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The Kiss2/GPR54 system stimulates the reproductive axis in male black porgy, *Acanthopagrus schlegelii*☆

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ABSTRACT

Although it is well established that the Kiss1/GPR54 system stimulates the reproductive axis in mammals, its functional roles, especially in male reproduction of non-mammalian species, is less clear. In this study, we have isolated the full-length *kiss2* and *gpr54* cDNAs from black porgy (*Acanthopagrus schlegelii*). The Kiss2 precursor expressed from *kiss2* comprises 124 amino acids and contains a highly conserved 10-amino acid sequence, Kiss2-10 (FNFNPFGLRF). GPR54 comprises 375 amino acid residues and contains distinct characteristics of G protein-coupled receptors. Real-time PCR analysis indicated that *kiss2* and *gpr54* were expressed highly in the brain regions. Moreover, intraperitoneal injection of porgy Kiss2-10 could stimulate genes expression of the *gpr54, gnrh1, gnrh3, fshβ, lhβ, p450c17, star, and ar*, and the serum testerone level in male black porgy. Our findings demonstrate that the Kisspeptin stimulates the male reproductive axis in black porgy.

1. Introduction

Kisspeptin, a member of the RFamide peptide family, has recently received considerable attention as a potential key player in the neuroendocrine regulation of reproduction in various vertebrate species (Oakley et al., 2009). In mammals, kisspeptin is encoded by the *kiss1* gene and was first found in melanoma and breast cancer cells as a metastasis suppressor (Lee et al., 1996; Lee and Welch, 1997). Kisspeptin-54, Kisspeptin-14, Kisspeptin-13, and Kisspeptin-10, which are generated by proteolytic cleavage of the Kisspeptin precursor, share a distinct structural Arg-Phe-amide motif in the C-terminus, which allows them to bind to their cognate G protein-coupled receptor, Kiss1r/GPR54 (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). In humans, a mutation of *gpr54* or *kiss1* leads to idiopathic hypogonadotropic hypogonadism (IHH) syndrome (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). In mice, kiss1 and gpr54 genetic knockout models show significant deficits in pubertal development and reproductive function (Funes et al., 2003; Seminara et al., 2003; Lapatto et al., 2007).

Recent research suggests that unlike mammals expressing only the *kiss1* gene, some teleosts such as zebrafish (*Danio rerio*) (Biran et al., 2008; van Aerle et al., 2008; Kitahashi et al., 2009; Servili et al., 2011), medaka (*Oryzias latipes*) (Kanda et al., 2008; Kitahashi et al., 2009), goldfish (*Carassius auratus*) (Li et al., 2009; Yang et al., 2010; Kanda et al., 2012), European seabass (*Dicentrarchus labrax*) (Felip et al., 2009; Alvarado et al., 2013; Escobar et al., 2013), and chub mackerel (*Scomber japonicus*) (Selvaraj et al., 2010; Ohga et al., 2013; Ohga et al., 2015) have two paralogous genes, *kiss1* and *kiss2;* however, the evidence obtained thus far has shown the absence of the *kiss1* gene in a few perciform fishes, including tiger puffer (*Takifugu rubripes*), green puffer (*Tetraodon nigroviridis*), grass puffer (*Takifugu niphobles*), orange-spotted grouper (*Epinephelus coioides*), and three-spined stickleback (*Gasterosteus aculeatus*) (Selvaraj et al., 2010; Ohga et al., 2013; Ohga et al., 2015), indicating that the *kiss1* gene may have been lost during evolution in these species. With regards to the *kiss* receptor, 4 different subtypes of *kissr* have been found in vertebrates (Migaud et al., 2010; Migaud et al., 2012; Zohar et al., 2010). Only *kissr4* and *kissr2* have been described in teleosts (Akazome et al., 2010; Tena-Sempere et al., 2012); *kissr4* has been reported in many teleosts and is considered to be the most predominant and functionally active form (Akazome et al.,

Abbreviations: ar, Androgen receptor; erβ, Estrogen receptor beta; fsh, Follicle-stimulating hormone; GnRH, Gonadotropin-releasing hormone; GPR54, G protein-coupled receptor-54; HPG, hypothalamus-pituitary-gonad; lh, Luteinizing hormone; LHRH-A2, Luteinizing hormone-releasing hormone-A2; MS-222, 3-Aminobenzolic acid ethyl ester methanesulfonate; ORF, Open reading frame; star, Steroidogenic acute regulatory protein; UTR, Untranslated region

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Table 1
The primers used in the present study.

Sense Primer	5′-3′	Anti-sense Primer	5′-3′
Primers for bpkiss2 and bpgpr54 partial cDNAs			
kiss2 F1	ATGAGACTCGTGGCTCTGGT	*kiss2* R1	GTCCTGGCTGTTTTAACTGC
gpr54 F1	CATCATCTTCTTGGTGTGC	*gpr54* R1	ATCTCAGCRTTGGCAGTC
Primers for bpkiss2 and bpgpr54 5′ end			
kiss2 5′GSP1	AGCCATTGTAGCGTTTCCCAAAGCG	*kiss2* 5′GSP2	AGGAGCTGCCGCTGGTCTTCATTCT
gpr54 5′GSP1	CGTAGCAGCGGTCTCCACTCATAGC	*gpr54* 5′GSP2	GAAGGGGACGCAGCACACCAAGAAG
Primers for bpkiss2 and bpgpr54 3′ end			
kiss2 3′GSP1	GGTGGTGTGCGGGCTGATTGTTG	*kiss2* 3′GSP2	CTGAGAGAGAATGAAGACCAGCGGCA
gpr54 3′GSP1	AGGAGCAAAGTGTCCAAGATGGTGGTG	*gpr54* 3′GSP2	GTACAAAATCAAGACGTGGGCCAACTGC
Primers for ORF of kiss2 and gpr54			
kiss2 F2	TAGTTCCTGCTGAGTAGGTGAACAC	*kiss2* R2	TGTAAATGTAGCCGTTGTAGCGT
gpr54 F2	GCCGCTTAGCCTCATAT	*gpr54* R2	TGCCACTCAGTCCCTTAC
Primers for reference genes			
18S F	CCTGAGAAACGGCTACCACATCC	18S R	AGCAACTTTAGTATACGCTATTGGAG
β-actin F	GAAATCGCCGCACTGGTT	β-actin R	TCCTCAGGGGCAACTCTCAG
Primers for RT-PCR			
RT-kiss2 F	AGGAGAGTTTTTGGCGGAG	RT-kiss2 R	GCCGAACGGGTTGAAGT
RT-gpr54 F	CATCTTCTTGGTGTGCTGCGT	RT-gpr54 R	TGTCGGAGAGATTTCAGAGGGTAG
gnrh1 F	GGTGGTGATGATGATGATGATGTC	*gnrh1* R	AATGTTGCCCAGCGTGTCC
gnrh3 F	GCGAGCAGCAGAGTGACG	*gnrh3* R	TTCTCTTCCCACCTGGTAGCC
fshβ F	GCCATCCAACCAACATCAGC	*fshβ* R	GGTCCTTTGTTCAGCCCAGT
lhβ F	ATGTTGGGTTCCTTCCTGG	*lhβ* R	CTGGGTCCTTGGTGATGC
p450c17 F	TGGGAAAACCCTGAGCACTT	*p450c17* R	TGGCGTTCACTTTGTACTTGACT
star F	AGGTCCCAGCCCCAGTAA	*star* R	CTCCATTCGCAGCCACAG
ar F	GCATTATTCCAGTCGAGGGTT	*ar* R	GAGTCCAGTAGTCGGGTGAGC
erβ F	ACTACATCTGCCCAGCAACCA	*erβ* R	GGAGCGATTAACGGACCCA

2010). Both *kissr4* and *kissr2* have been identified in species such as zebrafish, medaka, goldfish and sea bass with two *kiss* genes.

