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Molecular identification of GnIH and its potential role in reproductive physiology and male pregnancy of the lined seahorse (*Hippocampus erectus*)☆

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ABSTRACT

The gonadotropin-inhibitory hormone (GnIH) plays a negative role in the hypothalamic-pituitary-gonadal (HPG) axis by inhibiting gonadotropin secretion in vertebrates. Male pregnancy and ovoviviparous behavior are unique phenomena among vertebrates. To better understand the neuroendocrine regulatory mechanisms in ovoviviparous fish with male pregnancy, we identified the orthologous GnIH gene in the lined seahorse (*Hippocampus erectus*). The full-length cDNA of the GnIH precursor was 658 base pairs with an open reading frame of 528 base pairs that encoded a 175-amino acid prepro-GnIH peptide. The seahorse GnIH precursor contained two putative LPXRFamide peptides. Both seahorse LPXRFa-1 and LPXRFa-2 were found to be unique among vertebrates. The synteny blocks of GnIH gene loci were conserved in mammals and teleosts. Tissue distribution analysis revealed that seahorse GnIH mRNA was mainly expressed in the hypothalamus, with relatively high levels observed in the brood pouch. The expression patterns of seahorse GnIH during different reproductive stages and pregnancy stages were also detected, and GnIH mRNA expression was significantly reduced during the early puberty stage. In addition, GnIH mRNA expression was significantly increased during the pregnancy stage compared to non-pregnancy stages. In summary, our results reveal the existence of GnIH in ovoviviparous fish and suggest its involvement in regulation of reproductive behavior and male pregnancy in the male seahorse.

1. Introduction

In vertebrates, the hypothalamus-pituitary-gonadal axis (HPG axis) plays an essential role in regulating reproductive behavior. This axis is regulated by gonadotropin-releasing hormone and kisspeptin in the hypothalamus to stimulate the synthesis and release of gonadotropins (luteinizing hormone and follicle-stimulating hormone) from the pituitary (Ohga et al., 2018). Researchers have suggested that neuroendocrine factors play a negative role in regulating gonadotropin release. In 2000, a novel hypothalamic neuropeptide named as gonadotropin-inhibitory hormone (GnIH) was first discovered in birds (Japanese quail, *Coturnix japonica*) and was found to directly act on the pituitary via a novel G protein-coupled receptor (GnIHR) to inhibit gonadotropin synthesis and release (Tsutsui et al., 2000).

Since then, GnIH orthologs have been identified in other vertebrates from fish to humans. In mammals, most studies have shown that GnIH orthologs are involved in inhibition of gonadotropin secretion (Clarke et al., 2008; Kriegsfeld et al., 2015; Tsutsui and Ubuka, 2018). However, several studies in mammals have shown that GnIH also has stimulatory effect in the HPG axis (Ubuka and Parhar, 2017). In teleosts, the GnIH gene was first cloned in goldfish and found to regulate pituitary hormone release (Sawada et al., 2002). Furthermore, GnIH precursors were cloned, and their functions were identified in orange-spotted grouper (Wang et al., 2015), European sea bass (Paullada-Salmeron et al., 2016), and clownfish (Choi et al., 2016). These findings indicate that GnIH and its orthologs have conserved roles in the negative regulation of gonadotropin release across species.

The structure of GnIH varies among vertebrates, such as mammals (RFamide-related peptides), amphibians (growth hormone-releasing peptide), and teleosts (LPXRFa). However, all identified peptides possess a C-terminal Leu-Pro-Xaa-Arg-Phe-NH_2 motif (Xaa = Leu or Gln). Thus, these peptides have been designated as LPXRFamide peptides. LPXRFa peptide precursors in vertebrates are composed of two or four LPXRFa peptides: two in mammals (RFamide-related peptide-1 and -3), three in avians (GnIH, GnIH-RP-1 and -2), four in amphibians (frog GH-releasing peptide (GRP) and GRP-related peptides, GRP-RP-1, -2, and

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-3), and two or three in teleost fishes (Osugi et al., 2012). LPXRF peptides form a large group in the RFamide peptide family (Tsutsui et al., 2009).

