General and Comparative Endocrinology 281 (2019) 126–136

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

NKB/NK3 system negatively regulates the reproductive axis in sexually immature goldfish (*Carassius auratus*)

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ARTICLE INFO

Keywords:
Neurokinin B
Tachykinin 3 receptor
Expression patterns
Reproductive function

ABSTRACT

To ascertain the significance of the Neurokinin B/Tachykinin 3 receptor (NKB/NK3) system in goldfish reproduction, two cDNAs encoding *tachykinin 3 receptors*, namely *tacr3a* and *tacr3b*, were cloned. Subsequent studies revealed that the downstream signalling of both Tac3rs can be activated by different NKB peptides, suggesting that the cloned receptors are biologically functional in goldfish. RT-PCR analysis showed that *tacr3s* are widely expressed in brain regions. During the gonadal development, *tacr3a* and *tacr3b* exhibited different expression patterns in the hypothalamus and pituitary. The actions of NKB peptides on reproductive axis was further investigated *in vivo*. Intraperitoneal injections of NKB peptides significantly reduced the expression of *kiss2* and *gonadotropin releasing hormone 3* (*gnrh3*) in the hypothalamus, and the expression of *luteinizing hormone beta subunit* (*lhb*) and *follicle stimulating hormone beta subunit* (*fshb*) in the pituitary in sexually immature goldfish. Taken together, our findings revealed that NKB/NK3 system plays a negative role in the reproductive axis of immature goldfish.

1. Introduction

Neurokinin B (NKB) which is encoded by *Tachykinin 3* (*TAC3*), via binding to Tachykinin 3 receptor (TAC3R), plays a critical role in human reproduction (Guran et al., 2009; Topaloglu et al., 2009). In recent years, many studies indicated that NKB/NK3 system regulates reproductive axis by controlling the gonadotropin-releasing hormone (GnRH) and gonadotropin (GtH) release in mammals (Billings et al., 2010; Navarro et al., 2009b; Ramaswamy et al., 2010; Wakabayashi et al., 2010). However, negative or null effects on luteinizing hormone (LH) secretion after administration of NKB peptides or senktide, an agonist of TAC3R, were also observed in the rodents (Corander et al., 2010; Kinsey-Jones et al., 2012). Intraventricular injection of senktide significantly reduced the serum LH level in the ovariectomized rats (Sandoval-Guzman and Rance, 2004). Senktide increased the LH level at the follicular phase while has no effect at the luteal phase in the ewes (Billings et al., 2010). Studies in ovariectomized goats and mice also obtained similar results that senktide treatment led to the reduction of LH (Navarro et al., 2009a; Wakabayashi et al., 2010).

In teleosts, several studies have demonstration that NKB/NK3 system is involved in the regulation of reproduction. NKB peptides treatment could promote the LH release in mature zebrafish and tilapia (Biran et al., 2012; Biran et al., 2014). Intraperitoneal injection of NKB synthetic peptide significantly stimulated the mRNA expression of *gnrh* and *gths* in mature female goldfish (Qi et al., 2015). Similar to mammals, some studies also found the negative role of NKB/NK3 system in fish reproduction. NKB administration reduced the *kiss1* and *kiss2* mRNA levels, and the Gnrh1 pituitary content in striped bass (Zmora et al., 2017). The neurokinin B related peptide (NKBRP) showed an inhibitory effect on the expression of GTH subunits in mature female tilapia (Jin et al., 2016). These studies indicated that NKB/NK3 system is functional multiplicity in vertebrate reproduction.

Based on what has been learned from the current data, in this study, we ask the question of whether the different actions of NKB/NK3 system in reproductive axis are associated with the gonadal development status of the experimental animals. We cloned the cDNAs of *tacr3a* and *tacr3b* in goldfish, studied the ligand-receptor interactions, and investigated the expression patterns of *tacr3s* during different gonadal stages. Finally, using the sexually immature goldfish, we investigated the physiological effects of NKB peptides on the expression of *kiss2*,

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https://doi.org/10.1016/j.ygcen.2019.05.020
Received 3 February 2019; Received in revised form 19 May 2019; Accepted 31 May 2019
Available online 01 June 2019
0016-6480/

gonadotropin releasing hormone 3 (*gnrh3*), *luteinizing hormone beta subunit* (*lhb*) and *follicle stimulating hormone beta subunit* (*fshb*) *in vivo*.

2. Materials and methods

2.1. Animals and chemicals

Goldfish were obtained from a local fish farm in Guangzhou, China. Fish were anesthetized with MS-222 (Sigma-Aldrich) before sacrifice via decapitation, and tissue samples were collected immediately, snap frozen in liquid nitrogen, and stored at −80 °C until further use. All animal experiments were conducted in accordance with the guidelines and with approval of the respective Animal Research and Ethics Committees of Sun Yat-Sen University.

Peptides corresponding to goldfish NKBs (NKBa-13, NKBa-10, NKBb-13 and NKBb-10) were synthesized by GL Biochem, Shanghai, China. The purity was > 95% as determined by analytical HPLC.

2.2. Cloning and sequence analysis of tacr3a and tacr3b of goldfish

Total RNA was extracted from the goldfish brain using TRIzol (Invitrogen, USA). One microgram of isolated RNA was used to synthesize first-strand cDNAs using the SMART RACE kit (Clontech, USA). PCR was performed using primers corresponding to zebrafish *tac3r* cDNA sequences, followed by 5′ and 3′ RACE (rapid amplification of cDNA ends) with gene-specific primers. All primers used in this study are listed in Table1.

The PCR reactions were performed using Blend Taq DNA polymerase (TOYOBO, Japan) with an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s. The reactions were completed with a final extension of 10 min at 72 °C. The amplification products were separated via 1.5% agarose gel electrophoresis, and the band of the desired size was excised and purified using an E.Z.N.A. Gel Extraction Kit (Omega BioTek, USA). This purified product was then subcloned into PGEM-T Easy Vectors (Promega, USA).

Table 1
The nucleotide sequences of the primers used in the present study.