In vertebrates, including teleosts, reproductive processes are regulated by a complex and precise coordination of neuroendocrine hormones acting through the brain-pituitary-gonad (BPG) axis (Okuzawa, 2002; Weltzien et al., 2004; Du et al., 2005; Wu et al., 2008; Zohar et al., 2010; Selvaraj et al., 2012; Wu et al., 2016). In the hypothalamus, gonadotropin-releasing hormone (GnRH) plays a crucial role in the onset of puberty (Terasawa and Fernandez, 2001) and in controlling the secretion of pituitary-gonad axis hormones (Yaron et al., 2003). Kisspeptins in mammals are considered to be upstream endogenous regulators of GnRH neurons (Colledge, 2009; Hameed et al., 2011). Numerous studies suggest that their role in teleostean fish is also conserved (Elizur, 2009; Tena-Sempere et al., 2012). However, species differences exist in the relative potencies of Kiss1 and Kiss2 for the regulation of reproductive functions. Additionally, the neuroendocrine regulatory mechanism of kisspeptin remains unclear in fish. Kiss2 was shown to be more potent than Kiss1 to increase pituitary *lhβ* and *fshβ* mRNA expression when injected intraperitoneally into mature female zebrafish (Kitahashi et al., 2009). Kiss2 was also significantly more potent than Kiss1-10 in inducing LH and FSH secretion (Felip et al., 2009) in sea bass, but Kiss1-10, not Kiss2-10, increased serum LH levels after being intraperitoneally administered to sexually mature female goldfish(Li et al., 2009). Furthermore, both estrogen and androgen receptors have been observed in Kiss1-expressing neurons (Smith et al., 2005; Mitani et al., 2010), and the Kiss1/GPR54 system is considered to be a missing link for understanding the feedback regulation of GnRH secretion by sex steroids (Dungan et al., 2006; Adachi et al., 2007). Thus, Kiss1 neurons are now thought to be the direct target for estrogen feedback in both mammals and nonmammalian vertebrates (Mitani et al., 2010; Kanda et al., 2012).

Although the above studies indicating that the kiss/GPR54 system may be also functional in fish, less clear how this system is involved in male reproduction. In this study, we aimed to provide more accurate information on the reproductive function of the Kiss/GPR54 system in male black porgy, which is a marine protandrous, hermaphroditic fish of high commercial importance for aquaculture (Chang and Yueh, 1990; Yen et al., 2002). The spawning water temperature of black porgy is in the range of 14.5–24 °C. Because of the different water temperature in different regions in China, the breeding period also varies. The coastal areas of Shandong Province are from the first ten days of May to the second ten days of May, while the coastal areas of Guangdong Province are from the first ten days of December to the second ten days of April next year. Black porgy belongs to the type of multiple maturation and spawning.

Our data demonstrate that the *kiss2/gpr54* system stimulates the male reproductive axis in black porgy.

2. Materials and methods

2.1. Animals and chemicals

All of the male black porgy were obtained from Guangdong Daya Bay Fishery Development Center (Huizhou City, Guangdong, P.R. China). All of the fish were randomly distributed into 250 L tanks (10 fish/tank) in a flow-through supplied with sand-filtered seawater at flow rate of 2 L/min and were fed with a commercial fish feed twice per day under a simulated natural photoperiod and a water temperature of 28 ± 4 °C. Tissue samples were immediately collected from decapitated black porgy after the fish were anesthetized with tricaine methanesulfonate (MS-222) and were snap-frozen in liquid nitrogen. All animal experiments were conducted in accordance with the guidelines and approval of the Animal Research and Ethics Committees of South China Normal University.

Physiological saline (0.9%) was purchased from the campus hospital. The conserved black porgy Kiss-2 decapeptide (Kiss2-10: NH2-FNFNPFGLRF-CONH2) and [D-Ala6, Pro9NEt]-LH-releasing hormone (LHRH-A2) were synthesized by GL Biochem (Shanghai, China) Ltd and Ningbo Fish Hormone Factory (Zhejiang Province, China), respectively. The purity of both was > 98%, as determined by analytical high performance liquid chromatography, HPLC.

2.2. Molecular cloning of black porgy kiss2 and gpr54 cDNAs

Total RNA from the black porgy hypothalamus was prepared using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized with

the PrimeScript™ RT reagent kit (TaKaRa, Japan) using isolated RNA. Partial cDNA fragments were first obtained by PCR using degenerate primers or gene-specific primers designed according to the predicted sequences. Full-length cDNA sequences were obtained by RACE using the SMART RACE cDNA amplification kit (Clontech, USA). All primers used in the present study are listed in Table 1.

For all PCR reactions in this study, amplifications were performed with an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 48–65 °C for 30 s and 72 °C for 30–90 s. The reaction was ended by a further extension step of 10 min at 72 °C. The amplification products were purified using an E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, GA, USA) and ligated into the pMD18-T vector (TaKaRa, Japan). Three different individual positive clones were sequenced to confirm the sequence information on an ABI 3700 sequencer (Applied Biosystems).

2.3. Sequence analysis

The signal peptide and the neuropeptide prohormone cleavage sites were predicted using SignalP3.0 (Bendtsen et al., 2004) and NeuroPred software (Southey et al., 2006), respectively. Multiple sequence alignments were performed using Clustal W (Thompson et al., 1994), and phylogenetic trees were constructed with MEGA 6.0 using the neighbor-joining method (Tamura et al., 2013).

2.4. RT-PCR analysis for tissue distributions of kiss2 and gpr54 in sexually mature male black porgy

To detect the tissue expression profiles of *kiss2* and *gpr54* in sexually mature male black porgy, samples, including the, telencephalon, epithalamus, hypothalamus, optic tectum, cerebellum, pituitary, muscle, adipose tissue, gill, liver, heart, stomach, intestine, head kidney, kidney, spleen, and testis, were collected. Total RNA from different tissues was isolated using TRIzol reagent (Invitrogen). One microgram of total RNA from each tissue was digested with DNase I and reverses transcribed (RT) into cDNA using the PrimeScript™ RT reagent kit (TaKaRa, Japan). Mock RT reactions without reverse transcriptase were used as negative controls. RT-PCR condition was set as follows: 95 °C for 5 min; 40 cycles at 95 °C for 10 s, 60 °C for 30 s. After amplification, fluorescent data were converted to threshold cycle values (CT). The concentration of the template in the sample was determined by relating the Ct value to the standard curve. The target gene transcript levels were normalized against β-actin transcript level. Relative transcript of sample target gene $= 2^{-\Delta\Delta Ct}$, $\Delta\Delta C_t = (C_{t\ \text{sample target gene}}\text{-}C_{t\beta\text{-actin}})\text{-}(C_{t\ \text{Negative control target gene}}\text{-}C_{t\beta\text{-actin}})$.