The seahorse is an ovoviviparous fish with a unique reproduction strategy, including male pregnancy. The male seahorse incubates developing embryos in its brood pouch, which is similar to the mammalian placenta, where the embryo is aerated, protected, and provisioned before the hatching stage (Stolting and Wilson, 2007). Our previous study demonstrated that RFamide peptides, such as kisspeptin, play important roles in regulating the gonadotropic axis in the lined seahorse (*Hippocampus erectus*) (Zhang et al., 2018). To investigate whether GnIH negatively regulates gonadotropic secretion in the lined seahorse, we cloned the full-length cDNA of the GnIH precursor and detected its GnIH expression profiles during different reproductive stages.

2. Materials and methods

2.1. Animals and tissue sampling

Lined seahorse individuals were cultured at the Shenzhen Seahorse Center of the South China Sea Institute of Oceanology, Chinese Academy of Sciences. Animal ethics approval for experimentation was granted by the Chinese Academy of Sciences. The seahorses were maintained in recirculating holding tanks with seawater pumped from the South China Sea and treated with double sand filtration. The seahorses were fed twice per day (09:00 and 16:00 h) with frozen *Mysis* spp. The temperature, salinity, pH, light intensity, dissolved oxygen content, and photoperiod were maintained as follows (mean ± S.D.): 22 ± 0.5 °C, 25 ± 1.0%, 7.9 ± 0.4, 2000 lx, 6.5 ± 0.5 mg L^{-1}, and 16 h light: 8 h dark, respectively.

2.2. Cloning of seahorse GnIH

Total RNA from the seahorse brain was prepared using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. One microgram of isolated RNA was used to synthesize first-strand cDNA using the ReverTra Ace-α First-strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). The full-length cDNA sequence of seahorse GnIH was obtained using a combined technique of RT-PCR and random amplification of cDNA ends. The PCR amplification products were purified using the E.Z.N.A Gel Extraction Kit (Omega BioTek, Norcross, GA, USA) and sub-cloned into a PMD18/T vector (Takara, Shiga, Japan). Three different individual positive clones were sequenced using an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA). All primers used in the present study are listed in Table 1.

Table 1
Nucleotide sequences used in 5′RACE PCR 3′RACE PCR and real-time PCR for GnIH and the internal reference gene β-actin.

Primer sequence			
Gene	Purpose	Primer	5′-3′ sequence
GnIH	Partial cDNA	GnIH F1	CTGGAAGGACGCAGACACAC
		GnIH R1	TCGAAGAAGCCTCCAACACAG
	5′RACE	GnIH R2 (first)	CATCAGGAACACTTTGCCTACC
		GnIH R3 (nest)	GTGCTCTAAGTCTAAACTCC
	3′RACE	GnIH F2 (first)	GTGCCAAGTGTGTCGGAGAG
		GnIH F3 (nest)	CCAGAAGCACGTCAAAGATGG
	Real-time PCR	GnIH qF	GGTAGGCAAAGTGTTCCTGATG
		GnIH qR	CAGAAACTTGAAGACAGTGCTGG
β-actin	Real-time PCR	β-actin qF	TTCACCACCACAGCCGAGA
		β-actin qR	TGGTCTCGTGGATTCCGCAG

2.3. Structural, phylogenetic, and syntenic analysis of seahorse GnIH

The putative signal peptides were predicted by SignalP 3.0 (Bendtsen et al., 2004). Multiple sequence alignments of amino acids were performed with ClustalX2.0 (Larkin et al., 2007). Protein phylogeny analysis was performed with Mega6.0 software (Tamura et al., 2013) using the neighbor-joining method with 1000 bootstrap replicates. Genome syntenic analysis was performed by comparing genomic regions containing GnIH loci in human, zebrafish, medaka, platyfish, stickleback, fugu and lined seahorses. All GnIH orthologs in these vertebrates were identified by using their genome sequence information in Ensembl (http://www.ensembl.org).

2.4. Tissue distribution of seahorse GnIH

To detect the tissue distribution of GnIH in the lined seahorse, adult male (n = 6) and female (n = 6) seahorses were anesthetized with 0.05% MS222. Total RNA was isolated from brain regions, including the telencephalon, cerebellum, optic tectum-thalamus, hypothalamus, and pituitary, and peripheral tissues, including the gill, liver, intestine, heart, kidney, muscle, gonad, and pouch. One microgram of total RNA from each tissue was digested with a genome eraser and reverse-transcribed into cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Toyobo). Real-time PCR was performed on a Roche LightCycler 480 (Basel, Switzerland) using SYBR premix Ex Taq™ (Toyobo). The PCR parameters were as follows: 40 cycles of 94 °C for 20 s, 52 °C for 20 s, and 72 °C for 15 s. Fluorescence was measured at the end of each cycle, and the melting curve from 50 °C to 99 °C was obtained. The seahorse β-actin gene was amplified as a reference gene.

