioned (4–5 μm). Sections were then deparaffinized, rehydrated and microwaved (in TE buffer, pH = 9) for antigen retrieval. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol, and nonspecific

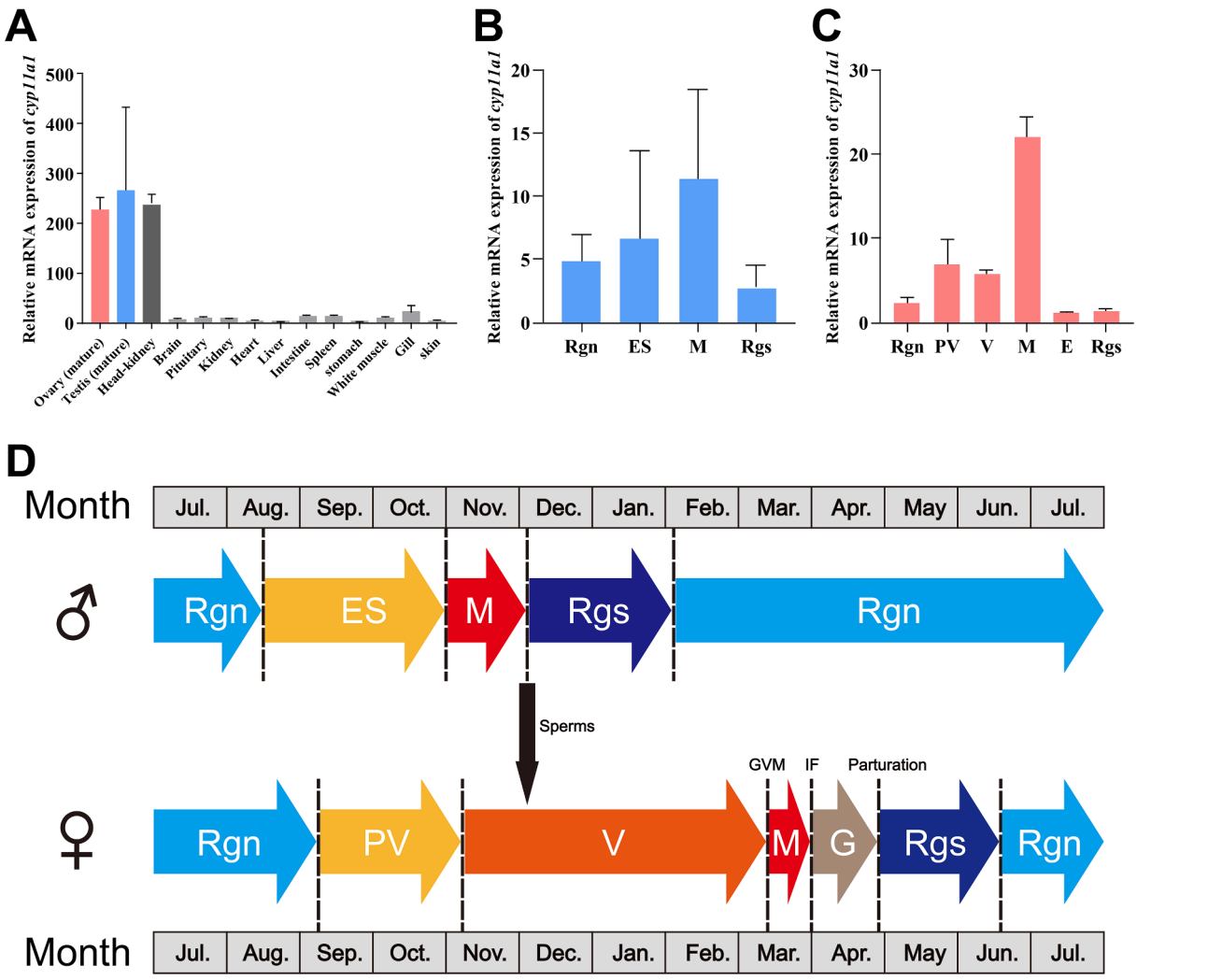

Fig. 3. Tissue distribution of *cyp11a1* (A). *Cyp11a1* expression in different developmental stages of testis (B) and ovary (C). Schematic diagram of black rockfish reproductive cycle (D). Male (♂): Rgn: regenerating; ES: early spermatogenesis; M: mature (late spermatogenesis); R: regressed. Female (♀): Rgn: regenerating; PV: previtellogenesis; V: vitellogenesis; M: mature (prefertilization); G: gestation (postfertilization); Rgs: regressed. GVM: germinal vesicle migration (germinal vesicle refers to oocyte nuclear, and GVM marks the beginning of oocyte maturation); IF: internal fertilization.