2.5. In vivo effects of Kiss2-10 on the expressions of related genes on the HPG axis and the serum testosterone (T) level of sexually mature male black porgy

In July 2015, sexually mature male black porgy at 1.5 years old (total body weight: 158.2 ± 25.4 g; standard length: 16.4 ± 2.3 cm, GSI = 0.62 ± 0.21) were acclimatized to the environment for 2 weeks and feed on commercially available fish foods without any supplemented hormones. The Kiss2-10 was dissolved in 0.9% physiological saline. Experimental group fish were intraperitoneally injected with 0.1 μg/g doses. The LHRH-A2 (0.1 μg/g doses) injected group was used as the positive control. Negative control fish were administered with 0.9% physiological saline only. Each group has 10 fish (n = 10). All the fish were anesthetized with 0.05% MS-222 before sampling. The HPG axis tissues (hypothalamus, pituitary, and testis) and blood were collected at 1 h, 3 h, 6 h, and 12 h after injections. The blood samples were centrifuged at 1200 × g for 15 min at 4 °C within 30 min of collection. The separated serum and tissue samples were stored at −80°C until measurement. RT-PCR analysis of kiss2, gpr54, gnrh1, and gnrh3 expression in the hypothalamus, fshβ and lhβ expression in the pituitary, p450c17, star, ar, and erβ expression in the testis was performed as described above.

Serum testosterone (T) levels were measured by ELISA (enzyme-linked immunosorbent assay) with Fish Testosterone (TESTO) Elisa kit (BlueGene Biotech, China).

2.6. Statistical analysis

All data were represented as the mean values ± SD. Significant differences were estimated by one-way ANOVA followed by Duncan's multiple range test, and a probability level < 0.05 ($p < 0.05$) was used to indicate significance and different letters in figures represent significant differences among treatments. All statistics were performed using SPSS 16.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Cloning and sequence analysis of kiss2 and gpr54

Using the methods described in the Materials and Methods section and the primers described in Table 1, full-length cDNAs encoding the *kiss2* and *gpr54* precursors were cloned from the black porgy hypothalamus (GenBank accession numbers KR610413 and KR610414, respectively). As shown in Fig. 1A, the cDNA of *kiss2* is 492 bp in length and contains a 60 bp 5′ untranslated region (UTR), a 57 bp 3′ UTR and an open reading frame (ORF) of 375 bp encoding a precursor protein of 124 amino acids (aa) with a predicted signal peptide of 15 aa. The cDNA of *gpr54* is 2166 bp in length and contains a 406 bp 5′ UTR, a 3′ UTR and an ORF of 1128 bp encoding a precursor protein of 375 aa with seven transmembrane domains (Fig. 2A).

Sequence comparison of the deduced aa sequences of the teleost Kiss2 precursor proteins revealed relatively low identity among the proteins (Fig. 1B). However, a BLASTx search reveals more than 80% sequence identity with other perciform Kiss2 sequences, including that of red seabream *(Pagrus major)*, European seabass and orange-spotted grouper. Moreover, the core peptide (Kiss2-10) and the C-terminal cleavage site (GKR) are also well conserved. This is reflected in the phylogenetic analysis of the same sequence in relation to Kiss2 proteins of other teleosts, perciformes clustering into one clade and black porgy, sharing the highest similarity with that of red seabream (Fig. 1C). GPR54 is more conserved than Kiss2 between species. The sequence identity with deduced perciform GPR54 aa sequences is greater than 90%. Phylogenetic analysis showed that GPR54 aa sequences are clustered into two separate clades, GPR54-1 and GPR54-2; the black porgy GPR54 is clustered with perciformes and is most closely related to sea bass and striped bass GPR54 (Fig. 2B).

3.2. Tissue distributions of kiss2 and gpr54

The amounts of *kiss2* and *gpr54* mRNAs in different tissues were examined by real-time PCR. Both *kiss2* (Fig. 3A) and *gpr54* (Fig. 3B) mRNAs were highly expressed in the brain, with the highest expression in the optic tectum and the hypothalamus, respectively. In peripheral tissues, the highest *kiss2* mRNA level was observed in the heart, and the *gpr54* was in the testis. Both *kiss2* and *gpr54* were expressed in the pituitary, heart, liver, stomach, muscle, testis and gill, but not in kidney and adipose tissues.

3.3. The expression of related genes on the HPG axis following injection of Kiss2-10

kiss2, gpr54, gnrh1, and gnrh3 in hypothalamus: The intraperitoneal injection of Kiss2-10 significantly increased all the expressions of *kiss2, gpr54, gnrh1* and *gnrh3* in hypothalamus (Fig. 4A–D). The mRNA level of *kiss2* rose to a maximum at 1 h postinjection of Kiss2-10, and then gradually decreased (Fig. 4A). At all the time we detected, the mRNA

A

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                                                        TAGTTCCTGCTGAGTAGGTGAACACTCACACTTATACCTTCAGCGTTTGAACACACAGAG 60
ATGAGACTCGTGGCTCTGGTGGTGGTGTGCGGGCTGATTGTTGGTCAGGATGGAGGCAGCGTGGGAGCAGCTCTGCCAGGATTTGACTCT 150
 M  R  L  V  A  L  V  V  V  C  G  L  I  V  G  Q  D  G  G  S  V  G  A  A  L  P  G  F  D  S  30
GCACAGAGGACACATGTGACAGGATCAGTCCTCTCAGCGCTCAGGAGGAGCAGCGCAGGAGAGTTTTTGGCGGAGGATTCCAATCCCTGT 240
 A  Q  R  T  H  V  T  G  S  V  L  S  A  L  R  R  S  S  A  G  E  F  L  A  E  D  S  N  P  C  60
TTCTCCCTGAGAGAGAATGAAGACCAGCGGCAGCTCCTGTGCAACGACCGCAGGAGTAAATTCAACTTCAACCCGTTCGGCCTGCGCTTT 330
 F  S  L  R  E  N  E  D  Q  R  Q  L  L  C  N  D  R  R  S  K  F  N  F  N  P  F  G  L  R  F  90
GGGAAACGCTACAATGGCTACATTTACAGGAGAGCAGTTAAAACAGCCAGGACAAATAAGTTTCCCCCCCTTTCTCTCTTCTCTAGAGAA 420
 G  K  R  Y  N  G  Y  I  Y  R  R  A  V  K  T  A  R  T  N  K  F  P  P  L  S  L  F  S  R  E  120
CTGGATGAGCACTGATACTTCTGTGAATAAAACTTTTGTACCAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                492
 L  D  E  H  *                                                                              124
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B