2.5. Expression profile of GnIH during sexual development stages

The expression profile of seahorse GnIH was analyzed in the whole brain of male seahorses during four sexual development stage, including juvenile (JUV), early puberty (EP), advanced puberty (AP), and mature (MAT) (n = 8), by real-time PCR. Seahorse gonadal tissues at different stages were fixed in Bouin's solution for 21 h. Subsequently, the gonads were embedded in paraffin, cut into 10 μm sections, and stained with hematoxylin and eosin. Classification of gonad developmental stages was determined by light microscopy.

2.6. Expression profile of GnIH during different pregnancy stages

The expression profile of seahorse GnIH was analyzed in the whole brain of seahorses at different pregnancy stages by real-time PCR. Adult seahorses were allowed to mate freely before being subjected to a standardized assessment of pregnancy status based on courtship behaviors. Pregnant seahorses (P stage) (n = 8) were maintained in the tank before sacrifice to sample the brain tissue. Mature adult seahorses were considered as the pre-pregnancy group (PreP stage) (n = 8), and post-parturition (PostP stage) seahorses, in which the larval seahorses had hatched from the brood pouch, were considered as the post-parturition group (n = 8). The expression levels of each target gene analyzed by real-time PCR were determined using the comparative quantification method $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

2.7. Statistical analysis

Real-time PCR data were analyzed using Prism 6.0 software (GraphPad, Inc., La Jolla, CA, USA). All data were expressed as the mean ± standard error of mean and evaluated by one-way analysis of variance followed by the Duncan's multiple-range test. Differences between groups were considered significant at $P < 0.05$.

```
1   TTGACGTCTGCAAGGGTGAAGATGGTGACGGGAATCTTACTGTCACTTCTCTTGATGCTGGGAGGTTTTGACACCGAGACCGCATCCAAC 90
1                          M  V  T  G  I  L  L  S  L  L  L  M  L  G  G  F  D  T  E  T  A  S  N  23
                                                Signal peptide
91  CCAAAGGTCAGTAGGAAATCCAGCTGCTGTGGCAAAGACCCGCAGAGGTGCAATCAGCGATGGCACACTGGAAGGACGCAGACACACAAT 180
24   P  K  V  S  R  K  S  S  C  C  G  K  D  P  Q  R  C  N  Q  R  W  H  T  G  R  T  Q  T  H  N  53
181 CAGAAAAAAAGTGAAGCGTTGAGGAGTTTAGACTTAGAGCACTTCAAAATAGATGTGGTCCCGACCACAAGCGAAACGAGCCTCCCTGCC 270
54   Q  K  K  S  E  A  L  R  S  L  D  L  E  H  F  K  I  D  V  V  P  T  T  S  E  T  S  L  P  A  83
271 GCTGTTGGGCTCCACCCTCCTGATGCAAAACCCCTCCACCTGCTCAAAAACATGCCCCAACGCTTTGGTAGGCAAAGTGTTCCTGATGAT 360
84   A  V  G  L  H  P  P  D  A  K  P  L  H  L  L  K  N  M  P  Q  R  F  G  R  Q  S  V  P  D  D  113
                                  LPXRFa-1
361 GCAAAGTCACCTCCCAACAGCCCCCAAAGGTTTGGAAGATCCTGGGAGGTGATCCCAAAATGTGCCAAGTGTGTCGGAGAGATTCTGAAT 450
114  A  K  S  P  P  N  S  P  Q  R  F  G  R  S  W  E  V  I  P  K  C  A  K  C  V  G  E  I  L  N  143
            LPXRFa-2
451 CCAGAAGCACGTCAAAGATGGACAAGAAACATTCTGTGTTGGAGGCTTCTTCGAACATTAACCAGAGCCAAGTTGTGGGAAACAGACTTG 540
144  P  E  A  R  Q  R  W  T  R  N  I  L  C  W  R  L  L  R  T  L  T  R  A  K  L  W  E  T  D  L  173
541 CAATGGTAGGAAAATCTAAAGGCTATTCATCACATTTTTCTGTTAAAAAAGAATGTTTATATCCAGCACTGTCTTCAAGTTTCTGACTCG 630
174  Q  W  *                                                                                    175
631 TGGAAAACATTCTGCTCATTAGTTTACA                                                                658
```

