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Research paper

Melanocortin-4 receptor regulation of reproductive function in black rockfish (*Sebastes schlegelii*)

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ABSTRACT

Melanocortin-4 receptor (MC4R) is a G protein-coupled receptor with multiple functions in mammals. However, the functions of MC4R in fish have not been investigated extensively. The purpose of this study was to determine potential regulation of reproduction by the MC4R. We cloned the black rockfish MC4R and analyzed its tissue distribution and function. The results showed that black rockfish *mc4r* cDNA consisted of 981 nucleotides encoding a protein of 326 amino acids. The quantitative PCR data showed that *mc4r* mRNA was primarily expressed in the brain, gonad, stomach and intestine. In the brain, *mc4r* was found to be primarily located in the hypothalamus. Both α-MSH and β-MSH increased *gnih* expression and decreased *sgnrh* and *cgnrh* expression ($P < 0.05$). α-MSH and β-MSH had opposite effects on *kisspeptin* expression. In contrast, α-MSH and β-MSH increased the expression of *cyp11, cyp19, 3β-hsd* and *star*. In summary, our study shows that MC4R in black rockfish might regulate reproductive function and that the effects of α-MSH and β-MSH might differ.

1. Introduction

Melanocortins are products of the precursor protein proopiomelanocortin (POMC), namely, α-melanocyte stimulating hormone (MSH), β-MSH, γ-MSH, and ACTH (Smith and Funder, 1988). They have important roles in the skin, stress response and immune system and influence feeding behavior and sexual function (Catania et al., 2000; Getting, 2006). Melanocortins contain the pharmacophore His-Phe-Arg-Trp, which is essential for binding to receptors. Melanocortin receptors (MCRs) comprise five members (MC1R to MC5R), all of which belong to the G protein-coupled receptor (GPCR) superfamily. MCRs have seven transmembrane domains (TMDs) connected by alternating extracellular and intracellular loops, with the N terminus located extracellularly and the C terminus located intracellularly (Cone, 2006; Tao, 2017). MC1R is the classic MSH receptor that regulates pigmentation in skin and hair. MC2R is the classic ACTH receptor that regulates adrenal steroidogenesis and cell proliferation in the adrenal cortex. MC3R and MC4R are expressed primarily in the central nervous system and regulate energy homeostasis (Tao, 2005). MC5R is involved in regulating exocrine gland function (Chen et al., 1997). In addition, MCRs also regulate several diverse physiological functions, such as immunomodulation, cardiovascular regulation and sexual function (Cone, 2006; Tao, 2017).

MC4R has received significant attention because it mediates leptin action on energy homeostasis and body weight (Tao, 2010). Cerdá-Reverter et al. and Schjolden et al. reported that intracerebroventricular (ICV) injection of the MC4R agonist NDP-MSH or MTII inhibits food intake in goldfish (*Carassius auratus*) and rainbow trout (*Oncorhynchus mykiss*), while the antagonist HS024 increases food intake in these species (Cerda-Reverter et al., 2003; Schjolden et al., 2009). In addition, an *mc4r* mutation has been found to contribute to enhanced appetite, growth, and starvation resistance in cavefish (*Astyanax mexicanus*) (Aspiras et al., 2015). However, in addition to regulating energy homeostasis, leptin also plays an important role in the reproductive system by mediating the secretion of related hormones in the hypothalamic-pituitary–gonadal (HPG) axis (Pérez-Pérez et al., 2015). For

Abbreviations: 3β-HSD, 3β-Hydroxysteroid dehydrogenase; Dd, Area dorsalis telencephali pars dorsalis; Dm, Area dorsalis telencephali pars medialis; GnRH, Gonadotropin-releasing hormone; GnIH, Gonadotropin-inhibitory hormone; HPG, Hypothalamus-pituitary–gonadal; LH, Luteinizing hormone; MSH, Melanocyte-stimulating hormone; NAT, Nucleus anterior tuberis; NLT, Nucleus lateral tuberis; NPO, Nucleus preoptic; NTP, Nucleus posterioris thalami; OTec, Optic tectum; POMC, Proopiomelanocortin; PRL, Prolactin; StAR, Steroidogenic acute regulatory protein; Vs, Area ventralis telencephali pars supracommissuralis; Vv, Area ventralis telencephali pars ventralis

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Table 1
Primers used for cloning and quantitative PCR of reproduction-related genes.

Primer name	Primer sequence (5′–3′)	Tm (°C)	Efficiency (%)
mc4r-5′race-R1	GGCGAGTGAAGGTTCTTGTTT	60–50	–
mc4r-5′race-R2	GGCTGATAAGTCTTTGTTGAGTGG	55	—
mc4r-3′race-F1	CCATCACCCTCACCATCCTC	60–50	—
mc4r-3′race-F2	GCCAGGAGATGAGGAAGACC	55	—
UPM long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	60–50	—
UPM short	CTAATACGACTCACTATAGGGC		
NUP	AAGCAGTGGTATCAACGCAGAGT	55	—
mc4r-complete-F	ATGAACACCACAGAGCACCATG	66	—
mc4r-complete-R	TCACACACACAAGAGAGCGTG		
mc4r-F	AGAAGGACTCGTCGGCAGGATG	60	100.76
mc4r-R	ACGATAGCAGCAACAACCAGGATG		
cgnrh-F	TGCTGCTTGGGCTGCTTCTATGT	62	95.36
cgnrh-R	CCTCTGAAACCTCTGATGTGCCG		
sgnrh-F	GTGTTGTTATTGGCGTTGGT	56	93.93
sgnrh-R	AAGTCTCTCTTGGGTCTGGG		
gnih-F	GCTTCGGACGGGAGATTAG	62	98.97
gnih-R	CTCGGACAACAGGGCACTC		
kisspeptin-F	ATCAGGAAATACTCAAAGCCC	56	92.00
kisspeptin-R	AGGAGTTGAGGTTGTATGAGG		
cyp11a1-F	AACAAATGGACCACGGACCTC	60	96.32
cyp11a1-R	CTGGGTAGGTCTTTGGAGTGC		
cyp19a1a-F	GCACCGCCAGCAACTACTACA	62	108.63
cyp19a1a-R	GCCAAACTGTCCAGGTCGTCC		
3B-hsd-F	CTTTCTTCTGCTACGATGACTC	56	109.21
3B-hsd-R	AGCAGCGGTGTGTAGTTATGAA		
star-F	CTGGCATCTCCTATCGGCA	58	92.60
star-R	CTCCACACTATCTGTCCCA		
18 s-F	CCTGAGAAACGGCTACCACAT	56	106.48
18 s-R	CCAATTACAGGGCCTCGAAAG		
mc4r-probe-F	CGCATTTAGGTGACACTATAGAAGCGCACCCTGCGAAGAGCGATG	64	—
mc4r-probe-R	CCGTAATACGACTCACTATAGGGAGACACACACAAGAGAGCGTGCGAG		

example, leptin modulates luteinizing hormone (LH) and prolactin (PRL) release (Schiöth and Watanobe, 2002). Recent studies have suggested that MC4R is involved in modulating reproductive function (Khong et al., 2001; Van der Ploeg et al., 2002). Studies on rats suggest that MC4R mediates the effects of leptin on LH and PRL secretion (Watanobe et al., 1999) and the preovulatory PRL surge (Watanobe et al., 2001). *Mc4r* knockout female mice show advanced reproductive aging with increased numbers of cystic follicles (Sandrock et al., 2009).