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Black porgy Kiss2                MRLVALVVVCGLIVGQDGGSVGAALPGFDSAQRTH------VTGS-VLSALRR--------SSAGE-FLAEDSNPCFSLRE
Red seabream Kiss2               MRLVALVVVCGLIVGQDGGSVGAALPGSDSAQRTH------VTGS-VLSALRR--------SSTGE-FLAEDSNPCFSLRE
Epinephelus coioides Kiss2       MRLVTLVVVCGLIVGQDGDSVGAALPGFDSAQRTH------ATES-ILSALRR--------RSTGE-FVAEDTSPCLSLRE
Epinephelus bruneus Kiss2        MRLVTLVVVCGLIVGQDGDGVGAALPGFDSAQRTR------ATGS-ILSALRR--------RSTGE-FVAEDTSPCLSLRE
Morone saxatilis Kiss2           MRLVALVVVCGLIVGQDGGSMGAALPGLDSAQRTG------ATGS-LLSALRR--------RTAGE-FFGEDSSPCFSLRE
Seriola lalandi Kiss2            MRLVALVVVCGLIVGQDGGSVGAALPGYDSAQRTRATGSGSGSGS-VLSALSR--------RTAAD-FLDQDPNLCFSLRE
Sea bass Kiss2                   MRLVALVVVCGLILGQDGGSVGAALPELDSAQRTG------ATGS-LLSALRR--------RTAGE-FFGEDSSPCFSLRE
Solea senegalensis Kiss2         MRLVALAVVCGLMVGQDGASVGVDLPRYDT-QRTR------ATGSDLLSAHRRGGAAAAAAAAAD-FVAEEPSLCFSLRE
Thunnus thynnus Kiss2            MRLVALVVVCGLMLGLDGGSLAAALPGFDSEQRTR------GTAS-VLSALRG--------RTAGD-FLE-DPNLCFSLRE
Tilapia Kiss2                    MRLLALAVVCALIAIQDGGSVGAALPGVDPAQRTH------ATGA-VSSAFR---------RTAGD-FLAEDPSLCFSLRE
Chub mackerel Kiss2              MRLVALVVVCGLMLGHDGGSLAAALPGFDSEQRTQ------GRAS-VLSALR---------RSSAD-YLE-DPNLCFSLRE
Chrysiptera cyanea Kiss2         MRLVAVVVVCSLMAAQ-----AADLPGFDSAQRRH------ETGE-VLPALRR--------RTSGD-LLQDDPNLCFSLRE
Takifugu niphobles Kiss2         MRVLVLLLVLAVAPDRGG--------AHATMQVTG------GSGS---VQLRR--------GTAGQLQLLQESNPCLTFRD
Medaka Kiss2                     MTRAVVLVLCALIAAQDGGRAAAGLAARDSGRGTH------ATGV--LWILRR---------SEDD-SAAGGAGLCSSLRE
Rainbow trout Kiss2              MRMLAFFLVCAITRQYGTWGTPTHGFGWTDIPGQTP-----AKVQVGHSVVRR--------SEIVSPNLPGDANLCYLLK-
Goldfish Kiss2                   MKIKALILFMSAMICQSTALRASFTDMDISDSEPVP-----DSKQHYLSVERR---------QFDEPSSSDDASLCFFFQE
Zebrafish Kiss2                  MNTRALILFMSAMVSQSTAMRAILTDMDTP--EPMP-----DPKPRFLSMERR---------QFEEPSASDDASLCFFIQE
Gobiocypris rarus Kiss2          MKIKALILFLSAMICQSTATRAPFTDMDTP--EPIP-----DSKQTYLSMERR---------QFDEPNASDDGSLCFFIQE
                                 *  .. :. .                                                .      . *  ::

Black porgy Kiss2                NEDQRQLLCND---RRSKFNFNPFGLRFGKRYNGYIYRRAVKTARTNKFPPLSLFSRELDEH--
Red seabream Kiss2               NEDQRQLLCND---RRSKFNFNPFGLRFGKRYNGYIYRRAVKTARTNKFSPLSLFSRELDEH--
Epinephelus coioides Kiss2       NEEQRQLLCND---RRSKFNFNPFGLRFGKRYNGYIYRRAVKTARTNKFSPFSLFSRELEVPS-
Epinephelus bruneus Kiss2        NEEQRQLLCND---RRSKFNFNPFGLRFGKRYNGYIYRRAVKAARTNKFSPFSLFSRELEVPS-
Morone saxatilis Kiss2           NEEQRQLLCND---RRSKFNFNPFGLRFGKRY---IYRRALKRARTNKFSPLSLFSRELEVPI-
Seriola lalandi Kiss2            NDDQRQLLCND---RRSKFNFNPFGLRFGKRYNGYIYRRAVQRARTNKFSPLSLFPRELEVPN-
Sea bass Kiss2                   NEEQRQLLCND---RRSKFNFNPFGLRFGKRY---IYRRALKRARTNRFSPLFLFSRELEVPT-
Solea senegalensis Kiss2         NEEPRQLLCND---RRSKFNFNPFGLRFGKRFT---YRRAVTRARTNKFSPLSLFSRELEVPT-
Thunnus thynnus Kiss2            NDDQRQLLCND---RRSKFNFNPFGLRFGKRYNGYIYRRAVKRARTNKYSPVALFPRELEVPI-
Tilapia Kiss2                    NEDQRQLLCND---RRSNFNYNPLSLRFGKRYNGYIYRRAVKRARTKKFSPFSLFLRELEVPT-
Chub mackerel Kiss2              NDDQRQLLCND---RRSNFNFNPFGLRFGKRYNGYIYRRAVKRARTDQFTPVSLFPRELEVPT-
Chrysiptera cyanea Kiss2         NEEQRQLLCND---RRSKFNFNPFGLRFGKRY---IYRRAVKRARTDKFSPFSLFSPQLEVPT-
Takifugu niphobles Kiss2         NED--QLLCN-----RSKFNLNPFGLRFGKRF---IYRRAMKQARTHTRSPVS-----QEVPT-
Medaka Kiss2                     DDE--QLLCAD---RRSKFNYNPFGLRFGKRAP---PPRGAHRARAMKLPLMSLF---QEVPT-
Rainbow trout Kiss2              DEDQQQMICKHRLTRTSKFNVNPFGLRFGKRDKFDPSKASLIRSRTSKLLPILLYLRELEVPA-
Goldfish Kiss2                   KDESTHISCQHR-LPRSKFNYNPFGLRFGKRNE--APT---DRP--KHLLPMMI-YLRKQSETS
Zebrafish Kiss2                  KDETSQISCKHR-LARSKFNYNPFGLRFGKRNE--ATTSDSDRLKHKHLLPMML-YLRKQLETS
Gobiocypris rarus Kiss2          KDESSKISCKHR-LTRSKFNYNPFGLRFGKRDK--ATTTDSDRPSHKHLLPMMLFYLRKPLETS
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Fig. 1. (A) Nucleotide and deduced amino acid sequences of black porgy Kiss2. The putative signal peptide is underlined, and the mature core peptide (kissspeptin-10) is boxed. (B) A comparison of the amino acid sequences of Kiss2 precursors from different species. The signal peptides are shaded in gray, and mature peptides are boxed. Sequences were aligned using the ClustalW program. Identical sequences are indicated by asterisks. Gaps (indicated by hyphens) are introduced in some sequences to maximize the alignment. (C) Phylogenetic analysis of Kiss2 precursors in teleosts. The phylogenetic tree was constructed by MEGA 6.0 using the neighbor-joining method with 1000 bootstrap replicates. The number shown at each branch indicates the bootstrap value (%). GenBank accession numbers for Kisspeptin: Red seabream Kiss2 (BAL44206), Orange-spotted grouper Kiss2 (ACT65993), Grass puffer Kiss2 (BAJ15497), Senegalese sole Kiss2 (ADK92427), Longtooth grouper Kiss2 (ADF59545), striped bass Kiss2 (ADU54201), sea bass Kiss2 (ACM07423); Yellowtail amberjack Kiss2 (AEF32394), Chub mackerel Kiss2 (ADE21655), Medaka Kiss2 (AB439562), *Thunnus thynnus* Kiss2 (AGN05228), Tilapia Kiss2 (AEN69450), *Chrysiptera cyanea* Kiss2 (BAO23489), Rainbow trout Kiss2 (AFP99912), Zebrafish Kiss2 (AB439561), *Gobiocypris rarus* Kiss2 (AHH83758.1), and Goldfish Kiss2 (ACS34769).