Primers	Sequence (from 5′ to 3′)
Primers for 3′ RACE	
tac3ra-RACE-F1	ACCCTCTGAAGCCTCGTCT
tac3ra-RACE-F2	CTGTCCGTGTTGTGGTTGG
tac3rb-RACE-F1	CCTGGCGTTCCCACTCTGCTT
tac3rb-RACE-F2	TACAGCAGGGTCGGACTCAC
UPMA long	CTAATACGACTCACTATAGGGCAAGCAGT GGTATCAACGCAGAGT
UPMA short	CTAATACGACTCACTATAGGGC
NUPA	AAGCAGTGGTATCAACGCAGAGT
Primers for 5′ RACE	
tac3ra-RACE-R1	AGACGAGGCTTCAGAGGGT
tac3ra-RACE-R2	GCCAGGTTGAGCAAGAAAT
tac3rb-RACE-R1	TGAGCACCAGAGGCAGAAG
tac3rb-RACE-R2	CCAGGAACGCAGCCAGAAT
5′CDS	(T)25VN
Primers for real-time PCR	
tac3ra-real-F	ATCTGTCCGTGTTGTGTGT
tac3ra-real-R	GGAATGTGGGCTCTCTGTG
tac3rb-real-F	TTCCCACTCTGCTTCTATTC
tac3rb-real-R	TTCCCACTCTGCTTCTATTC
kiss2-real-F	GCAGTTTGACGAGCCCAGTTC
kiss2-real-R	AAATCATCATTGGCAGCAGGT
sgnrh-real-F	GCACTGGTCATACGGTTGG
sgnrh-real-R	TCTCATAGGCTCCAAGGGTT
lhβ-real-F	CCTGTGAGCCAGTTAATGAG
lhβ-real-R	ACAGGGTAGGTGATGTGGG
fshβ-real-F	GTTTACCGTAGCCCACTGATG
fshβ-real-R	GGGGTGTTTAGTTTCCTTGTCT
ef1a-real-F	GAAGAACGTGTCTGTCAAGG
ef1a-real-R	GTTCAGGATGATGACCTGAG

Putative amino acid sequences were predicted using BioEdit software (Hall, 1999), and the putative seven-transmembrane domains were predicted using the TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Multiple sequence alignments of Tac3rs were conducted with Clustal W 1.83. The protein phylogenetic analyze was performed with MEGA 6.0 using the neighbor-joining method and was re-sampled with 1000 bootstrap replicates (Tamura et al., 2013).

2.3. Cell culture, transfection and functional assays

The open reading frame (ORF) of goldfish *tacr3a* and *tacr3b* was subcloned into pcDNA3.1 expression vector (Invitrogen, USA). Prior to transfection, 293-T cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Thermo Scientific, USA). Twenty hours before transfection, 1×10^5 cells/well were seeded into 24-well tissue-culture plates. Then, 500 ng of pCRE-Luc or pSRE-Luc reporter plasmids, 300 ng of pcDNA3.1-*tacr3a* or pcDNA3.1-*tacr3b* and 50 ng of pRL-CMV (to normalize transfection efficiency) containing Renilla luciferase were transiently co-transfected into the cells in 500 ml DMEM (10% FBS) using Lipofectamine reagent 3000 (Invitrogen, USA). Six hours after transfection, cells were treated with vehicle or various (from 10^{-10} to 10^{-6} M) concentrations of NKBs. After additional 24 h incubation, cells were harvested, and luciferase activity assays were carried out using a Dual-Luciferase kit (Promega, USA). Parallel control transfection experiments were performed with only pcDNA3.1, CRE or the serum response element (SRE) promoter and an internal control, pRL/CMV.

2.4. Tissue distribution of tacr3a and tacr3b in goldfish

Quantitative real-time PCR was conducted to detected the expression patterns of *tacr3a* and *tacr3b* in male (n = 4) and female (n = 4) adult sexually mature goldfish. Total RNA was extracted from telencephalon, optic tectum thalamus, cerebellum, hypothalamus, pituitary, eye, kidney, intestine, spleen, gill, liver, testis and ovary, respectively. One microgram of total RNA from each tissue was digested with DNase I (Invitrogen) and reverse-transcribed (RT) into cDNA, using the ReverTra Ace-First-strand cDNA Synthesis Kit (Roche Diagnostics, Germany). Mock RT reactions without reverse transcriptase were used as negative controls.

2.5. Expression profiles of tacr3a and tacr3b at different stages of gonadal development

Goldfish were maintained at a local farm (Guangzhou, China), and samples at different stages of gonadal development were collected. The gender and gonadal stages were checked by histological analysis. The gonadal histology process and result were showed in our previous study (Liu et al., 2018). The hypothalamus was removed and promptly frozen in liquid nitrogen. Quantitative real-time PCR was performed to detect the expression levels of *tacr3a* and *tacr3b* in the hypothalamus and pituitary.

2.6. In vivo effects of NKB peptides on the expression of kiss2, gnrh3, lhb and fshb mRNA in sexually immature goldfish

The sexually immature goldfish, 80 to 100 g body weight, were selected for *in vivo* experiment. The gonad of female and male fish was at early vitellogenic oocyte stage and early spermatogenesis stage (supplementary Fig. 1). The fish were kept in indoor tanks with circulating water at temperatures between 26 °C and 28 °C. The fish were acclimatized to the environment for 10 days, and then were intraperitoneally injected with NKBa-10 and NKBa-13 peptides (500 ng/g body weight), respectively. The negative control group were injected with the equal volume of saline (0.7% NaCl) only. Samples