antibody binding sites were blocked with 5% BSA in TBST. Sections were incubated with primary antibody (1% BSA in TBST, 1:700) overnight at 4℃ followed by incubation with secondary antibody (1:400) for 1 h at room temperature. The peroxidase reaction was then developed using a diaminobenzidine chromogenic kit (Jiancheng, China). Finally, sections were counterstained with hematoxylin, dehydrated and coverslipped with mounting medium for microscopic observation. Negative control sections were processed with the primary antibody replaced by antibody dilution buffer alone, and none of the negative control sections showed positive staining.

2.8. Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons using GraphPad Prism 8. P values less than 0.05 were considered to be significant.

3. Results

3.1. Sequence analysis of cyp11a1

Data mining of the black rockfish genome found that the *cyp11a1* gene is located on chromosome 5 and contains 9 exons (Fig. 1a). The ORF of *cyp11a1* is 1,554 bp (GenBank accession number: MW000347), encoding a precursor protein of 517 amino acids (Fig. S1 A). The predicted mitochondrial targeting peptide (MTP) is aa 1–39. The protein molecular weight of the precursor and mature protein is 59.64 kDa and 55.25 kDa, respectively. The theoretical pI value of the precursor and mature protein is 9.26 and 9.10, respectively. To simplify the research, the amino acids of black rockfish Cyp11a1 and human CYP11A1 were renumbered according to precursor instead of mature protein in this study. Further, the ORF of *fdx1* is 504 bp (GenBank accession number: MW000348), and the ORF of *fdx1* encodes a protein (Adx) of 167 amino acids (Fig. S1 B) with a predicted MTP (aa 1–47).

3.2. Phylogenetic analysis and homology analysis of S. schlegelii Cyp11a1 (P450scc)

Phylogenetic analysis of Cyp11a indicated that Cyp11a1 is clustered with Cyp11a with other teleosts. Most vertebrates only have one copy of Cyp11a, except for zebrafish with two copies (Fig. 1b, 2a). The alignments of Cyp11a1 amino acid sequences revealed that the *S. schlegelii* Cyp11a1 (Ss-Cyp11a1) amino acid sequence is highly homologous to *Oreochromis niloticus* (89.90%), *Takifugu rubripes* (88.54%), *Oryzias latipes* (83.14%) and *Oncorhynchus mykiss* (80.81%). In addition, the two copies of *Danio rerio*, Cyp11a1 and Cyp11a2, are homologous to Ss-