However, only a few studies have been conducted on MC4R regulation of reproduction in basal vertebrates. Lampert et al. and Volff et al. proposed that sequence polymorphisms in the *mc4r* genes due to *mc4r* copies located on the X and Y chromosomes including functional and nonfunctional mutations modulate the onset of puberty in platyfish (*Xiphophorus maculatus*) and swordtail (*Xiphophorus nigrensis*) (Lampert et al., 2010; Volff et al., 2013). The results of both in vitro and in vivo studies on spotted scat, *Scatophagus argus*, have shown that activation of MC4R increases the expression of *gnrh* (in the hypothalamus) and of *fshb* and *lhb* (in the pituitary gland) (Jiang et al., 2017).

Gonadotropin-releasing hormone (GnRH) plays a crucial role in the HPG axis, incorporating internal and external signals to regulate gonadotropin secretion in vertebrates (Zohar et al., 2010). Another peptide, gonadotropin-inhibitory hormone (GnIH), inhibits the reproductive process by suppressing GnRH neurons to decrease gonadotropin synthesis and release (Kriegsfeld et al., 2006; Murakami et al., 2008). Kisspeptin encoded by the *KiSS1* gene in the hypothalamus acts upstream of GnRH and is sensitive to feedback from sex steroid induction (Pinilla et al., 2012). Kisspeptin regulates puberty onset, gonadotrophin secretion, and fertility (Dungan et al., 2006).

3β-Hydroxysteroid dehydrogenase (3β-HSD) and steroidogenic acute regulatory protein (StAR) are required for the biosynthesis of all classes of steroid hormones, such as progesterone, androgens and estrogens (Tsuchiya et al., 2003; Simard et al., 2005). Mutations in *StAR* cause lipoid congenital adrenal hyperplasia, with impaired steroidogenesis in the adrenal gland and gonad (Bose et al., 1996). The cholesterol side-chain cleavage enzyme P450scc, which is encoded by *CYP11A1*, is involved in the first step of the steroidogenic pathway (Storbeck et al., 2007), and cytochrome P450 aromatase, encoded by *CYP19a1a*, is a terminal enzyme in the estrogen biosynthetic pathway that converts androgen into estrogen (Simpson et al., 1994; Conley and Hinshelwood, 2001).

Black rockfish (*Sebastes schlegelii*), an important commercial species, is a typical marine ovoviviparous fish in which yolk accumulates in oocytes during vitellogenesis to serve as an energy source (Boehlert and Yamada, 1991). The black rockfish mainly inhabits the coasts of Korea, Japan and China. However, studies on the reproductive physiology of ovoviviparous species are scarce. Previous studies have shown that MC4R might participate in the reproductive process by affecting the secretion of reproductive hormones. In our study, we investigated the MC4R-mediated regulation of reproductive functions in black rockfish by cloning and analyzing the tissue distribution and localization of *mc4r* in the brain and ovaries. To further study whether MC4R is involved in regulating reproduction, we incubated brain and ovary tissues in vitro with α- and β-MSHs and determined changes in the expression of reproduction-related genes.

2. Materials and methods

2.1. Animals

Female black rockfish were purchased from a local fish market in Qingdao, China, approximately every two months. All the fish were killed after anesthetization in a 0.01% solution of tricaine methanesulfonate (MS-222), and fish sexual maturity was determined by examination of ovarian morphology. Ovaries at six different maturity stages (stage II (perinucleolar oocyte stage), stage III (primary yolk stage), stage IV (secondary yolk stage), stage V (tertiary yolk stage), stage VI (gestational ovary stage), and stage VII (degenerative ovary stage)) were collected for seasonal studies. All animal experiments were

conducted in accordance with the national guidelines and were approved by the Animal Research and Ethics Committee of Ocean University of China (Permit Number: 20141201).

2.2. Cloning and sequence analysis of mc4r

Total RNA was extracted from black rockfish brains using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. One microgram of RNA was used to synthesize first-strand cDNA with a SMART™ RACE cDNA amplification kit (Takara Bio, Mountain View, CA, USA). Two-round PCR was performed using gene-specific primers designed according to the partial MC4R cDNA sequence from the transcriptome database (unpublished data) to amplify the cDNA fragments of the black rockfish MC4R gene, and 5′- and 3′-rapid amplification of cDNA ends (RACE) was then performed. All primers used in this study are listed in Table 1.

PCR was performed using Taq DNA polymerase (Takara Bio) following touchdown PCR cycling conditions. The program began with a denaturation step at 94 °C for 3 min that was followed by 20 cycles of 94 °C for 15 s, a range of annealing temperatures from 60 to 50 °C (decreasing 0.5 °C per cycle) and 72 °C for 40 s; after the 20 cycles, the program ended with 10 min at 72 °C for extension. The first-round PCR amplification products were used as templates for second-round PCR, which was performed with a denaturation step at 94 °C for 3 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 15 s and 72 °C for 40 s. The reaction was terminated with extension for 5 min at 72 °C. The products were purified using a TIANgel Midi DNA Purification Kit (Tiangen, Beijing, China), subcloned into the T1 vector (Transgen, Beijing, China), propagated in *E. coli* DH5α (Transgen) and sequenced using an ABI3700 sequencer.

The putative TMDs were predicted with TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Multiple amino acid sequence alignment was performed using ClustalX and was further modified with GeneDoc software. A phylogenetic tree was constructed using the amino acid sequence of black rockfish MC4R and those of MC4Rs in several vertebrate species downloaded from the NCBI database. The phylogenetic tree was constructed using MEGA 7.0 software based on the neighbor-joining (NJ) method and the Jones-Taylor-Thornton model with 1000 bootstrap replicates (Kumar et al., 2016).

2.3. In situ hybridization (ISH) of mc4r in black rockfish brain and ovary

Female black rockfish were killed after anesthetization. Their brains and ovaries were collected, fixed in buffered 4% paraformaldehyde for 12 h at 4 °C, dehydrated using a series of graded ethanol solutions (70–100%), cleared with xylene and embedded in paraffin. Transverse serial sections were cut to 6 μm with a rotary microtome and dried at 37 °C overnight. Sense and antisense digoxigenin (DIG)-labeled riboprobes were synthesized from the open reading frame (ORF) sequence of the black rockfish *mc4r* using a DIG RNA labeling kit (Roche Diagnostics, Basel, Switzerland). Before hybridization, the sections were briefly cleared, rehydrated, washed with phosphate-buffered saline (PBS), and rinsed with 0.1 M HCl for 10 min before being digested with proteinase K (10 μg/mL) for 20 min at 37 °C. Next, they were washed in 2× standard saline citrate (SSC) for 10 min and pre-hybridized at 55 °C for 1 h. The sections were hybridized with DIG-labeled riboprobes diluted to 1600 ng/mL in hybridization buffer at 55 °C for 16 h in a humidified chamber. After hybridization, the sections were washed with graded SSC (2–0.1×) and blocked with blocking buffer for 30 min. DIG was detected through incubation with an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics; diluted 1:2000) and chromogenic development with an NBT/BCIP stock solution (Roche Diagnostics). Finally, the sections were examined by light microscopy.