A

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                                                                                                                    TTAAGCAGTGGTATCAACGCAGAGTACGCGGGGGGGG 37
ATCGAGCGCAGTACGATGCGCACGAGGACGTGCCAGATCTATGCTTGACACTATAGTAGAATAAATATATGCCTCTGATCAAATCCTATCTGTTTAAGGAGGATATCTAATAAGTTTATC 157
ATGGCTGGTCCTCAGAGCGGCTCAAACGTAGCGCGTAATTTCACAAATCAGTTCGTGCAGCCGCCGTGGCGCGTCGCGATCTGGTCGGTCGCGTACAGCTCCGTGCTGGCGGTCGCTGTG 277
  M  A  G  P  Q  S  G  S  N  V  A  R  N  F  T  N  Q  F  V  Q  P  P  W  R  V  A  I  W  S  V  A  Y  S  S  V  L  A  V  A  V
TTCGGGAACCTCATCGTTATGTGGATCATTTTGGCTCACAAACGGATGCGCACCGTGACCAACTATTTCTTGCTCAACCTGGCGTTCTCCGACGCCTCGATGGCCGCCTTCAACACGCTC 397
  F  G  N  L  I  V  M  W  I  I  L  A  H  K  R  M  R  T  V  T  N  Y  F  L  L  N  L  A  F  S  D  A  S  M  A  A  F  N  T  L
ATCAACTTCATTTACGCCACGCACGGAGAGTGGTACTTTGGAGAGGTTTACTGCAAGTTCCACAACTTCTTTCCGGTGACCGCTGTGTTTGCCAGCATTTACTCCATGACTGCGATTGCA 517
  I  N  F  I  Y  A  T  H  G  E  W  Y  F  G  E  V  Y  C  K  F  H  N  F  F  P  V  T  A  V  F  A  S  I  Y  S  M  T  A  I  A
GTCGACAGGTACATGGCCATAATTCACCCTCTGAAGCCTCGTCTGTCAGCTACCGCTACCAAAGTGGTGATTGTCTGTATTTGGGCCCTGGCTGTGATTTTGGCCTTCCCGCTGTGTTTC 637
  V  D  R  Y  M  A  I  I  H  P  L  K  P  R  L  S  A  T  A  T  K  V  V  I  V  C  I  W  A  L  A  V  I  L  A  F  P  L  C  F
TACTCGACCACGAGAACCATGCCTCGCAGAACCGTCTGTTACGTTGCTTGGCCGAGACCTTCTGAGGATTCGTTCATGTATCATATCATAGTAACAGTGCTGGTGTACATGCTGCCCCTA 757
  Y  S  T  T  R  T  M  P  R  R  T  V  C  Y  V  A  W  P  R  P  S  E  D  S  F  M  Y  H  I  I  V  T  V  L  V  Y  M  L  P  L
GTGGTCATGGGTATCACCTACACTATAGTCGGGGTGACACTATGGGGAGGAGAGATTCTTGGAGATTCGTCGGACAATTATGTTGGACAATTACATGCTAAAAGGAAGGTGGTGAAGATG 877
  V  V  M  G  I  T  Y  T  I  V  G  V  T  L  W  G  G  E  I  L  G  D  S  S  D  N  Y  V  G  Q  L  H  A  K  R  K  V  V  K  M
ATGATCGTGGTGGTGGTGACCTTTGCCCTCTGCTGGTTGCCCTATCACATCTATTTCATTGTGACGGGCCTAAACAAGCGTCTGAACAAGTGGAAGTCCATCCAGCAGGTGTATCTGTCC 997
  M  I  V  V  V  V  T  F  A  L  C  W  L  P  Y  H  I  Y  F  I  V  T  G  L  N  K  R  L  N  K  W  K  S  I  Q  Q  V  Y  L  S
GTGTTGTGGTTGGCCATGAGCTCCACCATGTATAACCCCATCATTTACTGCTGTCTGAATGGCAGATTTAGGGCAGGTTTCAAACGGGCCTTCAGGTGGTGTCCCTTCATCCATATCTCC 1117
  V  L  W  L  A  M  S  S  T  M  Y  N  P  I  I  Y  C  C  L  N  G  R  F  R  A  G  F  K  R  A  F  R  W  C  P  F  I  H  I  S
AGCTATGATGAGCTCGAGCTCCGTCCCACTCGTCTCCACCCACGCAACCAGAGCAGCATGTGCACCCTGTCCCGCATCGACACCAGCGTCCACGATGATGACCCGCGACGCAGCGACCGC 1237
  S  Y  D  E  L  E  L  R  P  T  R  L  H  P  R  N  Q  S  S  M  C  T  L  S  R  I  D  T  S  V  H  D  D  D  P  R  R  S  D  R
AAGAGCACCAGGTCCCTGCAGTGTCAGGTGGAGGTCAGAGACCAAAGCAGTGCAGCGACTAAACTCTGTCTTCACAGAGAGCCCACATTCCCAACCGAGCAGCTCAGCTGAAGGGCCAAG 1357
  K  S  T  R  S  L  Q  C  Q  V  E  V  R  D  Q  S  S  A  A  T  K  L  C  L  H  R  E  P  T  F  P  T  E  Q  L  S  *
GGCCAGACCAGACGTGCATCGGATTGTGGTTCTGTTAGAGGTGCTCCATTACATTCCAAAAAAAAAAAAAAAAAAAAAAAAAAA                                1441
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B

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                                                                                                                    GTCTATTTCATCGTGACGGGGGGGGGGAAATCAGTCTCATCACGGAGAAGAGGTCCACTAGCGCATCATG 70
ATGTCCTCGTCGAGAAACTCCTCAAACTTCACCCACACCAACAGGTTCGCGCAGCCTCCGTGGCGCGTGGCGCTCTGGTCGCTCGCGTTCGCGCTGGTTCTGCTGGTGGCCGTCACCGGG 190
  M  S  S  S  R  N  S  S  N  F  T  H  T  N  R  F  A  Q  P  P  W  R  V  A  L  W  S  L  A  F  A  L  V  L  L  V  A  V  T  G
AACCTGATCGTCATCTGGATCATCGTGGCGCACAAGAGGATGAGGACCGTAACCAACTACTTTCTGCTCAACCTCGCGGCGTCTGACGTGTGCGTGGCCGCGCTCAACGCGCTGGTGAAC 310
  N  L  I  V  I  W  I  I  V  A  H  K  R  M  R  T  V  T  N  Y  F  L  L  N  L  A  A  S  D  V  C  V  A  A  L  N  A  L  V  N
TTTGTGTACGGCGCGCACGGAGACTGGTACTTCAGCAGCGCGTACTGCCGCTTCCAGAACTTCTACCCGGTGACCGCGGTGTTCGCCAGCATCTACTCCATGAGCGCCATCGCCTTGGAC 430
  F  V  Y  G  A  H  G  D  W  Y  F  S  S  A  Y  C  R  F  Q  N  F  Y  P  V  T  A  V  F  A  S  I  Y  S  M  S  A  I  A  L  D
AGGTACATGGCGATCATCCACCCGATGAAGCCACGCCTCTCAGCATCGGCCACGAAAGGCGTGATCGCATGCGTGTGGATTCTGGCTGCGTTCCTGGCGTTCCCACTCTGCTTCTATTCC 550
  R  Y  M  A  I  I  H  P  M  K  P  R  L  S  A  S  A  T  K  G  V  I  A  C  V  W  I  L  A  A  F  L  A  F  P  L  C  F  Y  S
ATAACTGAAGTGAGGCCGCACAGGACGGTGTGCTACGTGTCCTGGCCGCGGCGCGACGCAGACGCATTCATATATCACGTGATCGTGGCCGTGCTGGTGTATCTTCTGCCTCTGGTGCTC 670
  I  T  E  V  R  P  H  R  T  V  C  Y  V  S  W  P  R  R  D  A  D  A  F  I  Y  H  V  I  V  A  V  L  V  Y  L  L  P  L  V  L
ATGGCTGCCACCTACAGCAGGGTCGGACTCACTCTGTGGGGCGGAGGATTTCCAGGACATTCCTCAGAAAACTTCCAGGGTCACCTGCAGGCCAAGAGAAAGGTTGTGAAGATGATGGTG 790
  M  A  A  T  Y  S  R  V  G  L  T  L  W  G  G  G  F  P  G  H  S  S  E  N  F  Q  G  H  L  Q  A  K  R  K  V  V  K  M  M  V
ATCGTGGTGGTAACATTTGCCATCTGTTGGCTTCCATATCATGTGTATTTCATTGTAACGAGCTTCAACCAGAAGCTGAAGAAGATCAAGTCGATCCAGCAGGTGTATCTGTCAGTGCTG 910
  I  V  V  V  T  F  A  I  C  W  L  P  Y  H  V  Y  F  I  V  T  S  F  N  Q  K  L  K  K  I  K  S  I  Q  Q  V  Y  L  S  V  L
TGGCTCTCCATGAGCTCCTCCATGTACAACCCCATCATCTACTGCTGCCTCAACAGCAGGTTCCGCGCAGGCTTCAAACAGGTGTTTCGCTGGTGTCCCTTCATTCACGTGTCAGACAGT 1030
  W  L  S  M  S  S  S  M  Y  N  P  I  I  Y  C  C  L  N  S  R  F  R  A  G  F  K  Q  V  F  R  W  C  P  F  I  H  V  S  D  S
GATGAGCTGGAGCTCCAGATCACTCACTTCCAGCAGAACCGCCAGAGCAGCCTGTACACGGTCACGCGTGTGGAGTCCTGCCCCGACGCGAGCGCTAACCCCAGCAGACGCAAGAGCTCC 1150
  D  E  L  E  L  Q  I  T  H  F  Q  Q  N  R  Q  S  S  L  Y  T  V  T  R  V  E  S  C  P  D  A  S  A  N  P  S  R  R  K  S  S
AGCACCAGCCACCGCAGCAGCCTCATGGGCCAATCACGGCCCTCCGCACGACCCTCGCTCAACGGGGGTCTCCATGTTAACCCCGCGAGCCCCGGCGAGAAGGAGCTCAGCTGAGCTCAG 1270
  S  T  S  H  R  S  S  L  M  G  Q  S  R  P  S  A  R  P  S  L  N  G  G  L  H  V  N  P  A  S  P  G  E  K  E  L  S  *
CTGATACAGTAGATTCTGCAGTGCTGACCTTTTATGAAAAACTTTCACTTGATGCAATTGCCCTTTGTAAAATGGATCGAGAGAGAGAAGTAATATGTAGAAGGATAGCTGAAATTGGAC 1390
AATGGATAGCTAACAAAGATGGACAATAAAATTCATTACTAACGAAAAAAAAAAAAAAAAAAAAAAAAA                                                1459
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Fig. 1. The nucleotide sequences and the deduced amino acid sequences of goldfish *tacr3a* (A) and *tacr3b* (B). The seven transmembrane domains are underlined. Stop codons are indicated by asterisk.