Fig. 1. cDNA and deduced amino acid sequence of the lined seahorse GnIH precursor. The putative signal peptide is underlined. The putative LPXRFa-1 and LPXRFa-2 peptides are also underlined.

```
Zebrafish     --MSYFALLSLALGILSS--------FMLSEVTALRLPLSGERDLNGFTWGQFSENAQEIPRSLEIQDFTLNVAPTSGGA 70
Goldfish      --MSYFTLVFLALGTLSS--------FMLREVTALRWPLPDDSDPDRFTWGQFLENAQEIPRSLEIEDFTLNVAPTSGRV 70
Tilapia       MLVTMILSALLMLRGLGGSDVH----VFGKSVRSGKTLLSSNDGTYSVRKQPHQETKNEIHRSLDLESFNIRVAPTTSKF 76
Grouper       MLTTVFLSALLMLGGLGGAAAFN-RQVHGKSIHSDKTLPGSNDETHTVRTQPHQQTKNEISRSLDLESFSVHVTPTTSKI 79
Flounder      MLTTVFLSALLMLGGLGGAAVS---DLYGKSIHSDRTLLGGEDVRHTVRRQPHQQTKSEMRRSLDLESFNMRVTPATSKI 77
Seabass       MLTTVFLSTLLMLGGLGGAAVSD-LQVYGKSIHSDKTLLSSDDGRHTVRKQPHQQAKGEIRRSLDLESFNIHVSPTTSKI 79
Tonguesole    MLSTVFLSVLLMLGGPGGAAAAADFQVYGKSAYSDKSLPSSEEGRHTVRRQPLQQAKAVTRRSLDLERLNMRVTPTASKS 80
Takifugu      MLVTAFLAMLLMIAGIGGAAETD-LQVNGK--LNDRTL-SSREGRHNVRKQLRHQIKSNILRSLDMERINIQVSPTSGKV 76
Tetraodon     MLVTLFLAMLLMIAGLGKAAVSD-LQVTGK--VNDRTL-GSREGRHNMRKELHHQVKNNILRSLDLESLNIHVSPTS-KI 75
Seahorse      MVTGILLSLLLMLGGFDTETASN-PKVSRKSSCCGKDPQRCNQRWHTGRTQTHNQKKSEALRSLDLEHFKIDVVPTTSET 79
                    :    * :   -         -        :                  :      ***::: :-: * *::

                                      LPXRFa-1              LPXRFa-2                LPXRFa-3
Zebrafish     SSPTILRLHPIIPKPAHLHANLPLRFGRDAQPGTGDRAPKSTINLPQRFGRSCT---MCARSG---TGPSATLPQRFGRR 144
Goldfish      SSPTILRLHPKITKPTHLHANLPLRFGRDTENTPRERA-KSNINLPQRFGRSCT---MCARSG---TGLSATLPQRFGRR 143
Tilapia       SLPTIIRFYPPTVKPLHLHANMPLRFGRQS----DERTPNSSPNLPQRFGRSWEAIRVCAECPSVRRAPNQLLSQRFERN 152
Grouper       SLPSIIRLFPPTAKPFQLHANMPMRFGRESVPG-DDSAPNSTPNMPQRFGRSWEMCAKCLNVR--EAQN-PILPQRLGRR 155
Flounder      SLPTIIKLYPPTTKPVHMHANMPMRFGRQIDSN-EDKAPN-SPNMPQRFGRSR--GRICPKCPPVRIEPNPELPQRFGRS 153
Seabass       SLPTIIRLYPPTAKPLHLHANMPMRFGRDSNPG-DDRSPNSTPNMPQRFGRSWEVFQMCAECP--GVQEPPKSPQGLRRT 156
Tonguesole    SLPTIIKLYPPTVNP-HIHANMPMRFGREVEPE-DDQSHN-TPNMPQRFGRAWTFNRVCVKC---RGDADQVLPG----T 150
Takifugu      SLPTIVRLYPPTLQPRHQHVNMPMRFGRDGVQG-GDHVPNLNPKMPQRFGRSWKVIRLCEDCS----KVQGVLKHQVRYG 151
Tetraodon     SFPTIIRLYPPTPQPPLRHINMPMRFGRNSFHG-DDHNPNSAPNMPQRFGRSWKKIQLCEGCY----EAHRILKHRVKHA 150
Seahorse      SLPAAVGLHPPDAKPLHLLKNMPQRFGRQSVPD-DAKSP---PNSPQRFGRSWEVIPKCAKCVG--EILNPEARQRWTRN 153
              * *: : :-*   :*     *:* ****:            : ******:      *

Zebrafish     NIFALDPLRALALYTRTPESPSFPKERTQVHDYMFETVEDS-EETVKNTDYTALD 198
Goldfish      NIFPLDPFRALTLYKRTPESP-FPKERTQVHDYMLETVEDSVEETVKNKDYTVLD 197
Tilapia       SPYWK---------LLRTVASEQLLNTGLHWAENFDSTTTSDEETELEEKKSKE-- 197
Grouper       SPYWS---------LFRTLDNTWLLNTGLHWAEDFDFTTSS-EEVEMQEKSFKG-- 199
Flounder      SLYWS---------LLKTLATERLLKTGLHWAEDFTSSS------EVEEKSFEA-- 192
Seabass       SLYWS---------LLRTLANAELLNTGLHWAEDIDFTTSS-EEMQMQEKTFNG-- 200
Tonguesole    SLYWS---------LINSLAVEQFFNTDLHWAEDFDLTANA-EELEMERTSFKG-- 194
Takifugu      RNGQS---------LIRTLVNAQLLKTGLHW-------------------------- 173
Tetraodon     RNGQR---------FISTLLNAQLLKSALHW-------------------------- 172
Seahorse      ILCWR---------LLRTLTRAKLWETDLQW-------------------------- 175
                            :      : :   :
```