2.4. In vitro effects of α- and β-MSHs on the expression of several genes in female black rockfish brain and ovarian fragments

Black rockfish α- and β-MSHs were synthesized by GL Biochem (Shanghai, China). The purity was > 95% as determined by analytical HPLC. The peptides were dissolved to 10^{-3} M in dimethyl sulfoxide (DMSO) (cell culture grade) and diluted to the desired concentration in culture medium in preparation for the in vitro experiments.

Black rockfish were anesthetized with MS-222 before decapitation. Their brains and ovaries were collected, washed in PBS, cut into small pieces (< 1 cm^3) and washed with PBS three times. Then, the fragments were divided evenly into 24-well plates and starved for 2 h in 1 mL of medium 199 with 1% penicillin–streptomycin solution in an incubator without CO_2 at 27 °C. Three biological replicates were prepared for each group.

After starvation, the medium was replaced with fresh culture medium containing either α- or β-MSH (10^{-6}, 10^{-7} or 10^{-8} M) and 10% fetal bovine serum. Brain and ovarian fragments were collected after incubation for 3, 6 or 12 h and frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction and real-time PCR.

2.5. Quantitative PCR

Black rockfish brains and ovaries were placed into DEPC-treated centrifuge tubes, and total RNA was extracted using TRIzol according to the manufacturer's instructions. The RNA was assessed using a Biodropsis BD-1000 nucleic acid analyzer (OSTC, Beijing, China) and by 1.5% agarose electrophoresis. Total RNA (1 μg) was reverse transcribed into cDNA using a PrimeScript™ RT reagent kit (TaKaRa, Otsu, Japan) for quantitative PCR according to the manufacturer's guidelines. Quantitative PCR was performed to determine the expression levels of *mc4r, sgnrh, cgnrh, gnih, kisspeptin, cyp11, cyp19, 3β-hsd* and *star* in black rockfish brains or ovaries. The primers for all genes are listed in Table 1.

Each group consisted of three biological replicates. For quantitative PCR, a total volume of 20 μl was used that contained 10 μl of SYBR® FAST qPCR Master Mix (2 ×), 2 μl of cDNA, 6.8 μl of DEPC water, 0.4 μl of forward and reverse primers and 0.4 μl of ROX reference dye. The reaction conditions were as follows: initial denaturation for 60 s at 95 °C and 40 cycles of denaturation for 15 s at 95 °C, annealing for 30 s at 55 °C and extension for 30 s at 72 °C. The results from the melting curve analyses of the genes showed single peaks, confirming the specificity of the PCR results. The threshold cycle (Ct) values were measured for each sample, and 18S RNA was used to normalize the relative expression of the genes (Ma et al., 2013). The relative mRNA expression levels of the genes were calculated using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.6. Statistics

All data are presented as the mean ± SEM. Statistical significance was determined by one-way ANOVA and Duncan's method for multiple comparisons with SPSS20 software. A value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Cloning and sequence analysis of black rockfish mc4r cDNA

The full-length MC4R cDNA sequence was acquired from RACE and PCR cloning using black rockfish brain RNA as a template. The *mc4r* ORF was found to consist of 981 nucleotides encoding a protein of 326 amino acids (Fig. 1). MC4R was determined to be a typical GPCR composed of seven hydrophobic TMDs connected by alternating extracellular and intracellular loops with an extracellular N-terminus and an intracellular C-terminus. The cDNA sequence has been uploaded to the GenBank database (#MN082615).

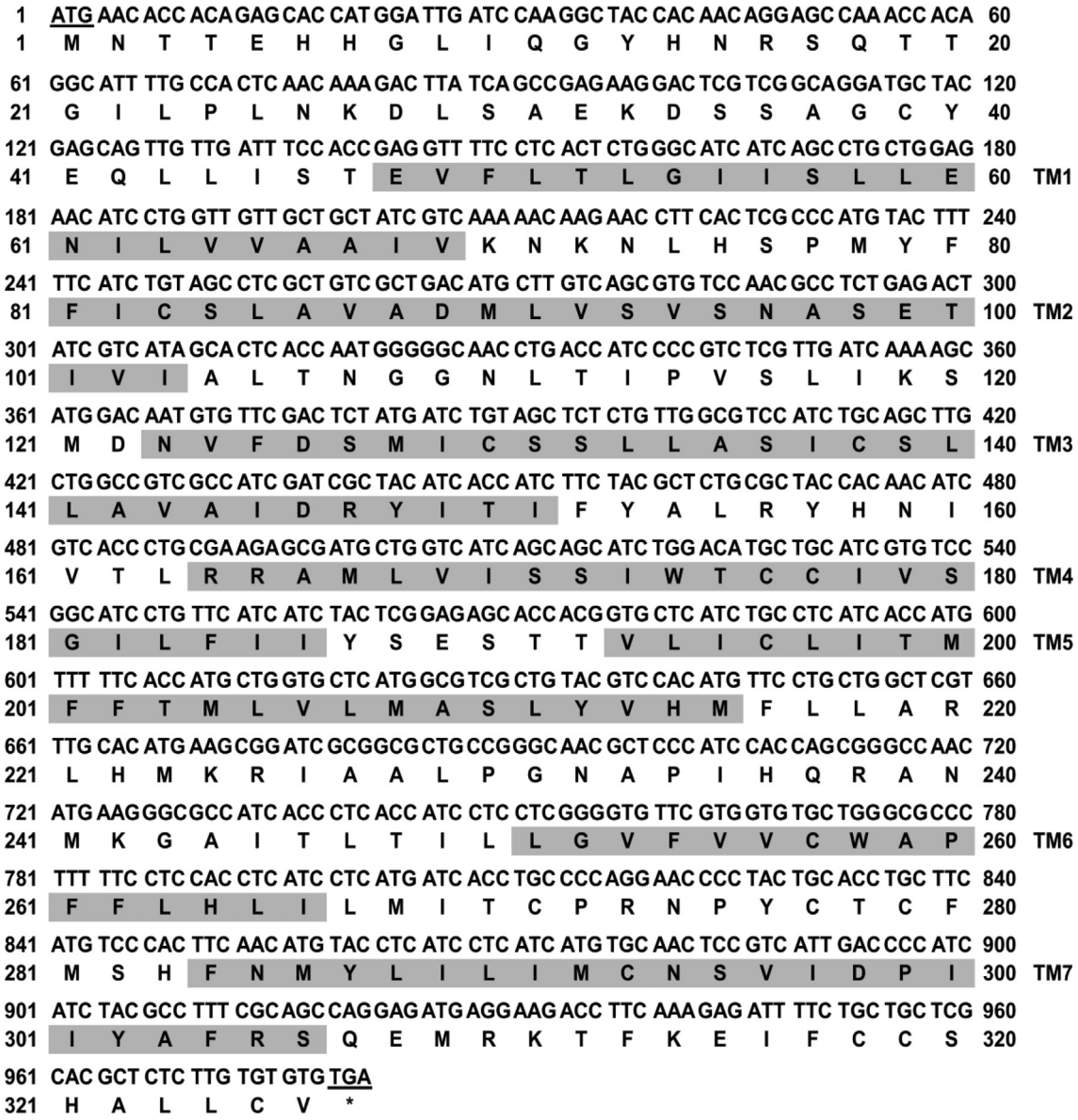

Fig. 1. Nucleotide sequence and deduced amino acid sequence of MC4R in black rockfish. The transmembrane domains are numbered on the right side of the figure, and their sequence compositions are shaded. The start codon and stop codon are underlined.