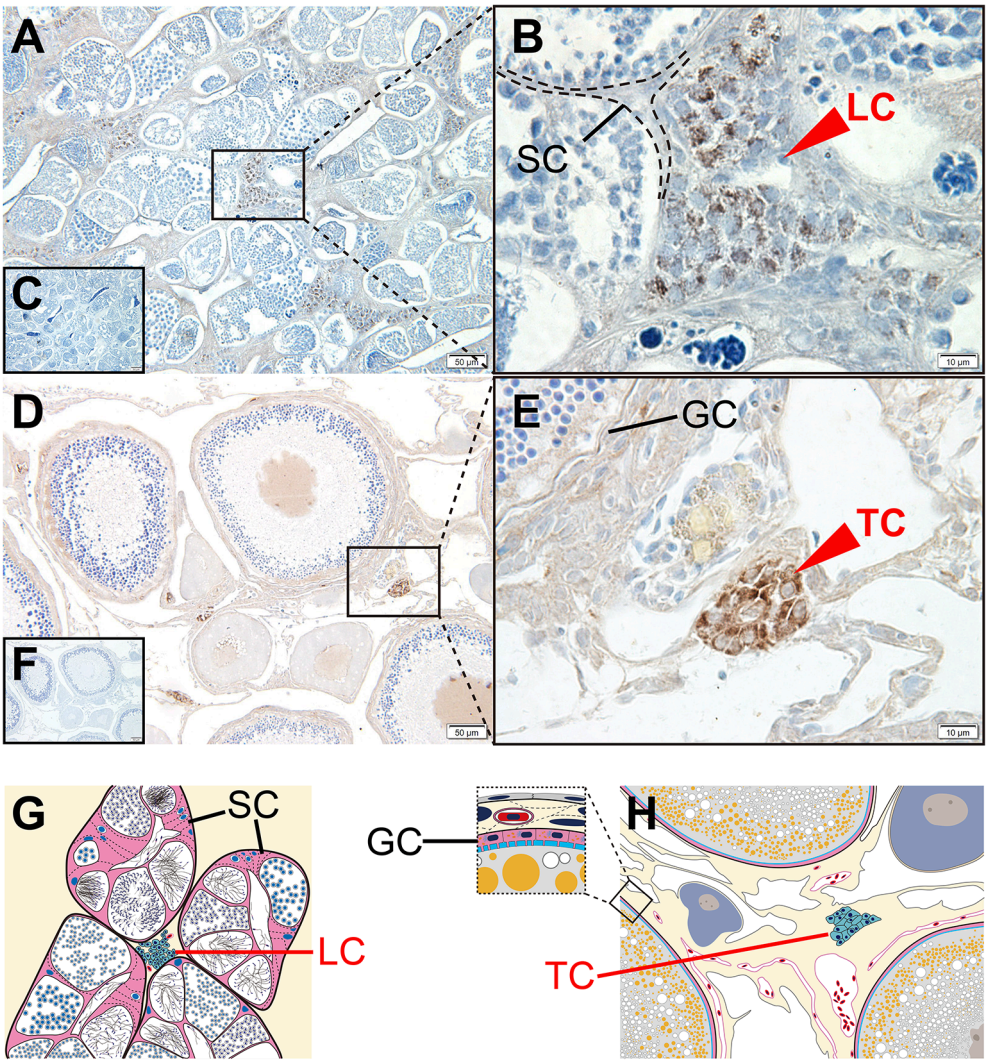

Fig. 4. Localization of P450scc in mature testis (A-C) and ovary at vitellogenesis stage (D-F). C, F: Negative control without primary antibody. G, H: Model of black rockfish gonad. LC: Leydig cell; SC: Sertoli cell; TC: theca cell; GC: granulosa cell. A, D: scale bar = 50 μm; B, E: scale bar = 10 μm.

Cyp11a1 with 65.96% and 74.47% sequence identity, respectively. Moreover, Ss-Cyp11a1 also shares homology with tetrapods, including *Xenopus laevis* (61.13%), *Gallus gallus* (60.16%), *Homo Sapiens* (52.55%) and *Mus musculus* (48.40%) (Fig. 1b).

As shown in Fig. 2b and Fig. 2c, the tertiary structure of black rockfish P450scc and the quaternary structure of P450scc-Adx heterodimer were modeled successfully. In the putative model, P450scc and Adx formed a heterodimer and bound to the substrate cholesterol. Multiple alignments in Fig. 1b indicated the presence of conserved regions. Although the conserved regions were limited, all P450scc proteins had a positively charged amphiphilicity (PA) region, which serves as an addressing presequence to mitochondrial inner membrane or matrix. Secondary structure analysis showed that Cyp11a1 lacks a transmembrane helix but that the A' helix and G' helix of Cyp11a1 are rich in highly hydrophobic amino acids. The ligand heme-, substrate cholesterol- and Adx-binding regions were predicted based on the crystal structure of human P450scc-Adx and sequence alignment. The amino acids forming the coordination bond or four salt bonds are highly conserved. However, the binding sites dependent on hydrophobic interactions, hydrogen bonds and water bridge are less conserved. In black rockfish and other nonmammalian vertebrates, some amino acids of these regions are replaced by similar ones, which may have little influence on P450scc functions (Fig. 1b, Table S1).

3.3. Expression pattern of *cyp11a1* mRNA in different tissues and reproductive cycle phases in black rockfish

qPCR analysis demonstrated that the expression level of *cyp11a1* varied in different tissues of adult black rockfish. The highest levels of *cyp11a1* transcript were detected in ovary, testis and head kidney, while lower expression levels were detected in brain, pituitary, kidney, liver and stomach (Fig. 3A).

In the Penglai sea area, the testis started to develop in mid-August and matured in late November followed by the mating behavior. Moreover, the ovary was maintained at the vitellogenesis stage and stored sperm from early November to mid-March. After oocyte maturation, sperms were activated, and pregnancy was initiated. Finally, larvae were labored by the end of April. The schematic diagram in Fig. 3D shows the reproductive cycle of black rockfish. The appearance and histological morphology of testes and ovaries at different stages are shown in Fig. S2.

The gonadal expression levels of *cyp11a1* were related to the reproductive cycle. In male black rockfish, the level was relatively low in regressed testis but increased gradually with spermatogenesis until maturation. At the end of spermatogenesis, the expression levels of *cyp11a1* sharply decreased though mature sperms could be observed in seminiferous tubules and the spermatic duct (Fig. 3B, Fig. S2).