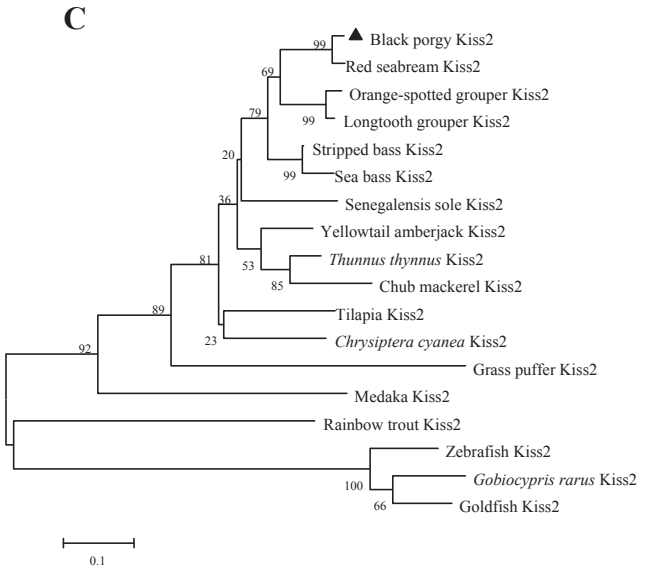

Fig. 1. (*continued*)

levels of *kiss2* and *gpr54* were significantly higher than the blank control group (Fig. 4A–B). For positive control group, the LHRH-A_2 group, the *kiss2* mRNA level was extremely higher than the blank control group and the Kiss2-10 group at all time we detected, and it peaked at 3 h postinjection (Fig. 4A). The expression of *gpr54* increased significantly after the injection of Kiss2-10, and peaked at 3 h (Fig. 4B). For LHRH-A_2 group, the *gpr54* mRNA level rose to a maximum at 1 h postinjection (Fig. 4B). The expressions of *gnrh1* and *gnrh3* were increased significantly after the injection of Kiss2-10 or LHRH-A_2, and peaked at 6 h (Fig. 4–-D). In brief, the expression of *kiss2, gpr54* and *gnrh* peaked at 1 h, 3 h and 6 h respectively after injection of Kiss2-10, showing a clear time sequence.

fshβ, lhβ in pituitary: The expression of *fshβ* in pituitary was significantly increased after the intraperitoneal injection of Kiss2-10 or LHRH-A_2, and peaked at 3 h. Furthermore, the increase degree of *fshβ* expression level in Kiss2-10 group is higher than that in LHRH-A_2 group, the positive control group (Fig. 4E). The *lhβ* mRNA level of Kiss2-10 group was lower than that of blank control group at 1 h after injection, while significantly higher than the blank control group at 3, 6, 12 h, and peaked at 6 h. For positive control group, the *lhβ* expression level significantly increased after injection of LHRH-A_2, and rose to a maximum at 3 h (Fig. 4F).

p450c17, star, ar and erβ in testis: LHRH-A_2 extremely significantly increased the expression of *p450c17,* while Kiss2-10 significantly decreased the expression of *p450c17* (Fig. 4H). We observed that Kiss2-10 had a stimulatory effect on *star* expression at 3, 6, and 12 h after injection (Fig. 4I) with a 32.0-, 10.3- and 6.5-fold increase, respectively, as compared to blank controls. The expression of *ar* in testis was stimulated by both Kiss2-10 and LHRH-A_2, up to a maximum at 6 h postinjection (Fig. 4J). Strangely, the mRNA level of *erβ* in testis increased at 3 h and then decreased at 12 h after injection of Kiss2-10 (Fig. 4K).

3.4. Effect of Kiss2-10 on serum testosterone (T)

The serum T level significantly increased after the intraperitoneal injection of Kiss2-10 in a clear dose-dependent manner (Fig. 5). At 1 and 3 h, there was no significant difference between the treated group and the blank control group. The significant difference among groups occurred at 6 h postinjection, the serum T level of the Kiss2-10 group and the LHRH-A_2 group began to rise. The 0.1 μg/g Kiss2-10 group, 1 μg/g Kiss2-10 group and 0.1 μg/g LHRH-A_2 group were significantly higher than blank control group. At 12 h, the serum T levels in treated groups were extremely significantly higher than that in the blank control group, and the serum T levels among different Kiss2-10 concentration groups were significantly different (Fig. 5).

4. Discussion

To understand the roles of Kiss/Gpr54 system in the regulation of reproduction in male fish, we cloned *kiss2* (Fig. 1) and *gpr54* (Fig. 2) cDNAs from male black porgy. The sequence detected from *kiss2* gene contains a core decapeptide (FNFNPFGLRF), which is well conserved with other species. Interestingly, we could not find *kiss1* and the other type of *gpr54*, indicating that black porgy may have only *kiss2* and one type of the *gpr54* gene. The phenomenon of gene loss in evolution occurs widely. For example, *kiss2* is not found in rodents or primates, and only *kiss2* and one type of *gpr54* are present in Fugu and pufferfish (Lee et al., 2009). This evidence might be described as the co-evolution of ligand/receptor pairs, considering that the loss of either the ligand or the receptor would lead to functional redundancy of its partner. Thus, both the ligand and the receptor would eventually be lost (Moyle et al., 1994). Black porgy and red seabream, belonging to the same species of Sparidae, only have *kiss2* gene (Shimizu et al., 2012). Different Gpr54 proteins share high aa sequence identity in the transmembrane regions and contain the NPxxY sequence in the TMH7 and DRY motifs, suggesting that these proteins belong to the rhodopsin-like GPCR family (Schwartz et al., 2006). Phylogenetic analysis revealed that black porgy Kiss2 or GPR54 is clustered into the branch with other perciform fishes (Fig. 1C; Fig. 2B).