The amino acid sequence of the black rockfish MC4R was aligned with those of MC4Rs in other species, and the results indicated that MC4R is conserved among fishes and several other vertebrates (Supplementary Fig. 1). The black rockfish MC4R was found to have high sequence homology with MC4Rs in other fishes, such as swamp eel, three-spine stickleback, and Panuco swordtail, and low sequence homology with MC4R in mammals such as goat, mouse and human (Supplementary Fig. 2).

3.2. Tissue expression and seasonal expression of *mc4r* mRNA in the brain and ovaries

The tissue expression of *mc4r* mRNA was analyzed in female black rockfish with ovaries in the perinucleolar oocyte stage. In addition to the ovaries, the following tissues were analyzed: brain, liver, spleen, kidney, stomach, intestine, skin, pituitary, muscle, head kidney, and heart. The results showed that *mc4r* was primarily expressed in the brain. It was also expressed at lower levels in the liver, ovary and stomach but was negligibly or not expressed in the other tissues (Fig. 2).

We used brains and ovaries at different stages of sexual maturity to investigate the seasonal expression of *mc4r* mRNA. The results showed that the expression of *mc4r* gradually increased from fish at stage II to those at stage IV (although the differences were not significant), reached the highest level in fish at stage V (statistically significant) and decreased at stages VI and VII (Fig. 3).

3.3. Localization of *mc4r* mRNA in the black rockfish brain and ovaries

The localization of *mc4r* mRNA in black rockfish brains and ovaries was determined using ISH with an antisense riboprobe, with a sense riboprobe used as the negative control. The brains were divided into the telencephalon, diencephalon, and opisthencephalon portions. Strong positive signals were observed in the tuberal hypothalamus (Fig. 4C). Almost all divisions of the tuberal hypothalamus were found to express *mc4r* mRNA, including the anterior part of the lateral tuberal nucleus (NLTA), the anterior tuberal nucleus (NAT) and the posterior part of the lateral tuberal nucleus (NLTP). Signals were also detected in the medial and lateral parts of the dorsal telencephalon (Dm and DL (Fig. 4A), respectively), the magnocellular neurons of the preoptic nucleus (NPO), the ventral part of the ventral telencephalon (Vv) (Fig. 4B) and the optic

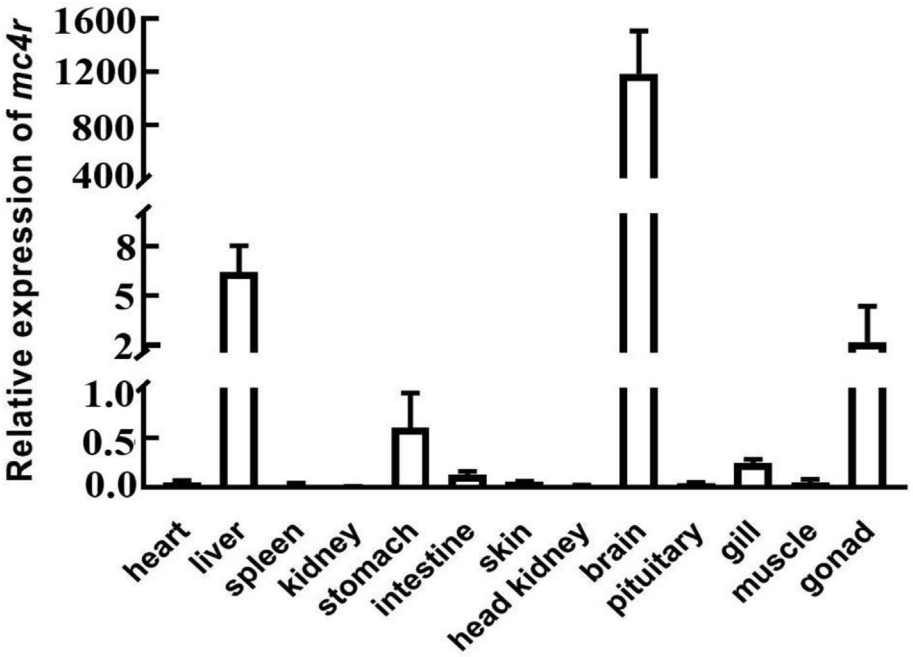

Fig. 2. Relative expression levels of *mc4r* in several tissues of black rockfish. The y-axis shows the relative expression level, and the x-axis shows the type of tissue. The data represent the mean ± SEM (n = 3) and were subjected to ANOVA followed by Duncan's multiple-range test.

tectum (OTec) (Fig. 4E). The sense riboprobe produced no signal (Fig. 4b, c, e). The localization of *mc4r* mRNA with the antisense riboprobe in the ovaries is shown in Fig. 4F, and the control is shown in Fig. 4f. Stronger *mc4r* signals were found in stage II ovaries than in stage III ovaries.

3.4. *In vitro effects of α-MSH and β-MSH on the expression of reproduction-related genes in black rockfish brains and ovaries*

Both α- and β-MSHs increased the expression of *mc4r*, although the effect of β-MSH was more sustained (Fig. 5A and 6A). Both α- and β-MSHs decreased the expression of *sgnrh* ($P < 0.05$) (#MN082617) and *cgnrh* (#MN082616) ($P < 0.05$), but increased that of *gnih* (#MN082618) (Fig. 5B, 5C and 5D for α-MSH, and Fig. 6B, 6C, and 6D for β-MSH). The effects of α- and β-MSH treatment gradually weakened with longer treatment, although the effects of treatment with 10^{-6} M α- or β-MSH sustained for 12 h.

α- and β-MSHs had opposite effects on *kisspeptin* (#AIZ68243.1) expression (Fig. 5E and Fig. 6E). α-MSH decreased *kisspeptin* expression ($P < 0.05$), whereas β-MSH increased *kisspeptin* expression ($P < 0.05$). Significant effects on *kisspeptin* expression were observed for both α- and β-MSHs administered at concentrations of 10^{-6} M and 10^{-7} M; however, no significant effect was observed after 10^{-8} M α- or β-MSH treatment compared to the control treatment.

α-MSH did not affect the gene expression of *cyp11a1* (#MN082619) or *3β-hsd* (#MN082620) but significantly increased the expression of *cyp19a1a* (#FJ594995.2) after 3 h treatment ($P < 0.05$) (Fig. 7). In contrast, β-MSH increased the gene expression of *cyp11a1, cyp19a1a, 3β-hsd,* and *star* ($P < 0.05$) (Fig. 8). β-MSH administered at a concentration of 10^{-6} M had the most significant effect on gene expression, especially that of *3β-hsd* ($P < 0.05$).