In females, the variation of the *cyp11a1* expression pattern in ovary

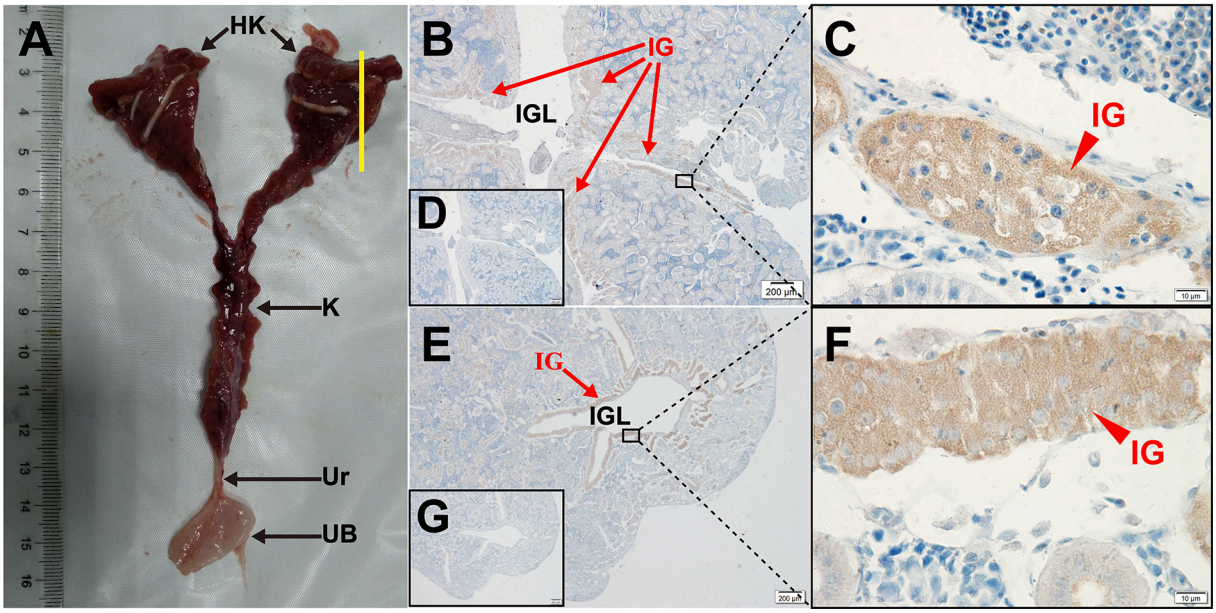

Fig. 5. Localization of P450scc in head kidney. A: Urinary system of adult black rockfish. Yellow line indicates cutting direction of tissue sections B-D; B, C, E, F: IHC staining of head kidney. D, G: Negative control without primary antibodies. B-D: head kidney of adult fish (over 3 years old); E-G: Head kidney of juvenile fish (5 month of age). HK: head kidney, K: kidney; Ut: ureter; UB: urinary bladder; IG: interrenal glands of head kidney; IGL: interrenal glands lumen. B, D, E, G: scale bar = 200 μm; C, F: scale bar = 10 μm.

was similar to that of testis. The expression level increased with vitellogenesis until oocyte maturation (prefertilization stage) when the highest level was detected and then decreased significantly after fertilization (Fig. 3C).

3.4. Localization of P450scc in gonad, head kidney and brain regions in black rockfish

The immunohistochemistry results of P450scc coincided with the *cyp11a1* mRNA expression level in gonad and head kidney. In late spermatogenesis testis, positive signals were observed in Leydig cells located in the interstitial tissue among seminiferous lobules. Similarly, positive signals were also located in Sertoli cells with weaker staining compared to that in Leydig cells (Fig. 4A–C). In the vitellogenesis ovary, positive signals were observed in theca cells located in the interstitial tissue among ovarian follicles, while no signal was found in granulosa cells in the inner layer of follicles (Fig. 4D-4F). Regarding the anatomy, black rockfish had two head kidneys connected to the kidney (Fig. 5A). Positive signals of P450scc were observed in the columnar epithelioid interrenal gland located along special hemopoietic tissues in the head kidney. In addition, immunostaining of interrenal glands was detected in both adult fish (Fig. 5B-5C) and juvenile fish (Fig. 5E-5G).