In the present study, *kiss2* and *gpr54* mRNAs were expressed mainly in the brain and all tissues of the HPG axis, including the hypothalamus, pituitary and testis (Fig. 3). Thus, the neurocrine functional region of Kiss2/GPR54 system is in the brain, especially in the hypothalamus and Kiss2/GPR54 system may participate in the regulation of reproduction in male black porgy. Similar expression pattern of *kiss2* and *gpr54* in the HPG axis were detected in some other fishes, such as zebrafish (Biran et al., 2008; van Aerle et al., 2008; Kitahashi et al., 2009; Felip et al., 2009), medaka (Kanda et al., 2008; Kitahashi et al., 2009; Felip et al., 2009), sea bass (Felip et al., 2009; Alvarado et al., 2013; Escobar et al., 2013), goldfish (Li et al., 2009; Yang et al., 2010) and grass buffer (Shahjahan et al., 2010). Both *kiss2* and *gpr54* mRNAs were widely expressed in the brain of black porgy, with relatively higher levels of *kiss2* in the optic tectum and cerebellum, and that of *gpr54* in the telencephalon, epithalamus and hypothalamus (Fig. 3). The expression patterns also have been reported in zebrafish (Biran et al., 2008; van Aerle et al., 2008; Kitahashi et al., 2009; Felip et al., 2009), goldfish (Li et al., 2009; Yang et al., 2010), medaka (Kanda et al., 2008; Kitahashi et al., 2009; Felip et al., 2009) and grass buffer (Shahjahan et al., 2010). These results are in agreement with the possible role of kisspeptin in regulating GnRH neurons. Moreover, the widely location of *kiss2* and *gpr54* in the brain suggests that kisspeptin has a role as neurotransmitter/neruomodulator in the central nervous system. In peripheral system, *gpr54* expressed highest in testis, and *kiss2* expressed highest in heart, also relatively high level in testis. The relatively higher expression of *kiss2* and *gpr54* in testis indicates that kisspeptin is possibly involved in testis function by autocrine/paracrine mechanisms.

In addition, *kiss2* and *gpr54* were co-expressed in the muscle, heart and gill (Fig. 3), indicating that kisspeptin may have been involved in regulation cardiovascular system as in humans (Mead et al., 2007). Neither *kiss2* nor *gpr54* was observed in adipose tissue of black porgy, in contrast to the research in rat (Brown et al., 2008), human (Brown et al., 2008), goldfish (Li et al., 2009), and orange-spotted grouper (Shi et al., 2010). Thus, kisspeptin may not participate in regulating energy balance or food intake in black porgy.

In hypothalamus, gpr54mRNA level was significantly up-regulated at 1, 3, 6, and 12 h postinjection of Kiss2-10 in male black porgy (Fig. 4B). In prepubertal hybrid bass, Kiss2 was more potent than Kiss1 in up-regulating gpr54-2 expression (Zmora et al., 2012). Thus, it is

A

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                                                                ACATGGGGGGCTGCCTGGAGGTTTTCTTATCCTGCAAGTCGTGCAC  46
ACAGCATGCGTTACAGCTCTGCAGGGCGGCACTTCCAATCCAACTTGAACTGAAGAGAAGAAGCACCGTCTCCCTATTTTCGTTCTCCTA 136
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 F  L  Q  Q  V  T  V_TM3Q  A  T  C  I  T  L  T  A  M  S  G  D  R  C  Y  V  T  V  Y  P  L  K  150
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 S  L  R  H  R  T  P  R  V  A  M  I  V  S  I  C  I_TM4W  I  G  S  F  I  L  S  T  P  I  L  I  180
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 L  Y  Q  F  I  A  A  Y  L  L_TM5P  V  L  T  I  S  F  C  Y  T  L  M  V  K  R  V  G  Q  P  T  240
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 K  F  V  A  A  E  E  G  N  N  N  N  A  L  N  *                                              375
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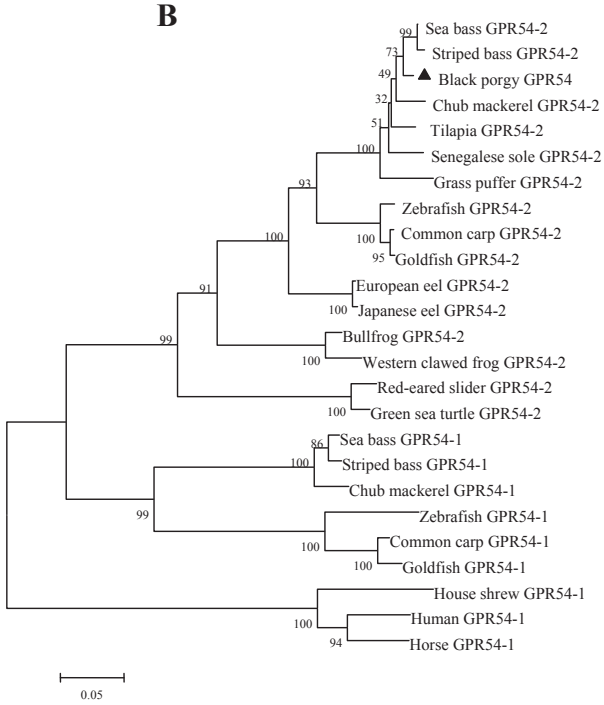

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Fig. 2. (A) Nucleotide and deduced amino acid sequences of black porgy GPR54. The seven predicted transmembrane domains (TM1-7) are underlined. The stop codon is denoted by an asterisk. (B) Phylogenetic analysis of GPR54 precursors in vertebrates. The phylogenetic tree was constructed by MEGA 6.0 using bootstrap replicates. The number shown at each branch indicates the bootstrap value (%). GenBank accession numbers for GPR54: sea bass (GPR54-1, AFK84355; GPR54-2, AFK84356), Striped bass (GPR54-1, AID62214; GPR54-2, ADU54205), Chub mackerel (GPR54-1, AGC30577; GPR54-2, AGC30578), Nile tilapia GPR54-2 (BAD34454), Senegalese sole GPR54-2 (ABW96362), Grass puffer GPR54-2 (BAJ15876), European eel GPR54-2 (CBV36798), Japanese eel GPR54-2 (BAM72049), Zebrafish (GPR54-1, ABV44613; GPR54-2, ABV44612), Common carp (GPR54-1, AFM08412; GPR54-2, AFM08411), Goldfish (GPR54-1, ACK77793; GPR54-2, ACK77792), Bullfrog GPR54-2 (ACD44939), Western clawed frog GPR54-2 (NP_001165296), Red-eared slider Gpr54-2 (BAN82579), Green sea turtle GPR54-2 (EMP23809), House shrew GPR54-1 (BAL04095), Human GPR54-1 (AF343725), and Horse GPR54-1 (AGU99579).