Fig. 2. Comparison of amino acid sequences of GnIH precursors from different teleost fish species. Identical sequences are indicated by asterisks. Gaps introduced in some sequences to maximize alignment are indicated by hyphens. The conserved terminal regions of LPXRFa peptides are boxed. The GnIH precursor sequences of other teleosts were downloaded from GenBank (Table 2).

Table 2
Amino acid sequence identities of the lined seahorse GnIH precursors with GnIHs of various vertebrates.

Species	Accession no.	Amino acid sequence identity of GnIH (%) Seahorse GnIH
Seahorse GnIH		100.0
Tetraodon	BAF34879.1	51.8
Takifugu	NP_001092115.1	51.9
Tongue sole	AMB48604.1	58.5
Sea bass	CEK03537.1	65.8
Flounder	XP_019946147.1	62.4
Grouper	Wang et al. (2015)	63.4
Tilapia	NP_001298256.1	60.4
Goldfish	BAC06473	35.4
Zebrafish	NP_001076418	36.1
Lamprey	BAL52329.1	9.8

3. Results

3.1. cDNA cloning and identification of seahorse GnIH

The full-length cDNA encoding the GnIH precursor was isolated from the brain of the lined seahorse (Approved GenBank Accession Number: MK450461). The seahorse GnIH precursor cDNA was 658 base pairs (bp) containing a short 5′-untranslated region (UTR) of 21 bp, open reading frame (ORF) of 528 bp, and 3′-UTR of 109 bp in length. The ORF encoded a precursor protein of 175 amino acids (aa) with a predicted signal peptide of 21 aa (Fig. 1). The seahorse prepro-GnIH is composed of two putative LPXRFamide (X = L or Q) peptides, with each peptide containing a classical amidation signal (GK/R) at the C-terminus. Multiple sequence alignment of the teleost GnIH precursors revealed three putative RFamide peptides in zebrafishes, goldfishes, tilapias, groupers, and flounders and two putative RFamide peptides in seabasses, tongue soles, *Takifugu*, *Tetraodon*, and seahorses (Fig. 2). LPXRFa-1 and LPXRFa-2 in the lined seahorse are unique among teleost species. The seahorse LPXRFa-1 included an –MPQRF motif, while –MPMRF or –LPLRF was found in other teleost fishes. Additionally, the seahorse LPXRFa-2 included an –SPQRF motif, while –MPQRF or –LPQRF was found in other teleost fishes (Fig. 2). Homology analysis revealed that seahorse GnIH had the highest sequence identities with European seabass (65.8%) and orange spotted grouper (63.4%) among Perciformes fish species (Table 2).