Although low mRNA expression level was detected in the brain and pituitary, P450scc immunopositive signals were still observed in some regions of the brain and pituitary. In the pituitary, strong P450scc-positive signals were only detected in somatolactin cells (SLCs), a type of adenohypophyseal cell close to neurohypophysis (Fig. 6A2, C1). In the brain, coronet cells of the saccus vasculosus (SV) (Fig. 6A4, B7-B9) and Purkinje cells (PCs) of the valvula cerebelli (VCe) and corpus cerebelli (CCe) (Fig. 6A4, A5, B1-B6) showed strong P450scc-positive staining. PCs presented as large neurons that distributed between the molecular layer and granular layer, and they spread their dendrites into the molecular layer (Fig. 6B1-B6). The epithelial tissue of SV included two cell types, namely coronet cells (CCs) and supporting cells (SuCs), and the CCs were comprised of a cell body, protrusion and globules (Fig. 6B7-B9). Immunostaining of black rockfish P450scc was only observed in the cell body and protrusion of CCs, whereas no signal was detected in the globules of CCs and SuCs (Fig. 6B7).

Positive cells were also located in the dorsal telencephalon (pallium, Fig. 6C2-C6), ventral telencephalon (subpallium, Fig. 6C7, C8), paraventricular organ (PVO, Fig. 6C10), radial glia cells of the optic tectum (OT, Fig. 6C11), diffuse nucleus of the inferior lobe (NDLI, Fig. 6C12), central nucleus of the inferior lobe (NCLI, Fig. 6C13), lateral nucleus of the valvula (NLV, Fig. 6C14), medial longitudinal fasciculus (MLF, Fig. 6C15) and medial octavolateral nucleus (MON, Fig. 6C16). The abbreviations of the brain regions are listed in Table 2.

4. Discussion

Cytochrome P450scc is the only known enzyme that catalyzes the conversion of cholesterol to pregnenolone, which is the precursor of all steroid hormones (Hu et al., 2004). In this study, we identified the full-length coding sequence of *S. schlegelii cyp11a1*. The deduced amino acid sequence of Cyp11a1 shares high identity with teleost Cyp11a1/2 but shares less identity with tetrapods Cyp11a1.

The precursor protein of black rockfish P450scc possesses an N-terminal cleavable presequence (aa 1–39) that guides the protein to mitochondria. A positively charged amphiphilicity (PA) region, an amphipathic helix with positively charged and hydrophobic amino acids, is necessary for mitochondrial targeting (Mossmann et al., 2012). P450scc locates to the matrix side of the inner membrane, which is similar to mitochondrial matrix proteins (Midzak et al., 2011; Minenko et al., 2008). The hydrophobic amino acids in the A' helix and F-G loop play an important role in membrane interactions (Headlam et al., 2003). As a cholesterol monooxygenase that contains a heme ligand, P450scc requires 2 electrons from the Fe_2-S_2 ligand of Adx in each monooxygenase step as the binding region (Mast et al., 2011; Strushkevich et al., 2011). In P450scc, the binding regions with heme, cholesterol and Adx are critical for electron transport and cholesterol side-chain oxidation. Structure analysis identified the key regions of P450scc, which are conserved among vertebrates, indicating a conserved function of the protein.

P450scc mainly plays a role in gonadal and adrenocortical/interrenal steroidogenesis in vertebrates, including fetuses and adults (Hu et al., 2002; Kim et al., 2008; Nan et al., 2020). Human *CYP11A1* is expressed in the adrenals, gonads and placenta under the control of