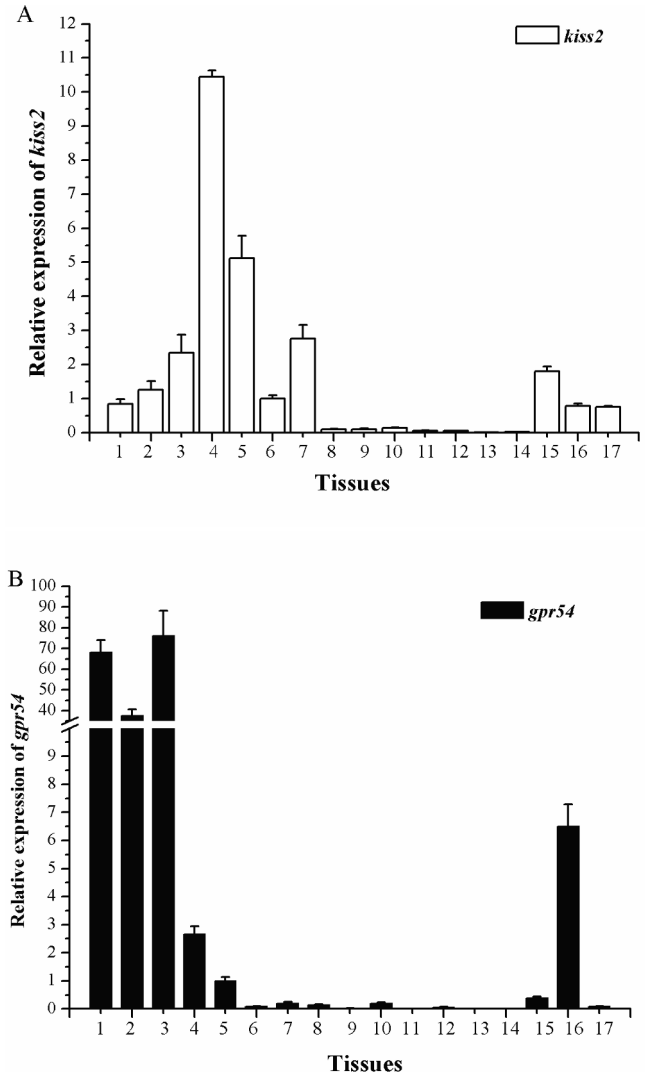

Fig. 3. Tissue distribution of *kiss2* (A) and *gpr54* (B). The expressions of *kiss2* and *gpr54* mRNAs in various tissues of black porgy were quantified by real-time RT-PCR. The tissues are labeled as following: 1 telencephalon, 2 epithalamus, 3 hypothalamus, 4 optic tectum, 5 cerebellum, 6 pituitary, 7 heart, 8 liver, 9 spleen, 10 stomach, 11 intestine, 12 head kidney, 13 kidney, 14 adipose tissue, 15 muscle, 16 testis and 17 gill. Different superscripts indicate significant differences between different tissues.

Kiss2 that can significantly induce its receptor, gpr54 mRNA expression in perciform fishes. There are three forms of GnRH in fish, which were previously referred to as sea bream GnRH (sbGnRH/GnRH1), Chicken GnRH-II (cGnRH-II/GnRH2), salmon GnRH (sGnRH/GnRH3) (An et al., 2008). GnRH1/sbGnRH was suggested to be the major hypothalamic hypophysiotropic hormone in red seabream (Okuzawa et al., 2003), grass puffer (Shahjahan et al., 2010), chub mackerel (Selvaraj et al., 2012), and European sea bass (Fornies et al., 2003), it may play a potent role in the reproduction. In goldfish and chum salmon (Lin et al., 1996), it is GnRH3/sGnRH that mainly stimulates GtH release from the pituitary gland. While GnRH2/cGnRH was believed to be related to the sexual behavior of musk shrews (Kauffman et al., 2005). Thus, in this study, we examined the effects of Kiss2-10 on only *gnrh1* and *gnrh3* but not *gnrh2* mRNA level in the hypothalamus of mature male black porgy. Our data demonstrated that the stimulatory effects of Kiss2-10 were both on *gnrh1* and *gnrh3* mRNA level in hypothalamus (Fig. 4C–D). Whereas, in orange-spotted grouper, the intraperitoneal injection of Kiss2-10 significantly increased *gnrh1* but not *gnrh3* mRNA levels in the hypothalamus at 6 and 12 h postinjection (Shi et al., 2010). Therefore, the effect of Kiss2 on *gnrh* expression in fish presents the specificity of the species.

In pituitary, *fshβ* and *lhβ* mRNA levels were significantly increased by Kiss2-10 in male black porgy (Fig. 4C–D), similar to other fishes. It is Kiss2 but not Kiss1 that significantly increased *fshβ* and *lhβ* mRNA levels in the pituitary of zebrafish (Kitahashi et al., 2009). Kiss2 peptides are more potent than Kiss1 peptides in inducing gonadotropin expression or secretion in sea bass (Felip et al., 2009) and hybrid bass (Zmora et al., 2012). These results indicated that Kiss1 and Kiss2 are potential regulators of reproduction and Kiss2 is the predominant regulator of gonadotropin synthesis in fish. The hypothalamus represents the primary site of kisspeptin action on reproduction, and in the tissue distribution profile, the gpr54 is extremely low express in the pituitary, thus the effects of Kiss2-10 on *fshβ* and *lhβ* may be indirectly on hypothalamus via GnRH in male black porgy.

In testis, P450c17, belonging to the cytochrome P450 family members, is androgen synthesis key steroidogenic enzymes. It has both 17 alpha hydroxylase and 17, 20- lyase activity, the former catalytic progesterone or progesterone transformation to 17 alpha hydroxyprogesterone, and the latter catalyzes 17 alpha hydroxyprogesterone converted to androstenedione, a testosterone precursor. In mice, the missing StAR protein can cause male sterility; when the expression of *star* gene was up-regulated, serum testosterone levels were significantly increased (Warita et al., 2013). Androgen receptor (AR) and Estrogen receptor beta subunit (ERβ) are affected by the level of androgen and estrogen in serum. In the present study, the in vivo administration of Kiss2-10 potently increased *star* and *ar* while decreased *p450c17*mRNA levels in the testis of black porgy. Studies have shown that there are two types of P450c17 in some fishes, namely P450c17-I and P450c17-II. These two types of P450c17 may play different roles in fish reproduction, the activity of 17a-hydroxylase and the activity of 17, 20 cleavage enzyme. In the fathead minnow (*Pimephales promelas*) testis, P450c17 gene expression was negatively correlated with gonadal development, but there was no obvious association between P450c17 gene expression and sexual development in the ovary. With the development of semi-smooth tongue sole (Cynoglossus *semilaevis Günther*) testis (GSI tends to increase and serum T level increases), the expression of P450c17-II tends to decrease, which is different from the expression pattern and function of P450c17-I, suggesting that there is no correlation between P450c17-I and androgen synthesis, but it increases significantly in stage VI testis, which may be related to the role of progesterone in spermatogonia ejaculation and induction of DNA replication in the next batch of spermatogonia. In our study, the p450c17 expression was negatively correlated with the serum T level of black porgy, suggesting that the p450c17 should be the P450c17-II type. Enzymatic studies are needed to investigate the possible role of P450c17 in neurosteroid production in teleosts.