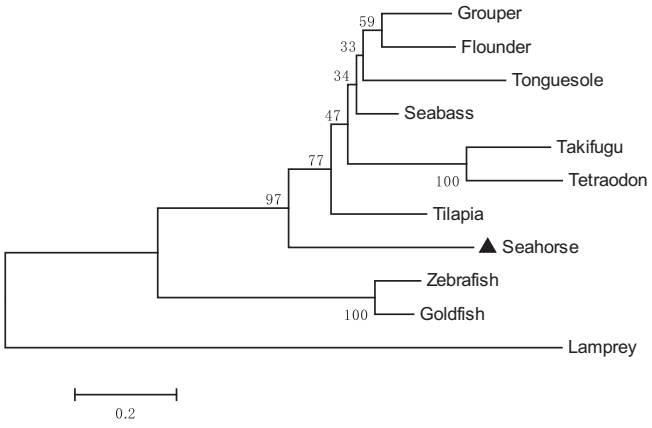

Fig. 3. Phylogenetic tree depicting evolutionary relationships between GnIH of lined seahorses and other vertebrates. The phylogenetic tree was constructed with MEGA6.0 software using the neighbor-joining method with 1000 bootstrap replicates. The tree was constructed from the deduced amino acid sequences of vertebrate GnIH listed in Table 2. Numbers at nodes indicate the bootstrap value (%).

3.2. Phylogeny and syntenic analysis of seahorse GnIH

A phylogenetic tree of seahorse GnIH and other previously identified GnIH precursors was constructed by the neighbor-joining method (Fig. 3). This tree demonstrated that seahorse GnIH clustered together with other teleost GnIH precursors. Synteny analysis of GnIH showed that the GnIH gene was typically positioned between the gene loci of *nfe2l3* and *cycs* (Fig. 4). The GnIH position is conserved among vertebrates, including teleosts and mammals. Interestingly, the organization of genes upstream of GnIH in zebrafish is similar to other teleost fishes, while the organization of downstream genes is comparable to humans.

3.3. Tissue distribution of seahorse GnIH

Using gene-specific primers designed from the cloned sequence, real-time PCR analysis was performed to examine the expression pattern of GnIH in various adult male and female seahorse tissues. As shown in Fig. 5, seahorse GnIH mRNA was mostly expressed in brain regions, with particularly high expression in the hypothalamus, with some expression in peripheral tissues, including the heart and muscle. Interestingly, GnIH mRNA was highly expressed in the brood pouch of male seahorses and minimally expressed in the gonadal tissues (testis and ovary).

3.4. Expression profile of GnIH during different sexual development stages

Real-time PCR was employed to quantify GnIH expression in the brain of male seahorses during different sexual development stages. As shown in Fig. 6, GnIH mRNA expression in the brain was significantly reduced during the early pubertal stage ($P = 0.0265$). However, the expression level gradually increased and returned to those seen during the juvenile stage.

3.5. Expression profile of GnIH during different pregnancy stages

To predict the role of GnIH in regulating male pregnancy in seahorses, the presence of GnIH was examined in the brain of male seahorses during different pregnancy stages by real-time PCR. As shown in Fig. 7, GnIH mRNA expression was increased significantly during the pregnancy stage (P stage) as compared to the non-pregnancy stages, including the PreP and PostP stages ($P = 0.0367$).

4. Discussion

In this study, the GnIH precursor gene was characterized and functionally evaluated in the lined seahorse. This is the first description of GnIH in an ovoviviparous fish, although it has been characterized in many types of oviparous fish species. The deduced amino acid sequence of the seahorse GnIH precursor shared low identity with other teleost fishes (35.4–65.8%). However, the core putative LPXRFamide peptides and GR cleavage site were conserved. The C-terminal of seahorse LPXRFa-1 is unique (MPQRF) among vertebrate LPXRFa-1 orthologs (MRMRF or LPLRF). Additionally, the C-terminal of seahorse LPXRFa-2 is unique (SPQRF), while the sequence of LPXRFa-2 in other vertebrates is MPQRF or LPQRF. Thus, the evolutionary rate was very rapid in seahorses. Moreover, at the genome level, the evolutionary rates of proteins and nucleotides in the seahorse were the fastest among teleost fish species with sequenced genomes (Lin et al., 2016).