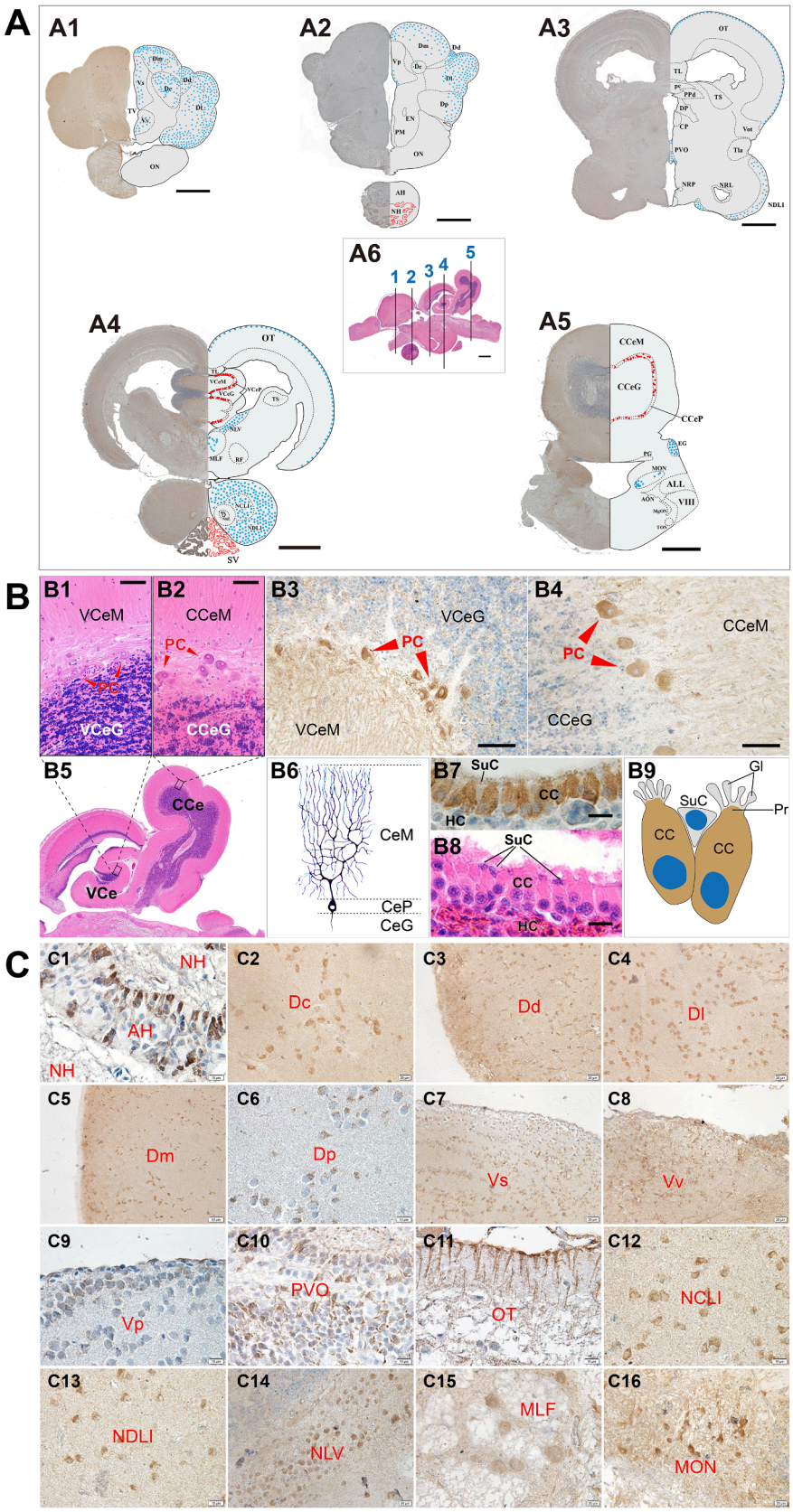

Fig. 6. Localization of P450scc in the brain and pituitary. A: Overview diagrams of the brain and pituitary. A2: anterior telencephalon; A2: posterior telencephalon and preoptic area; A3: mesencephalon, diencephalon and hypothalamus; A4: mesencephalon and pons; A5: cerebellum and pons; A6: longitudinal section of brain. The vertical lines indicate the cutting directions of A1-A5. The red points or lines indicate strongly positive cells, and blue points indicate weakly positive cells. Scale bar = 1 mm. B: P450scc localization in cerebellum (B1-B6) and saccus vasculosus (B7-B9). B1, B3: Purkinje cells in valvula cerebelli; B2, B4: Purkinje cells in corpus cerebelli; B5: cerebellum (longitudinal section); B6: Morphology of Purkinje cell (Bae et al., 2009); B7, B8: Epithelioid cells of saccus vasculosus; B9: Morphology of coronet cells and supporting cells (Nakane et al., 2013). B2, B4, B7: Immunostaining of P450scc; B1, B2, B5, B8: H-E staining. PC: Purkinje cell; CeM: molecular layer of cerebellum; CeP: Purkinje cell layer of cerebellum; CeG: granular layer of cerebellum; CC: coronet cell; SuC: supporting cell; HC: hemocyte. Pr: protrusion; Gl: globules. B1-B4: scale bar = 50 μm; B7, B8: scale bar = 10 μm. C: P450scc immunolocalization in the pituitary and brain. C2-C5, C6, C7, C14-C16: scale bar = 20 μm; C1, C6, C9-C13: scale bar = 10 μm.