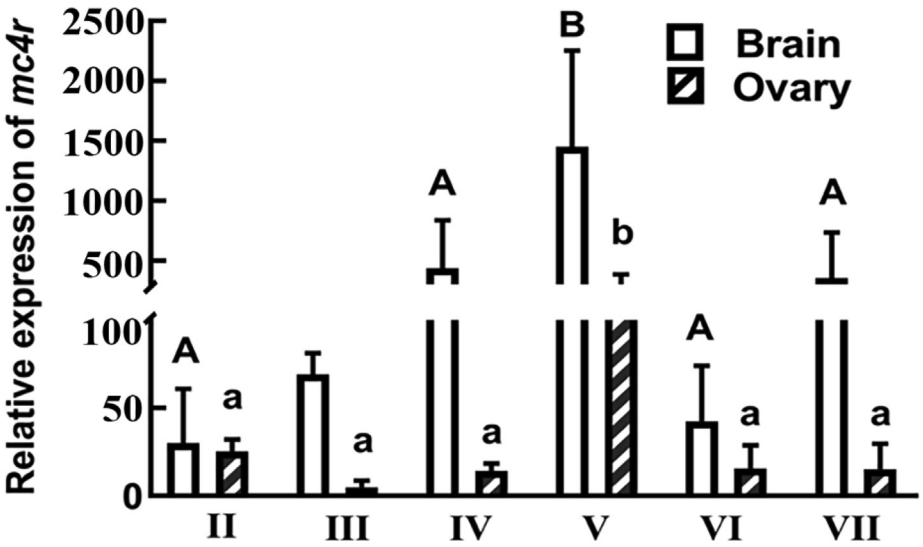

Fig. 3. Relative expression levels of *mc4r* in the brains and ovaries of black rockfish from different seasons. The y-axis shows the relative expression level of *mc4r*, and the x-axis shows the period of ovary development from II to VII. The data represent the mean ± SEM (n = 3) and were subjected to ANOVA followed by Duncan's multiple-range test. Significant differences are noted by different letters in each series ($P < 0.05$).

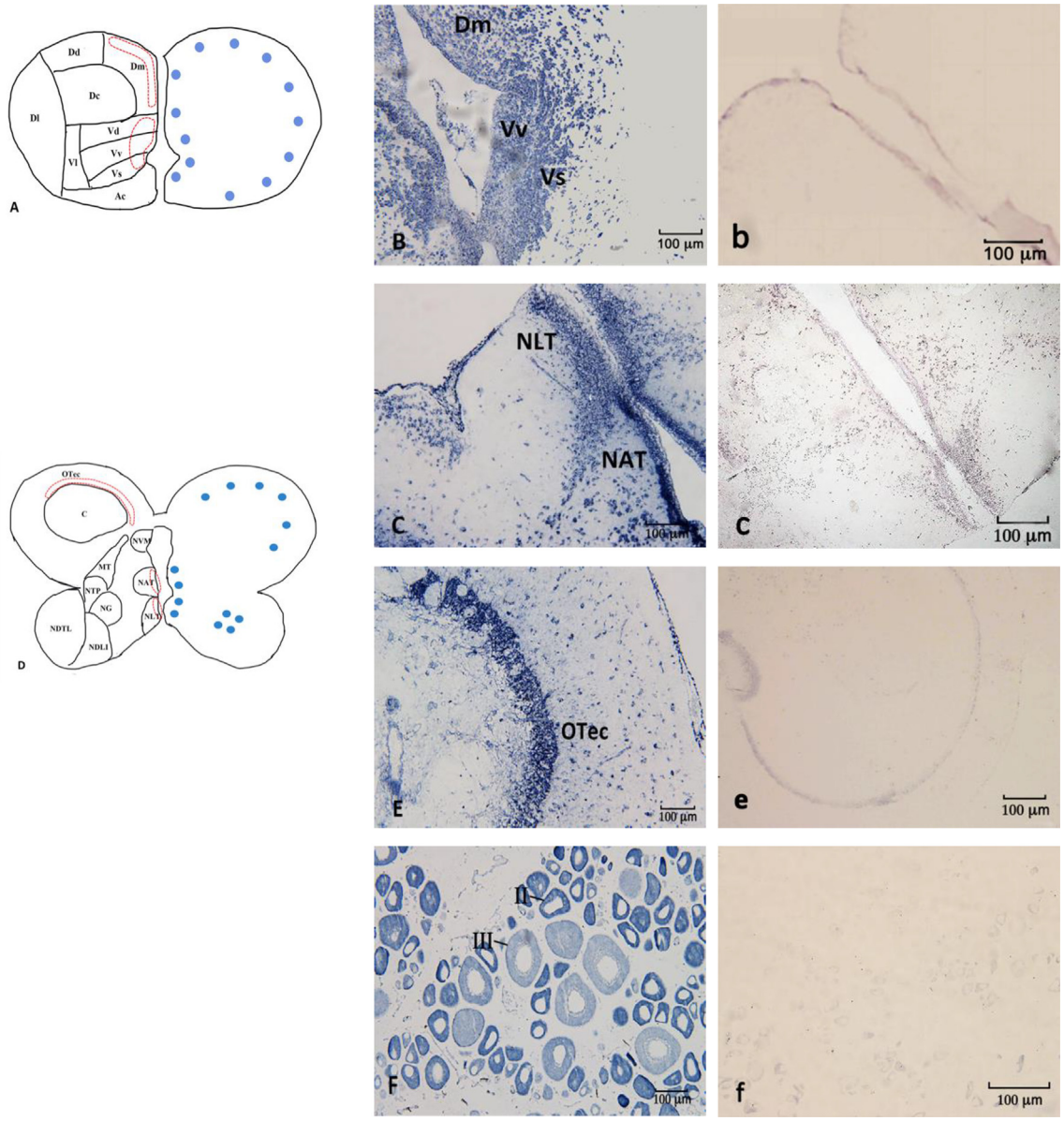

Fig. 4. Localization of *mc4r* in black rockfish brains and ovaries, as determined by in situ hybridization (ISH). Schematic illustrations of the brain are shown in A and D. For each section, the brain regions with nomenclature are displayed on the left side, with the signal regions marked by red lines; the mRNA signals as determined by ISH are represented by dots on the right side. The results obtained with brain anti-sense probes are shown in B, C, and E, and those obtained with sense probes are shown in b, c, and e. The results obtained with the ovary anti-sense probe and sense probe are shown in F and f, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

MC4R plays a crucial role in the regulation of energy homeostasis, including food intake and energy expenditure, in various mammals (Huszar et al., 1997; Cone, 2005; Tao, 2010). Mutations in *MC4R* genes have been identified in humans and other mammals (Fan et al., 2008; Tao, 2009; Yan and Tao, 2011; Hinney et al., 2013). Recently, several studies on MC4Rs in teleosts have been published (Ringholm et al., 2002; Cerda-Reverter et al., 2003; Jangprai et al., 2011; Wei et al., 2013; Li et al., 2016; Li et al., 2017; Yi et al., 2018; Rao et al., 2019; Wang et al., 2019; Zhang et al., 2019). However, studies on MC4R related to reproduction have been limited primarily to mammals, especially mice; there have been only a few studies on teleosts. Notably, *mc4r* is expressed in the gonad in several species, suggesting that MC4R may regulate reproduction.