(hypothalamus and pituitary) were collected at 6 h post-injection, frozen in liquid nitrogen and stored at −80 °C.

2.7. Quantitative real-time PCR

Quantitative real-time PCR analysis was used to determine the mRNA level of *tacr3a*, *tacr3b*, *kiss2*, *gnrh3*, *lhb* and *fshb*. The *ef1α* is used as the internal control. Primers for these genes are listed in Table 1. Quantitative real-time PCR was performed on a ABI 7900 T using the SYBR GreenIkit (TOYOBO, Japan) according to the manufacture's protocol. All standard curves of amplification were generated by serial dilutions of plasmid constructs. The 10 ul reaction contained 5 ul SYBR Green mix, 0.2 uM of each forward and reverse primer and 0.5 ul of RT product. PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 57 °C for 15 s, and 72 °C for 20 s. A melting curve analysis was performed with 1 cycle of 95 °C for 60 s, 55 °C for 30 s, and 95 °C for 30 s after amplification. Fluorescence 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.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software. All data are presented as the mean values ± S.E.M. Statistical differences were estimated using unpaired Student's t-tests or one-way ANOVAs followed by Tukey's tests, and a probability level less than 0.05 ($P < 0.05$) was used to indicate significance.

3. Result

3.1. Cloning and sequence analysis of goldfish tacr3a and tacr3b

The ORF region of goldfish *tacr3a* and *tacr3b* were cloned from the brain. As shown in Fig. 1, the ORF of *tacr3a* and *tacr3b* is 1191 bp and 1193 bp in length, encoding a protein of 397 and 398 amino acid (aa), respectively. Sequence analysis via TMHMM 2.0 showed that both Tac3ra and Tac3rb are typical G-protein couple receptors, containing an extracellular N-terminus, seven trans-membrane domains and a cytoplasmic C-terminus.

Sequence comparison showed that goldfish Tac3ra and Tac3rb share high identity with the zebrafish Tac3rs (Fig. 2). Phylogenetic analysis revealed that teleostean Tac3rs are clustered into two large separate groups, Tac3ra and Tac3rb. The Tac3ra group can be further divided into two clades, Tac3ra1 and Tac3ra2. The goldfish Tac3ra is clustered within the Tac3ra2 group, and the goldfish Tac3rb is clustered with zebrafish Tac3rb (Fig. 3).

3.2. Functional characterization of the goldfish NKB/NK3 system in cultured eukaryotic cells

The CRE and SRE gene assay was applied to investigate whether goldfish NKB peptides can functionally interact with goldfish Tac3rs. As shown in Fig. 4, NKB peptides could activate the CRE and SRE luciferase activity in the cells transfected with goldfish *tacr3a* with distinct affinities. Four NKB peptides were able to stimulate the CRE luciferase activity in a clearly dose-dependent manner. For the SRE promoter assay, high dose of NKBa-10, NKBa-13 and NKBb-13 peptides could sharply increase the luciferase activity in the *tacr3a*-expressing cells, but no significant post-receptor signaling could be detected in cells upon NKBb-11 peptide treatment (Fig. 4 C).

On the other hand, NKB peptides could not activate the CRE and SRE luciferase activity in the cells transfected with goldfish *tacr3b*, with the exception of a slight increase in SRE luciferase activity after NKBa-10 treatment (Fig. 4 B, D). Cells transfected with empty vectors did not show any response to NKB peptides (data not shown).

3.3. Tissue expression of goldfish tacr3a and tacr3b

A real-time quantitative PCR analysis was conducted to examine the tissue distribution patterns of *tacr3s* in male and female goldfish. As shown in Fig. 5, *tacr3a* is highly expressed in the brain areas, with moderate expression levels in the pituitary, eye and gonad (Fig. 5 A). *tacr3b* is widely expressed in central nervous system and peripheral tissues, with high expression levels in the telencephalon, optic tectum thalamus, intestine and testis, and appreciable expression levels in cerebellum, hypothalamus and pituitary (Fig. 5 B).

3.4. Expression profiles of tacr3s in the hypothalamus and pituitary during the gonadal development

As shown in Fig. 6A, the hypothalamic expression of *tacr3a* is high at the primary growth oocyte stage and late vitellogenic oocyte stage, but low at the early and middle vitellogenic stages. The *tacr3b* expression in the hypothalamus was not significantly changed during the ovarian development of goldfish. In male goldfish, both *tacr3a* and *tacr3b* mRNA expression in the hypothalamus was rather stable and showed little change during spermatogenesis.

As shown in Fig. 6B, *tacr3a* expression in the pituitary was significantly higher at the early vitellogenic stage and then decreased afterwards. In male goldfish, *tacr3a* expression in the pituitary was significantly decreased at the spermiation stage. NO significant change of *tacr3b* expression in the pituitary was observed during the vitellogenesis and spermatogenesis.

3.5. The in vivo effects of goldfish NKBa-10 and NKBa-13 on the expression of kiss2, gnrh3, lhb and fshb in the sexually immature goldfish

As shown in Fig. 7A, peripheral injection of NKBa-10 or NKBa-13 into female goldfish could decrease the mRNA expression of *kiss2* and *gnrh3* in the hypothalamus as well as the mRNA expression of *lhb* and *fshb* in the pituitary.