Table 2
Abbreviations of brain regions

AH	adenohypophysis	NRP	the nucleus of the posterior recess
ALL	anterior lateral line nerve	ON	optic nerve
AON	anterior octaval nucleus	OT	optic tectum
CCeG	granular layer of corpus cerebelli	pc	posterior commissure
CCeM	molecular layer of corpus cerebelli	PG	periventricular granular cell mass
CCeP	Purkinje cell layer of corpus cerebelli	PM	magnocellularis preoptic nucleus
CP	central posterior thalamic nucleus	PPd	dorsal periventricular pretectal nucleus
Dc	central part of the dorsal telencephalon	PVO	paraventricular organ
Dd	dorsal part of the dorsal telencephalon	RF	reticular formation
Dl	lateral part of the dorsal telencephalon	SV	saccus vasculosus
Dm	medial part of the dorsal telencephalon	TL	torus longitudinalis
Dp	posterior part of the dorsal telencephalon	Tla	nucleus of the torus lateralis
DP	dorsal posterior thalamic nucleus	TON	tangential octaval nucleus
EG	eminentia granularis	TS	torus semicircularis
EN	entopeduncular nucleus	VCeG	granular layer of valvula cerebelli
MgON	magnocellular octaval nucleus	VCeM	molecular layer of valvula cerebelli
MLF	medial longitudinal fascicle	VCeP	Purkinje cell layer of valvula cerebelli
MON	medial octavolateral nucleus	VIII	octaval nerve
NCLI	central nucleus of the inferior lobe	Vot	ventral optic tract
NDLI	diffuse nucleus of the inferior lobe	Vp	postcommissural nucleus of the ventral telencephalon
NH	neurohypophysis	Vs	supracommissural nucleus of the ventral telencephalon
NLV	lateral nucleus of the valvula	Vv	ventral part of the ventral telencephalon
NRL	the nucleus of the lateral recess		

pituitary hormones (Chung et al., 1997). Similar to mammals, zebrafish *cyp11a1* and *cyp11a2* are expressed in the gonads, brain and interrenal glands in adults (Hsu et al., 2002; Parajes et al., 2013). In medaka, P450scc expression has been detected in interstitial somatic cells throughout both testicular and ovary development (Nakamoto et al., 2010). In Japanese eel, P450scc mRNA has been continuously detected in the thecal cell layer throughout artificially induced maturation (Ijiri et al., 2006). In rainbow trout, *cyp11a1* is highly expressed in mature ovary (Bobe et al., 2004). Gonadal expression levels of *cyp11a1* in black rockfish have similar trends throughout gametogenesis and maturation despite asynchronous gonadal development. Different from oviparous teleosts, the gestation stage of black rockfish occurs after internal fertilization. Similar to oviparity, the expression of *cyp11a1* increased with gonadal development but decreased significantly in the pregnant ovary. The increase during gonadal development indicated the conserved reproductive function of P450scc, and the decrease during pregnancy may have been caused by a lower steroid demand.

In this study, black rockfish P450scc immunoreactivity was found in the Leydig cells of the testis, theca cells of the ovary and interrenal glands of the head kidney. In zebra finches, significant expression of *Cyp11a1* has been identified in large follicles (particularly the thecal cell layer), testicular interstitial area and adrenal (Freking et al., 2000). Cyp11a1$^{+/GC}$:R26-EYFP postnatal mice express Cre Recombinase in testicular Leydig cells, ovarian theca cells, adrenal cortex and hindbrain (O'Hara et al., 2014). Similar results have been reported in teleosts, such as Japanese eel and orange-red pygmygoby (*Trimma okinawae*), during gonadal development (Ijiri et al., 2006; Sunobe et al., 2005). Unlike birds or mammals, theca cells in black rockfish, eel and pygmygoby are present in clusters instead of forming a multilayer of cells around the ovarian follicle (Ijiri et al., 2006; Sunobe et al., 2005).