In the current study, we cloned black rockfish *mc4r* and showed that MC4R is highly conserved among vertebrates. Black rockfish MC4R was found to exhibit more than 90% amino acid identity with MC4R in some teleosts, including spotted scat, European seabass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*). In addition, the seven hydrophobic TMDs in the black rockfish MC4R were found to have higher homology with those of other species. The extracellular domains and portions of TMDs have been shown to be crucial for ligand binding, and the intracellular domains are known to be involved in G-protein recognition (Wess, 1997). In humans, mutations in MC4R residues within TM3 and/or TM6 decrease the binding affinity of the ligand (Yang et al., 2000; Huang and Tao, 2012). These findings suggest that high conservation of TMDs in MC4R ensures the consistency of ligand binding across species.

We further determined that *mc4r* is highly expressed in the black rockfish brain, followed by the liver, gonads, intestines and stomach (Fig. 2). MC4R has been found to be expressed in the gills, spleens, retinas, and ovaries of goldfish (Cerda-Reverter et al., 2003); to be

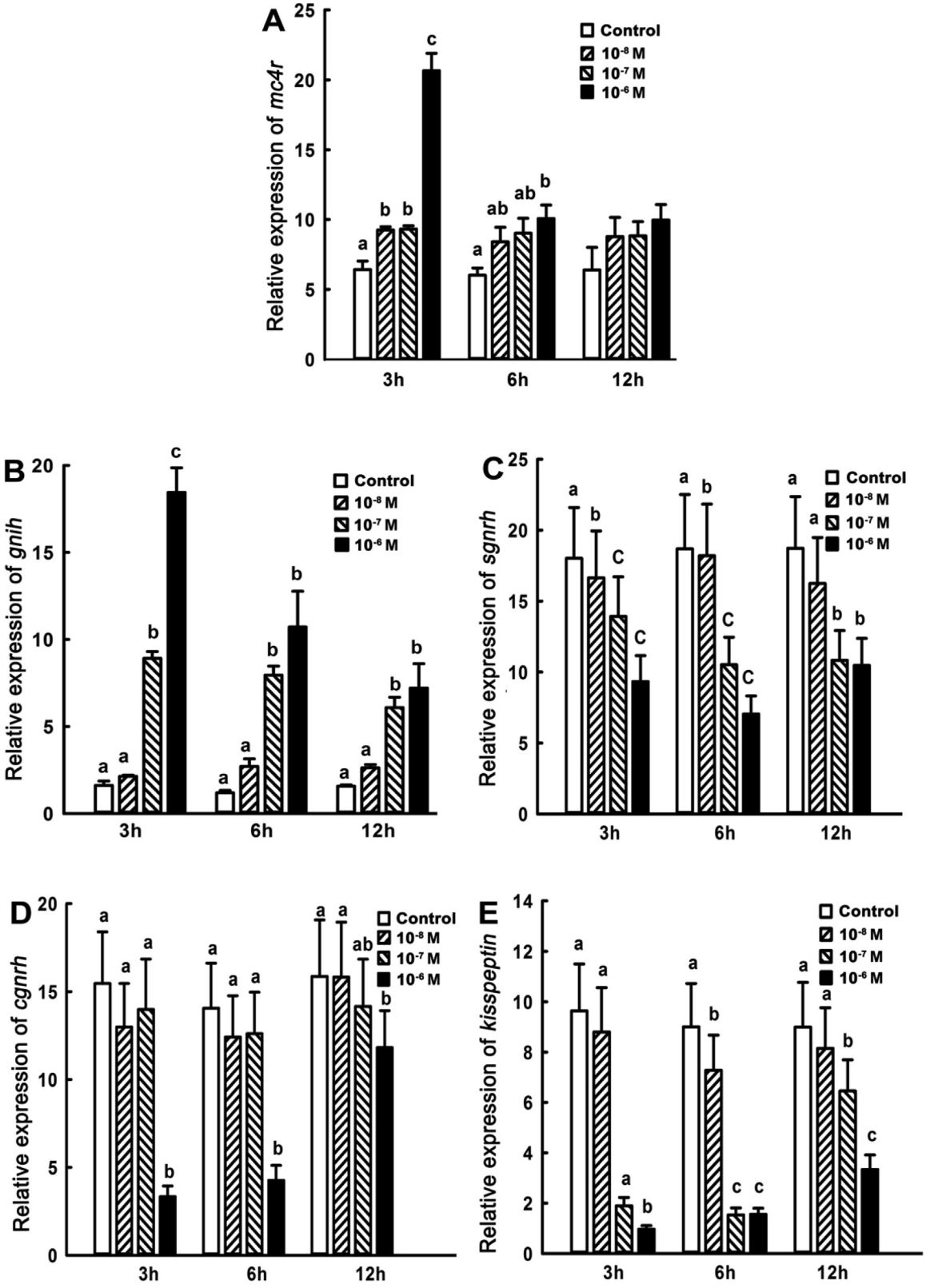

Fig. 5. Effect of α-MSH treatment on *mc4r, gnih, sgnrh, cgnrh,* and *kisspeptin* mRNA expression in female black rockfish brain fragments. The y-axis shows the relative expression level, and the x-axis shows the period of treatment. The data represent the mean ± SEM (n = 3) and were subjected to ANOVA followed by Duncan's multiple-range test. Significant differences are noted by different letters in each group ($P < 0.05$).

mainly expressed in the livers, ovaries, and testes of flounder (*Verasper moseri*) (Kobayashi et al., 2008); and to be expressed in the brain, pituitary, and gonads in both male and female spotted scat (Li et al., 2016). In addition, in this study, the relative expression level of MC4R increased with increasing ovary development. These findings suggest that MC4R might play a direct or indirect role in promoting the