In the male goldfish, NKBa-10 peptide could significantly reduce the mRNA expression of *kiss2* and *gnrh3*. NKBa-13 could also decrease the mRNA expression of *kiss2* but had no effect on the *gnrh3* expression. Downregulation of the mRNA expression of *lhb* and *fshb* in the pituitary were observed after NKBa-10 administration. However, injection of NKBa-13 at a dose of 500 ng/g bw could not significantly change the mRNA expression of *lhb* and *fshb* in the pituitary (Fig. 7B).

4. Discussion

There are at least two *tac3r* genes that have been identified in the fish genomes (Biran et al., 2012; Zhou et al., 2012). In the present study, the cDNAs of *tacr3a* and *tacr3b* were cloned from the brain of goldfish. Sequence analysis and phylogenetic tree revealed that the cloned receptors are seven transmembrane couple receptor and belong to the TACR3 family. Evolutionary analysis showed that there are two *tacr3a* genes (*tacr3a1* and *tacr3a2*) in teleosts (Zhou et al., 2012). The goldfish tacr3a shares a high degree of homology with zebrafish tacr3a2 and are clustered into the group of teleostean TACR3a2, suggesting that the goldfish *tacr3a* may be the homologue of *tacr3a2*. We tried to clone the cDNA of another *tacr3a* in goldfish but failed. Whether the goldfish possesses two *tacr3a* genes warrants further investigations. For *tacr3b*, our previous study showed that this gene was only identified in zebrafish, and might be lost in pufferfish, medaka and stickleback (Zhou et al., 2012). Here we cloned the *tacr3b* cDNA in goldfish, suggesting that this gene is not zebrafish specific, and may expressed in other fish species.

To further examine the biological activity of the cloned receptors in goldfish, we have studied the ligand-receptor interactions of the four goldfish NKB peptides and two goldfish Tac3rs. Our results showed that NKBa-10, NKBa-13 and NKBb-13 can activate the CRE and SRE

Fig. 2. Sequence alignment of goldfish Tac3rs and zebrafish Tac3rs. Sequences were aligned using ClustalW. The same amino acids are indicated by asterisk. The seven transmembrane domains are boxed.

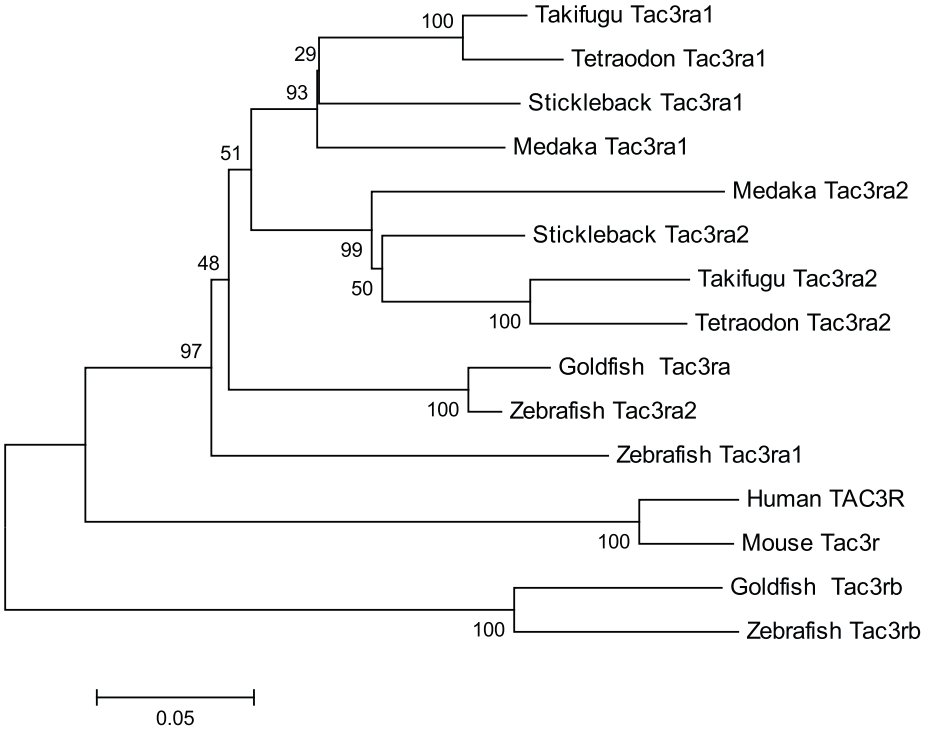

Fig. 3. Phylogenetic analysis of Tac3rs in vertebrates. The phylogenetic tree was constructed with MEGA 6.0 using the neighbor-joining method with 1000 bootstrap replicates. The number shown at each branch indicates the bootstrap value (%). GenBank accession numbers for the sequence are listed in the Supplemental data (Supplemental File1).