The synteny blocks of GnIH in different taxa of vertebrates are conserved. It has been reported that the GnIH precursor gene is near the *cycs* loci (Zhang et al., 2010). This conserved synteny was also found in the genome of the primitive vertebrate lamprey (Osugi et al., 2012). Phylogenetic analysis also showed that the seahorse GnIH precursor grouped with other teleost GnIH precursors. High bootstrap values supported this phylogenetic position. Based on these results, the seahorse GnIH precursor is presumed to be an ortholog of the LPXRFa

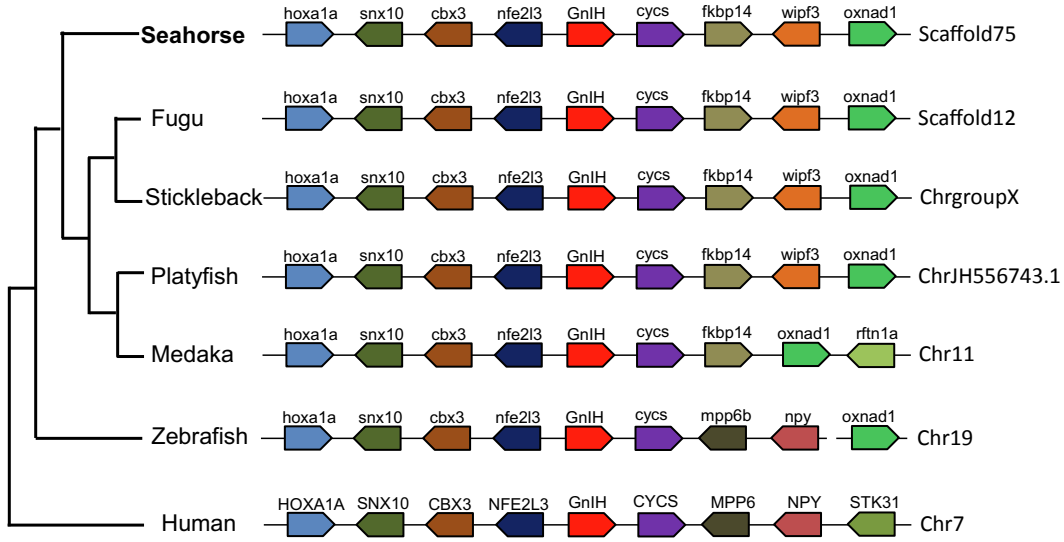

Fig. 4. Conserved synteny for the genomic region comprising the GnIH precursor gene. Gene loci organizations in the genomic region containing the GnIH gene were obtained from the Ensembl Genome Browser (http://www.ensembl.org).

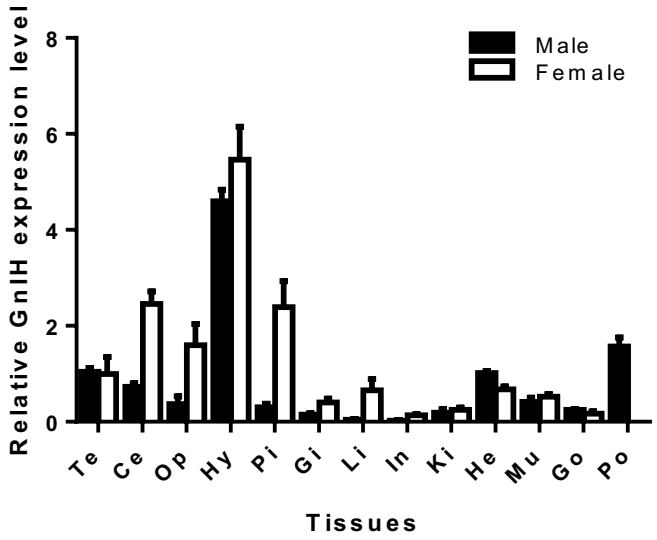

Fig. 5. Relative mRNA expression levels of the GnIH precursor gene in various tissues and brain regions of adult male and female seahorses. Te, telencephalon; Ce, cerebellum; Op, optic tectum-thalamus; Hy, hypothalamus; Pi, pituitary; Gi, gill; Li, liver; In, intestine; Ki, kidney; He, heart; Mu, muscle; Go, gonad; Po, pouch. The mRNA expression levels were determined by real-time PCR, normalized against the β-actin transcript, and presented as mean ± SEM.

peptide gene in vertebrates.