The expression or localization of P450scc has been described in the brain of mammals, birds, amphibians and teleosts (Do Rego and Vaudry, 2016). Even though the expression levels of *cyp11a1* in the brain are much lower than that in the adrenal cortex or head kidney (Parajes et al., 2013; Watzka et al., 1999), neurosteroids have been implicated in the control of several behavioral and metabolic activities, such as sexual activity, aggressiveness and food consumption. In the present study, P450scc immunopositive signals were widely distributed in the brain of black rockfish, indicating that the brain is a source of steroids, which may play a role in regulating reproductive behavior of black rockfish. In adult zebrafish, *cyp11a1* is mainly detected in the telencephalon and hypothalamus by whole-mount in situ hybridization of the brain (Hsu et al., 2002). Similarly, in situ hybridization has shown that zebrafish *cyp11a1* is expressed in the forebrain, spanning from the olfactory bulbs to more caudal parts of the brain, such as the cerebellum (notably in the subpallium, the anterior and hypothalamus) or the OT (Diotel et al., 2011). Japanese quail P450scc immunoreactive cells have been observed in the telencephalic, diencephalic and mesencephalic regions as well as cerebellar PCs (Mariko et al., 1995). The wide distribution of P450scc indicates an important role of steroidogenesis among vertebrates.

Strong P450scc-positive signals were found in the pituitary somatolactin cells (SLCs) of black rockfish. Somatolactin (SL) is a bony fish-specific peptide implicated in osmoregulation, energy metabolism, stress, reproduction and skin pigmentation (Ocampo Daza and Larhammar, 2018; Bertolesi and McFarlane, 2020). SL regulates gonadal steroidogenesis, and its secretion is also affected by plasma steroids (Planas et al., 1992; Mayer et al., 1998). SL is synthesized by cells located in the pars intermedia pituitary, which is regulated by the hypothalamus-neurohypophysis system (Cánepa et al., 2008; Tanaka et al., 2009). According to our study, SLCs are a type of steroidogenesis cells in the pituitary, but the steroidogenesis pathway of SLCs is still largely unknown. Further studies on SLCs are necessary to substantiate the potential connection between steroidogenesis and endocrine function in SLCs.

Strong P450scc-positive signals were also detected in the cerebellar PCs in black rockfish. In fact, teleost possesses cerebellar neurons similar to those of other vertebrates (Gruol et al., 2016). As the sole output neurons of the cerebellar cortex, PCs are important in establishing cerebellar zonal circuits in zebrafish and mouse, involving behavior control (Matsui et al., 2014; White et al., 2014). The mammalian PC has the capacity to synthesize progesterone and estradiol de novo from cholesterol (including P450scc activity), and P and E_2 are important for cell survival and synapse formation of PCs (Tsutsui, 2008). PCs of Japanese quail also show P450scc-positive staining (Mariko et al., 1995). Our study provides evidence that teleost PCs may have similar steroidogenesis functions as mammals and birds. The SV is a unique part of the fish brain (Bourne, 2012) as it is a sensor of seasonal changes in day length (Maeda et al., 2015; Nakane et al., 2013). The immunostaining of black rockfish P450scc was only observed in the cell body and protrusion of SV CCs. In teleost CCs, smooth endoplasmic reticulum mainly occurs in globules, while mitochondria are predominantly located in the cell body and protrusion (Lanzing and van Lennep, 1970). Steroids are usually synthesized by enzymes located in mitochondria and endoplasmic reticulum, and the distribution of mitochondria in CC is consistent with that of P450scc localization in black rockfish. However, studies on the steroidogenesis of SV are lacking, except that aromatase (Cyp19alb) immunoreactivity or mRNA is localized in the teleost SV (Forlano et al., 2001; Menuet et al., 2003). As a result, the CC is likely to be another powerful steroidogenesis cell type in the brain.

In summary, we cloned *cyp11a1* from viviparous black rockfish and determined its mRNA expression in tissues and gonadal developing stages. We demonstrated that P450scc is expressed in classic steroidogenic cells and tissues, including testicular Leydig cells, ovarian theca

cells, interrenal glands of the head kidney and several regions of the brain. These findings suggested that the expression pattern and localization of Cyp11a1 are relatively conserved among vertebrates. Moreover, we also found two types of fish-specific steroidogenic cells, including the coronet cell of saccus vasculosus and pituitary somatolactin cells. Our study provides interesting insights into the potential functions of Cyp11a1 in the asynchronized reproductive cycle of viviparous black rockfish, corticosteroid synthesis of interrenal glands and brain physiology.

CRediT authorship contribution statement

Xiaojie Wang: Writing - original draft, Data curation, Visualization. **Haishen Wen:** Supervision, Resources. **Yun Li:** Supervision, Resources. **Likang Lyu:** Software. **Min Song:** Validation. **Ying Zhang:** Formal analysis. **Jianshuang Li:** Data curation. **Yijia Yao:** Methodology. **Jifang Li:** Investigation. **Xin Qi:** Writing - review & editing, Funding acquisition, Project administration.

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Appendix A. Supplementary data

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