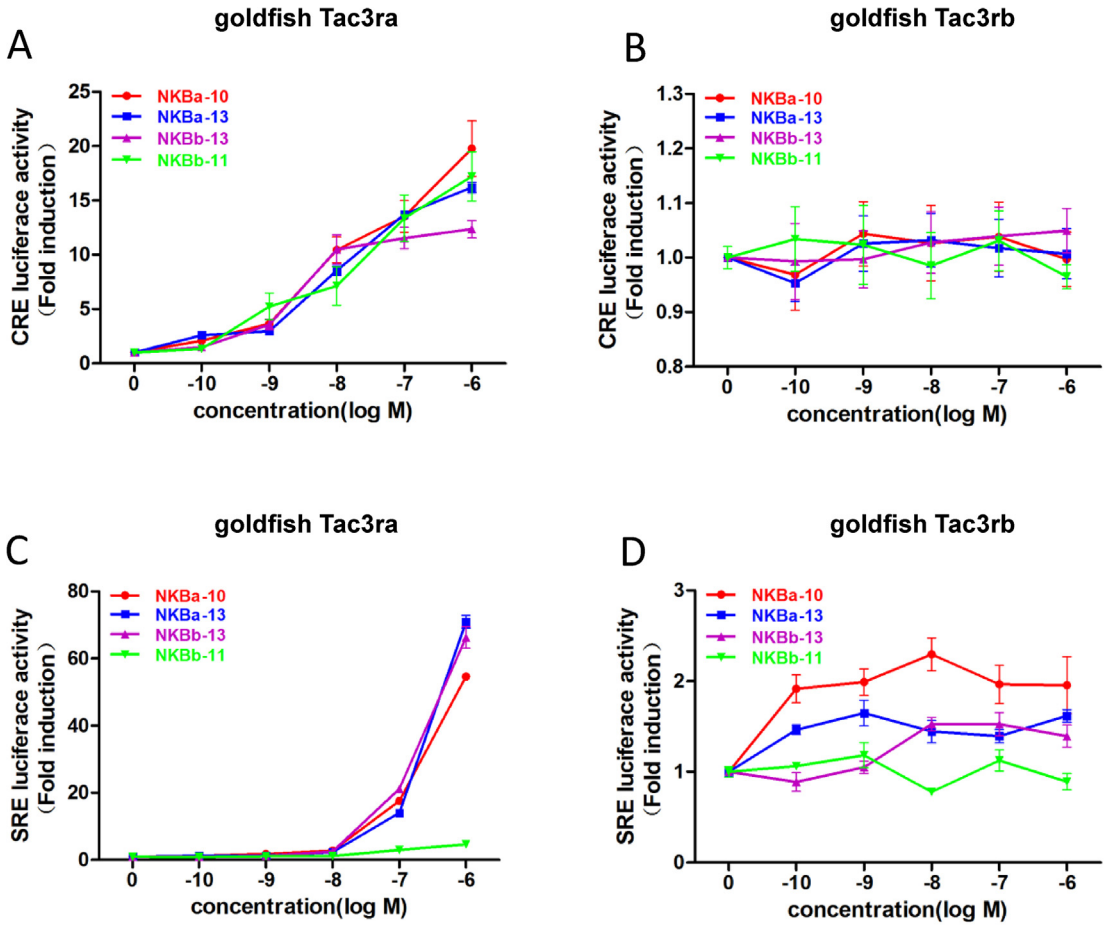

Fig. 4. Functional interaction between goldfish NKB peptides and Tac3rs. Induction of CRE-driven luciferase activities by different dose of NKB peptides in 293-T cells transfected with *tacr3a* (A) and *tacr3b* (B). Induction of SRE-driven luciferase activities by different dose of NKB peptides in 293-T cells transfected with *tacr3a* (C) and *tacr3b* (D). The results are mean values ± S.E.M. from four independent experiments, each conducted in quadruplicate, and are expressed as the ratio of the increase in luciferase activity relative to the control.

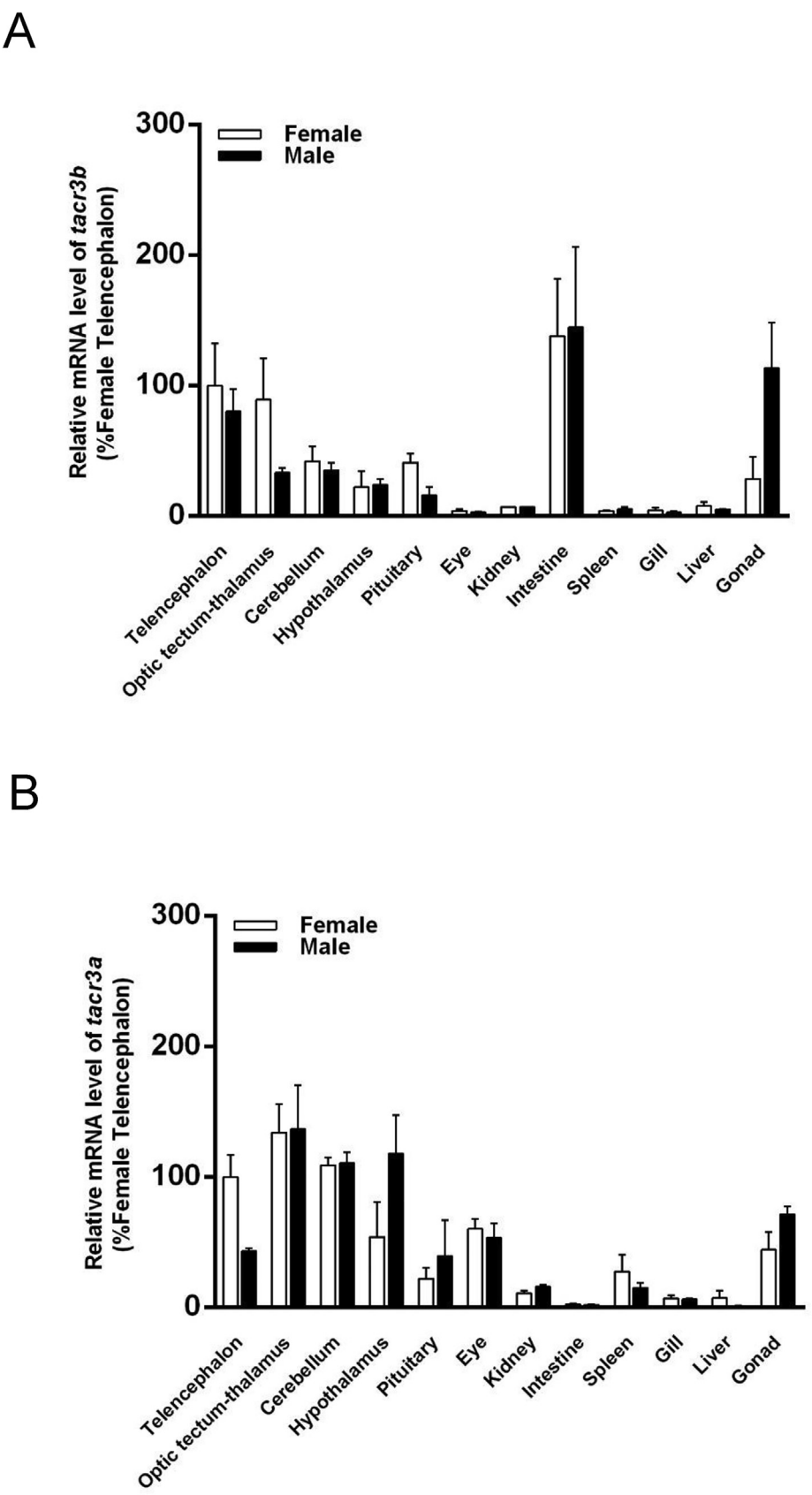

Fig. 5. Expression patterns of *tacr3a* (A) and *tacr3b* (B) in tissues of female and male goldfish. The amplification of *ef1a* was used as the house-keeping gene control. Data are represented as the mean ± SEM (n = 4) and expressed as the fold change relative to the mRNA level of the female telencephalon.