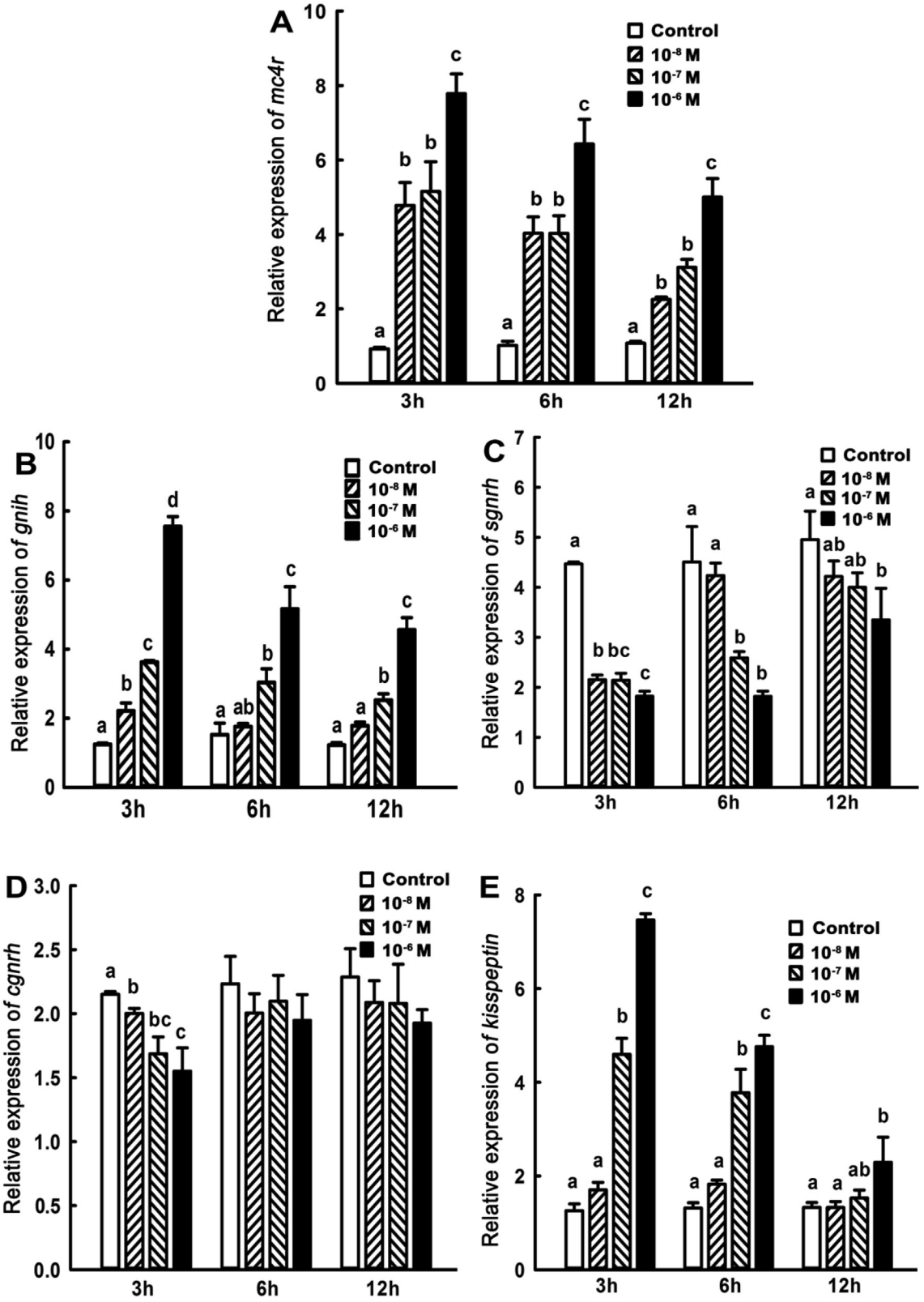

Fig. 6. Effect of β-MSH treatment on *mc4r, gnih, sgnrh, cgnrh,* and *kisspeptin* mRNA expression in female black rockfish brain fragments. The y-axis shows the relative expression level, and the x-axis shows the period of treatment. The data represent the mean ± SEM (n = 3) and were subjected to ANOVA followed by Duncan's multiple-range test. Significant differences are noted by different letters in each group ($P < 0.05$).

maturation of the ovaries.

To determine where *mc4r* is localized in the brains of the black rockfish, ISH was used. In the brain, *mc4r* mRNA was found to be widely expressed in the tuberal hypothalamus, dorsal telencephalon, and neurons of the NPO. Similarly, in a previous study, MC4R projections are found in the medial preoptic areas of mice where the key reproductive hormone GnRH is expressed (Ward et al., 2009). On the other hand, hypothalamic GT1-1 cells treated with NDP-α-MSH secrete

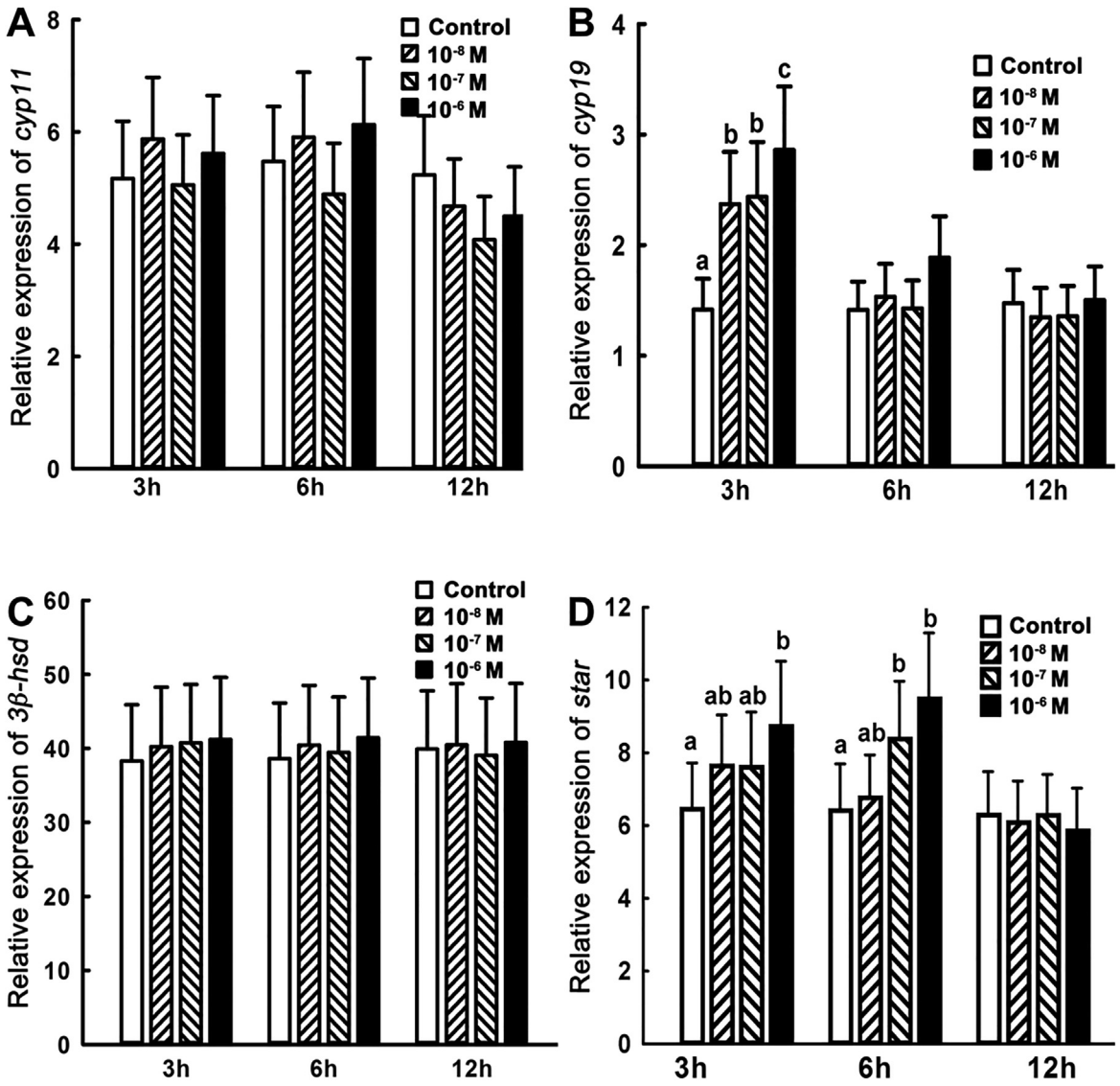

Fig. 7. Effect of α-MSH treatment on *cyp11a1, cyp19a1a, 3 β − hsd,* and *star* mRNA expression in female black rockfish ovarian fragments. The y-axis shows the relative expression level, and the x-axis shows the period of treatment. The data represent the mean ± SEM (n = 3) and were subjected to ANOVA followed by Duncan's multiple-range test. Significant differences are noted by different letters in each group ($P < 0.05$).

increased levels of GnRH, indicating that MC4R is coupled to GnRH release (Khong et al., 2001). These results suggest that black rockfish MC4R might regulate GnRH secretion.