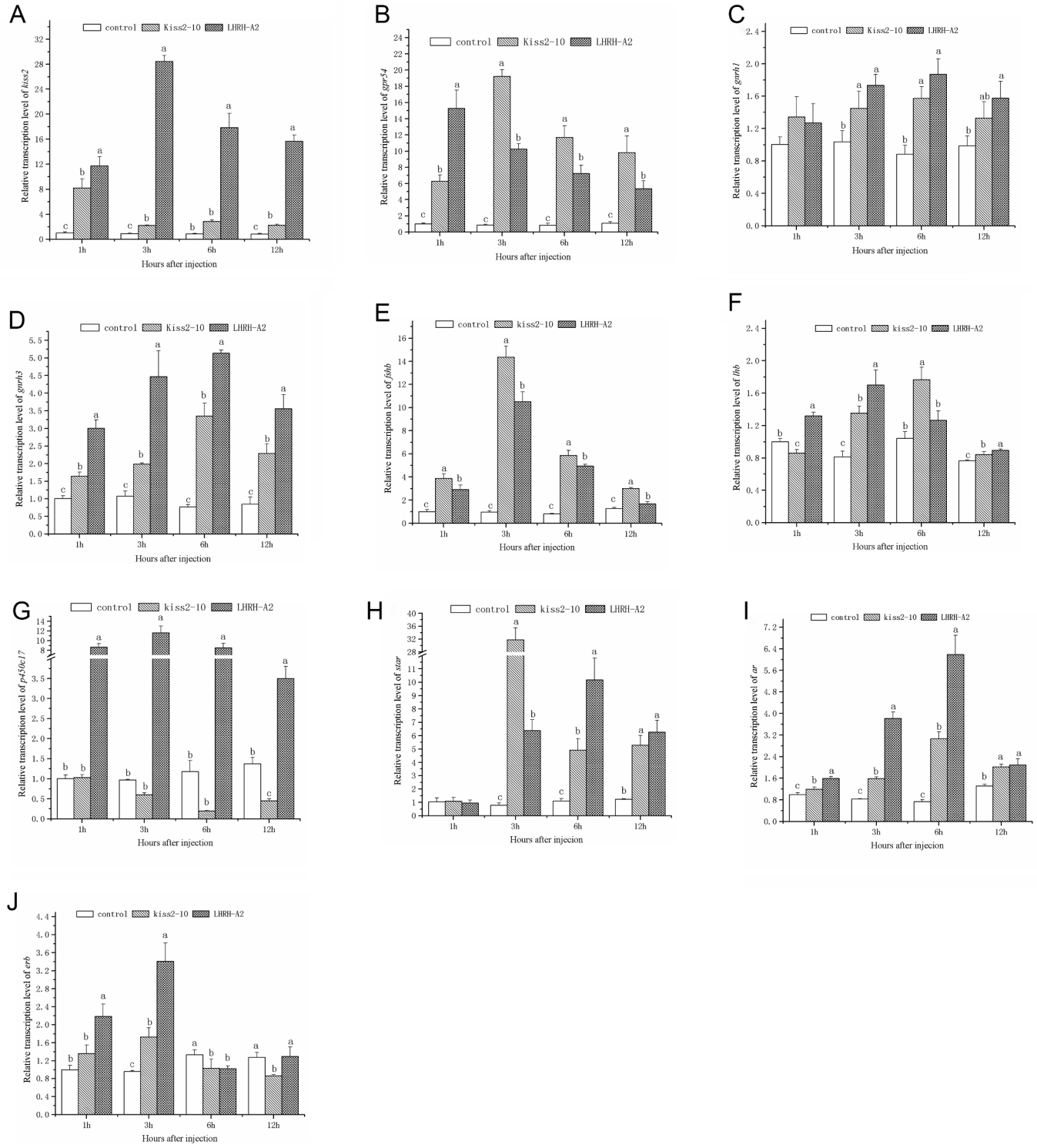

Fig. 4. Effects of Kiss2-10 on gene expression for *kiss2, gpr54, gnrh1, gnrh3, fshβ, lhβ, p450c17, star, ar* and *erβ* mRNAs in HPG axis of male black porgy at 1 h, 3 h, 6 h and 12 h. Beta-actin is used as the reference gene. Different superscripts indicate significant differences between different treatments at the same time.

Furthermore, the serum testosterone (T) levels were significantly increased after the intraperitoneal injection of Kiss2-10 in a clear dose-dependent manner. On one hand, Kiss2-10 may stimulate the secretion of GnRH in hypothalamus, and then GnRH stimulates pituitary release GtH, and then GtH function to the testis. On the other hand, Kiss2-10 may directly act on the GPR54 in testis, and then stimulate the expressions of *star*, *ar,* and the serum T level.

The deletion of either *kiss1* or *gpr54* in mice or the mutation of *gpr54* in humans results in uncompensated impairment of the reproductive axis (Funes et al., 2003; Seminara et al., 2003; Seminara et al., 2004; Lapatto et al., 2007; de Tassigny X et al., 2007). In a recent study, spermatogenesis and folliculogenesis as well as reproductive capability were not impaired in all mutant lines of *kiss* or *gpr54* genes in zebrafish (Tang et al., 2015). These findings indicate that there is no functional redundancy in the Kiss/GPR54 system in mammals but that this is not the case in some fishes. However, in this study, Kiss2-10 potently stimulated the mRNA expressions *of gnrh1*, *gnrh3* in hypothalamus, *fshβ*, *lhβ* in pituitary, *p450c17, star, ar* in the testis, and significantly

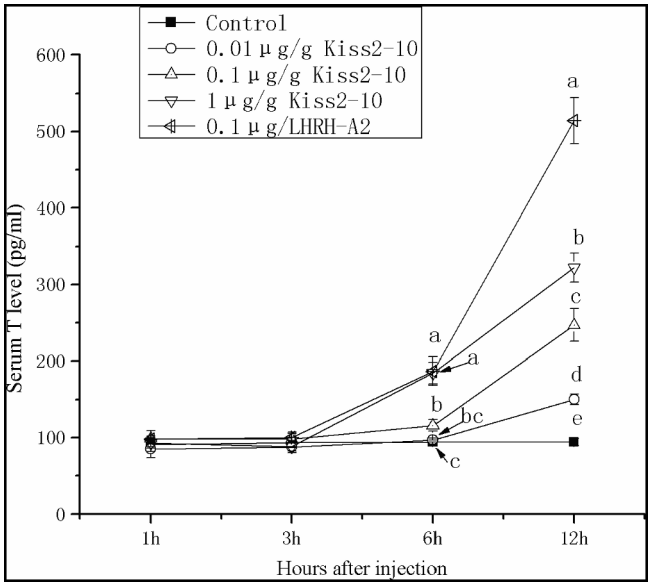

Fig. 5. Effects of Kiss2-10 on the serum testosterone level in male black porgy. Different superscripts indicate significant differences between different treatments at the same time.

increased the testosterone (T) levels in serum, suggesting that Kiss2-10 play a promotive role in HPG axis of male black porgy.

In summary, we cloned the *kiss2* and *gpr54* cDNA sequences from black porgy but could not obtain the *kiss1* and other *gpr54* gene sequences, indicating that black porgy may have lost the *kiss1* and other *gpr54* genes. These findings may pave the way for research on the evolution of *kiss* and its receptor in fish. Tissue distribution analysis showed that both genes were expressed extensively in the hypothalamus, pituitary, and testis, indicating a potential role in regulation of the HPG axis. By quantitative real-time PCR, we have also demonstrated that Kiss2-10 induces *kiss2, gpr54, gnrh1* and *gnrh3* expression in the hypothalamus, *fshβ* and *lhβ* expression in the pituitary, *star* and *ar* expression in the testis. Furthermore, Kiss2-10 can significantly elevate serum T level of male black porgy in a clear dose-dependent manner. Overall, these data suggested that the Kiss2/GPR54 system promotes the HPG axis in male black porgy.

Acknowledgments

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