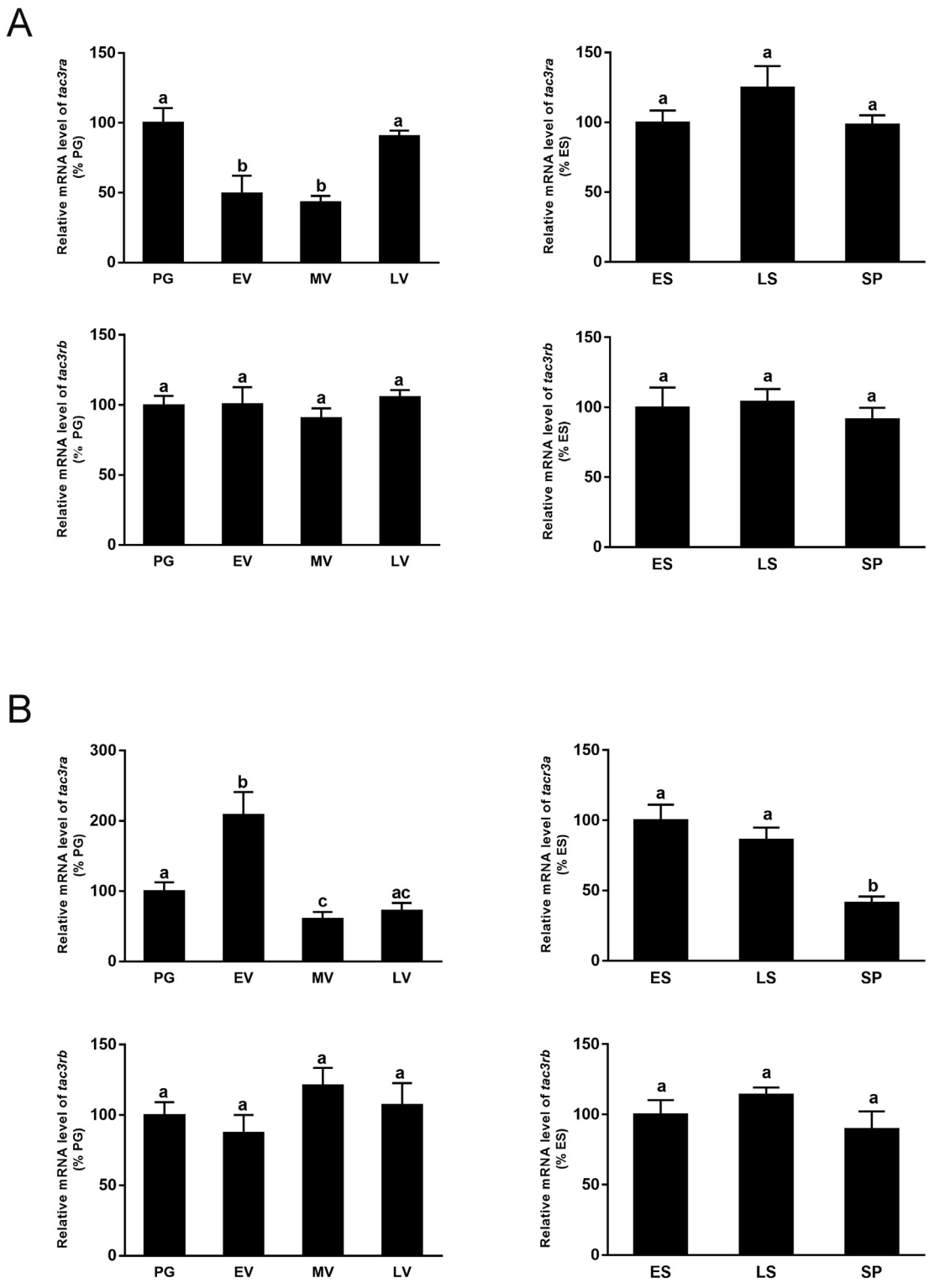

Fig. 6. The expression patterns of *tacr3a* and *tacr3b* in the hypothalamus (A) and pituitary (B) during gonadal development of goldfish. Relative expression of *tacr3s* at different gonadal stages was normalized against that of the PG stage in females or against that of the ES stage in males. Data are expressed as the mean values ± S.E.M. (n = 8). Different letters denote statistically significant differences ($P < 0.05$). PG, primary growth oocyte stage; EV, early vitellogenic oocyte stage; MV, middle vitellogenic oocyte stage; LV, late vitellogenic oocyte stage; ES, early spermatogenesis stage; LS, late spermatogenesis stage, SP, spermiation stage.

signaling pathways of Tac3ra. Interestingly, the goldfish NKBb-11 can also trigger the CRE promotor activity in *tacr3a*-expressing cells. The C-terminal pentapeptide (FVGLM) of NKB is critical for receptor activation. There is an amino acid mutation at the end of C-terminal pentapeptide (FVGLL) of NKBb-11, which was found to be unable to activate the downstream signal of Tac3rs in zebrafish (Zhou et al., 2012). Our data indicate that the NKBb-11 peptide is also biologically active in goldfish. Nevertheless, unlike the other three NKB peptides, NKBb-11 can not activate the SRE signalling pathway of Tac3ra, suggesting that the mutation at the end of C-terminal pentapeptide may weaken the ability of NKBb-11 in activating the Tac3r. On the other hand, a completely different picture is observed for the Tac3rb. Only NKBa-10 can