To further investigate whether MC4R in the brain participates in the regulation of reproduction, we determined the effects of α- and β-MSHs on the expression of reproduction-related genes (*mc4r, sgnrh, cgnrh, kisspeptin,* and *gnih*) in black rockfish brains in vitro (Figs. 5 and 6). Kisspeptin and GnIH, a stimulator and inhibitor of GnRH secretion, respectively, were chosen as the target genes. In our study, α-MSH increased the expression of *gnih* and decreased the expression of *kisspeptin*, *sgnrh* and *cgnrh*. These results are consistent with a report showing that LH and FSH levels in plasma increase significantly in mice injected intracerebroventricularly with Agouti-related protein, an antagonist of MC4R that stimulates the release of hypothalamic GnRH in vitro (Stanley et al., 1999). In mice in estrus, a negative correlation has been found between MC4R and estrogen (Cheung et al., 2001). Our results suggest that MC4R might stimulate GnIH secretion and simultaneously inhibit kisspeptin secretion, which in turn inhibits GnRH secretion. However, compared with α-MSH treatment, β-MSH treatment exerted an opposite effect on the expression of *kisspeptin*. The reason for these findings remains to be further investigated.

To investigate the possible function of *mc4r* in the ovaries, ISH was performed to localize the mRNA in the ovaries. The results showed that *mc4r* was localized in stage II and III follicles (Fig. 4F) and was more highly expressed when yolk accumulation was present (Fig. 3). In the in vitro culture system, stimulation with β-MSH was found to induce the expression of genes related to steroidogenesis, including *cyp11, cyp19, 3β-hsd,* and *star* (Fig. 8). MC4R has also been found to be involved in adrenal steroidogenesis in mice, in which it mediates action of Agouti-related protein (Doghman et al., 2007). However, *MC4R* has not been observed in human ovaries, indicating a species- and tissue-specific function in steroidogenesis (Pohlmeier et al., 2014). The results suggest that MC4R may regulate the synthesis and secretion of steroid hormones, and the effect may depend on the species.

In summary, we cloned black rockfish *mc4r* and found that it was evolutionarily conserved. MC4R was more highly expressed in the brain, stomach, liver and gonad than in other tissues. We identified the distribution of MC4R by ISH and determined the effects of α- and β-MSH treatment on reproduction-related genes. All of the findings suggest that MC4R might regulate reproductive function.

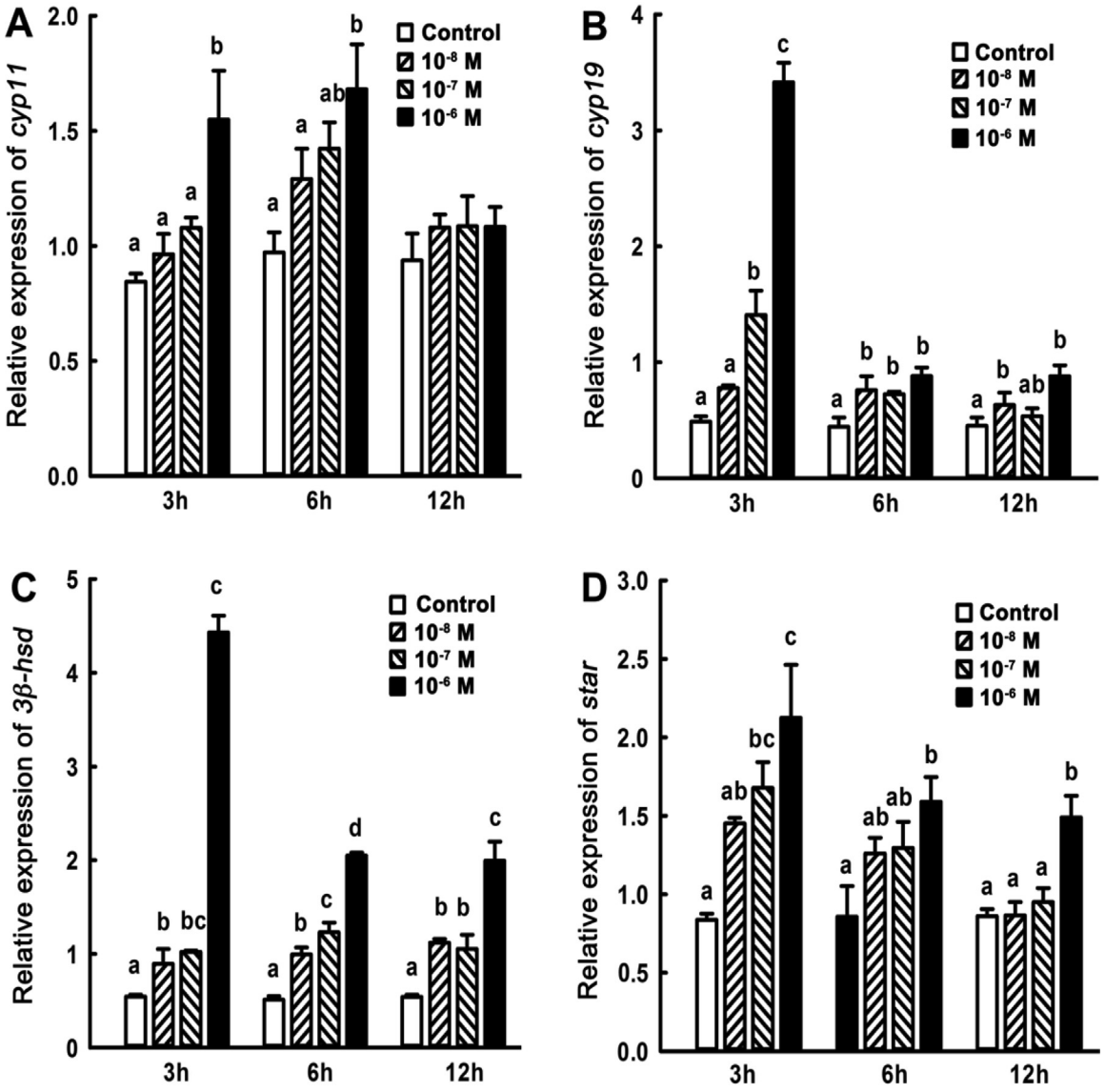

Fig. 8. Effect of β-MSH treatment on *cyp11a1, cyp19a1a, 3β − hsd,* and *star* mRNA expression in female black rockfish ovarian fragments. The y-axis shows the relative expression level, and the x-axis shows the period of treatment. The data represent the mean ± SEM (n = 3) and were subjected to ANOVA followed by Duncan's multiple-range test. Significant differences are noted by different letters in each group ($P < 0.05$).

CRediT authorship contribution statement

Ying Zhang: Writing - original draft, Data curation. **Hai-Shen Wen:** Resources, Supervision. **Yun Li:** Resources, Supervision. **Li-Kang Lyu:** Methodology. **Zhan-Xiong Zhang:** Data curation. **Xiao-Jie Wang:** Formal analysis. **Jian-Shuang Li:** Software. **Ya-Xiong Tao:** Writing - review & editing. **Xin Qi:** Project adminstration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2020.144541.

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