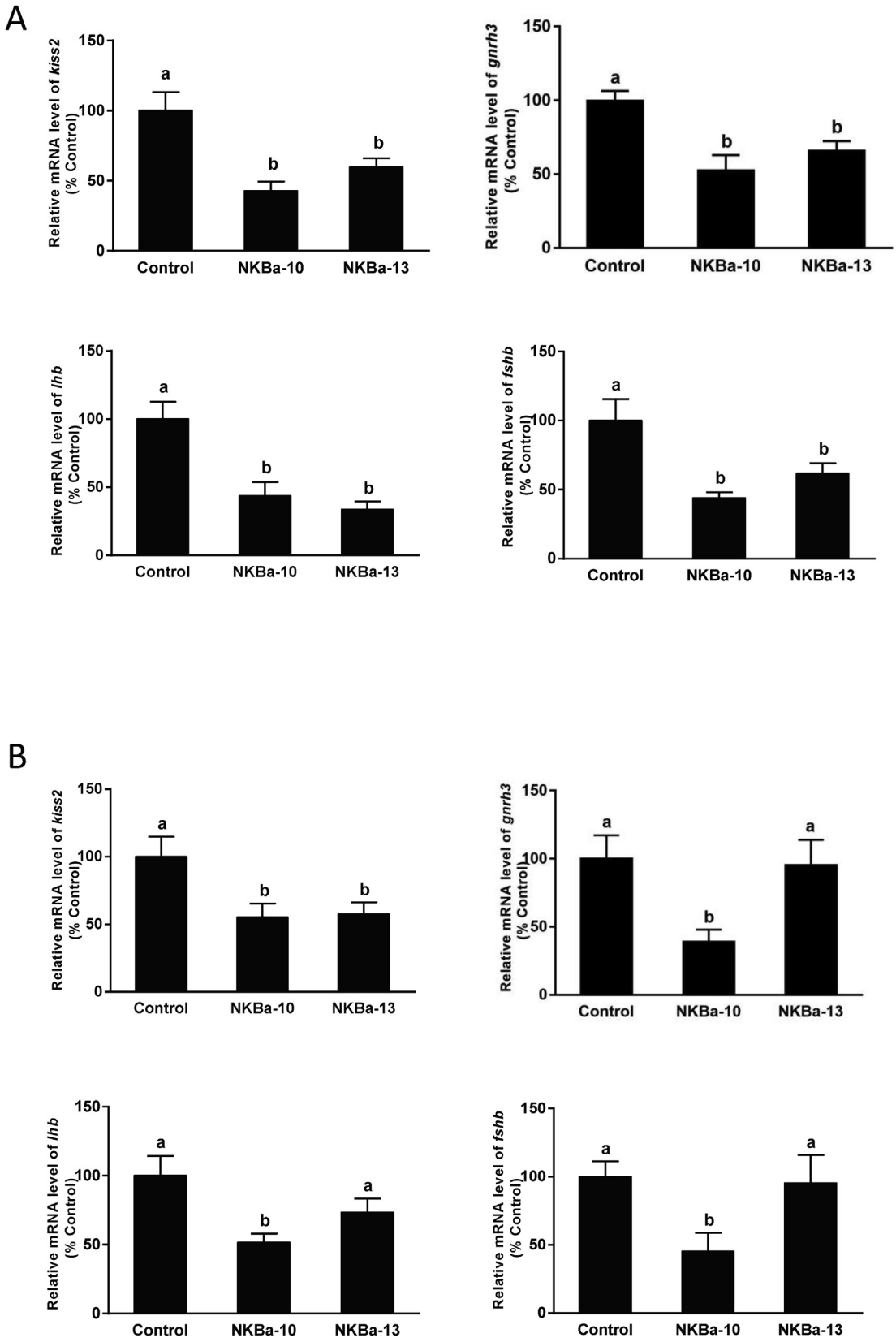

Fig. 7. *In vivo* effects of NKBa-13 and NKBa-10 on the mRNA expression of *kiss2*, *gnrh3*, *lhβ* and *fshβ* in female (A) and male (B) goldfish. Goldfish were injected intraperitoneally with 500 ng/g bw NKB peptides, and samples were collected 6 h post injection. Values are expressed as the mean values ± S.E.M (n = 8). Different letters denote statistically significant differences ($P < 0.05$) versus the corresponding control.

slightly activate the SRE signaling pathway of Tac3rb, indicating that the Tac3rb shows much less potency to NKB peptides activation compared to the Tac3ra.

Tissue expression analysis revealed that *tacr3a* and *tacr3b* is highly expressed in the central nervous system, suggesting that NKB/NK3R system is involved in extensive neural functions in goldfish. It is observed that two *tacr3s* are moderately expressed in the pituitary of goldfish, suggesting that NKB peptides can act directly on the pituitary. A study has demonstrated that NKB peptides could modulate the synthesis and secretion of pituitary hormones at the pituitary level in grass carp (Hu et al., 2014). In order to explore the reproductive function of *tacr3s* in goldfish, the expression patterns of *tacr3s* in the hypothalamus and pituitary was investigated during the gonadal development. In female goldfish, the hypothalamic expression of *tacr3a* decreased at the early and middle vitellogenic stages, and then increased at the late vitellogenic stage. Previous study showed that an increase of *tac3a* expression in the hypothalamus was observed at sexual maturation stage (Qi et al., 2015). The concomitant expression patterns of the ligand and the receptors at the late vitellogenic stage in goldfish is highly indicative of the physiological significance of the NKB/NK3R system in ovarian maturation. In the pituitary, *tacr3a* is abundantly expressed at the early vitellogenic stage and lowly expressed at the late stages of spermatogenesis. Current studies found that the actions of NKB on the pituitary are complex in different fish species. In tilapia, *tacr3* was found to be expressed in gonadotropic cells (Biran et al., 2014). NKB peptides treatment could promote the FSH and LH release from pituitary cells of tilapia and striped bass (Biran et al., 2014; Zmora et al., 2017). However, in grass carp, *tacr3* expression was found in somatolactin α (SLα) cells. NKB peptides did not influence the LH release or *lhb* mRNA levels but upregulated the PRL and SLα synthesis and secretion in carp pituitary cells (Hu et al., 2014). These data indicate that NKB/NK3R system exerts multiple roles in the pituitary. Here the significance of high expression of *tacr3a* in the pituitary of goldfish at the early vitellogenic stage is unclear and should be elucidated further. In striped bass, treatment with NKB antagonists did not affect the sperm volume. Base on their results, Zmora et al. hypothesized a possible inhibitory role of NKB in hindering sperm production in this fish (Zmora et al., 2017). The low expression of *tacr3a* at the late stages of spermatogenesis in goldfish may support this hypothesis. On the other hand, there is no significant change of *tacr3b* mRNA levels in the hypothalamus and pituitary during the gonadal development, suggesting that *tacr3b* may not involve in the regulation of reproductive function in goldfish.

As indicated in the introduction section, NKB or senktide exerts distinct actions on LH release and expression in mammals and teleost fish. Here we hypothesized that the physiological role of NKB/NK3 system in reproductive axis may related to the gonadal status of the experimental animals. This hypothesis is supported by the evidences that NKB peptides significantly downregulated the mRNA levels of *kiss2*, *gnrh3*, *lhb* and *fshb* in sexually immature male and female goldfish, as previous findings showed that injection of NKB peptides stimulated the mRNA expression of *gnrh3*, *lhb* and *fshb* in sexually mature goldfish (Qi et al., 2015). This phenomenon is not rare. Neuropeptide Y treatment was found to produced either a stimulatory or inhibitory effect on gonadotropin release in mammals, depending on the hormone milieu (Kalra et al., 1992; Sahu et al., 1987; Sandoval-Guzman and Rance, 2004). Estrogen exerts positive and negative feedback regulation on the *gonadotropin-inhibitory hormone* (*gnih*) expression by acting through different estrogen receptors at different stages of ovarian development in goldfish (Qi et al., 2017). These data indicate the regulation of reproductive neuroendocrine system is highly sophisticated. In this study, the physiological significance of different effects of NKB peptides on reproductive axis is unclear. The hypothalamus-pituitary–gonadal axis (HPG axis) in vertebrates is inseparable. The gene expression of HPG axis and the levels of gonadal steroid hormones change at different gonadal status. The sensitivity of neuroendocrine system to the treatment of exogenous peptides or the feedback of steroid hormones are also different. In female goldfish, the expression of *tac3s* and *tacr3a* were low at the early vitellogenic stage. When exogenous NKB peptides were injected into the fish at this stage, the balance of HPG axis might be broken, and evoked the inhibitory effects. So further studies on the NKB/NK3 system should focuses on animal gonadal status to reveal the physiological significance and the mechanisms of these distinct regulations.

In conclusion, we have cloned the cDNAs of *tacr3a* and *tacr3b* in goldfish, examined their biological activities and investigated their expression patterns. Furthermore, we demonstrated that NKB peptides could functionally inhibit the *kiss2, gnrh3*, *lhb* and *fshb* mRNA synthesis in sexually immature goldfish. These findings enhance our understanding of NKB/NK3 system in the regulation of reproductive axis in teleost fish species.

Acknowledgements

This work was supported by Science and Technology Planning Project of Guangzhou (201804020013), and the Guangdong Provincial Natural Science Foundation (2017A030313109).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.05.020.

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