Tissue distribution analysis revealed that the seahorse GnIH precursor gene is expressed in the hypothalamus but not in the pituitary. Similar findings were observed in other teleost fishes, including zebrafish (Zhang et al., 2010), goldfish (Sawada et al., 2002), tongue sole (Wang et al., 2018), and grouper (Wang et al., 2015). The expression level of GnIH in seahorses was detected in several peripheral tissues to some extent, such as the heart and muscle. High expression of GnIH in these peripheral tissues was also reported in tongue sole (Wang et al., 2018) and zebrafish (Zhang et al., 2010). Interestingly, GnIH precursor mRNA was highly expressed in the brood pouch, which is a seahorse sexual tissue. The brood pouch can protect embryos and provide oxygen and nutrients (Scobell and MacKenzie, 2011). This suggests that GnIH plays a role in regulating reproductive behaviors in the seahorse.

In this study, we observed that mRNA expression levels of seahorse GnIH in the brain varied during different reproductive stages. The GnIH mRNA expression level was reduced significantly during the early puberty stage compared to the juvenile stage and mature stage. This result indicated that GnIH may be involved in the regulation of reproductive function during early puberty and sexual differentiation. In contrast, the mRNA expression of kiss2 in the lined seahorse was increased significantly during the early puberty stage (Zhang et al., 2018). This result indicates that kisspeptin and GnIH play inverse roles in the lined seahorse and suggests that seahorse GnIH may play a negative role in the regulation of reproductive physiology and behavior.

Our study showed that GnIH mRNA expression in the brain of pregnant seahorses was significantly higher than non-pregnant seahorses, including the pre-pregnancy and post-pregnancy stages. This indicates that GnIH is involved in regulating pregnancy in the lined seahorse. In vertebrates, including mammals, birds, and teleost fishes, GnIH plays a negative role in regulating gonadotropin secretion and testosterone release (Ubuka et al., 2006; Tsutsui et al., 2010). In our previous study, kisspeptin was found to play a positive role in regulating gonadotropin secretion and promote testosterone release (Zhang et al., 2018). In male Syngnathid fishes, the period of androgen synthesis was prolonged during spermatocyte proliferation and brood pouch development, but suppressed during the pregnancy stage (Mayer et al., 2011). Up-regulation of GnIH and down-regulation of *kiss2* mRNA were consistent with the androgen level during the pregnancy stage. Considering that GnIH plays a negative role in the regulation of gonadotropin secretion, seahorse GnIH and kiss2 may co-regulate gonadotropin secretion and decrease plasma testosterone levels during the pregnancy stage.

In conclusion, we cloned the GnIH precursor cDNA sequence from the lined seahorse and investigated its expression profiles in various tissues and different pregnancy stages. The putative LPXRFa peptides and cleavage sites were conserved among vertebrates, while the sequences of seahorse LPXRFa-1 and LPXRFa-2 were found to be unique. Tissue distribution analysis showed that the GnIH precursor transcript was highly expressed in the hypothalamus and brood pouch. Additionally, GnIH mRNA in the brain increased significantly during the pregnancy stage. Our results suggest that GnIH plays a negative role in regulating gonadotropin secretion and male pregnancy.

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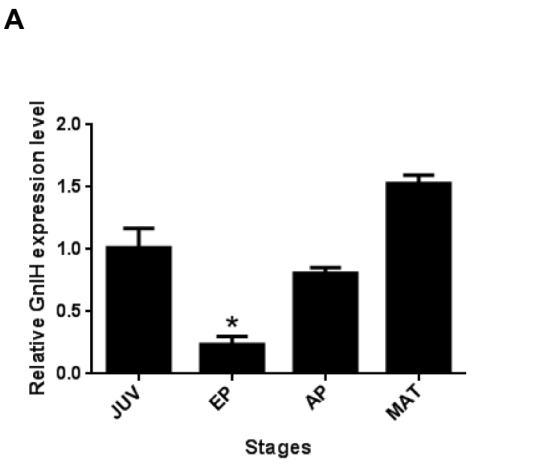

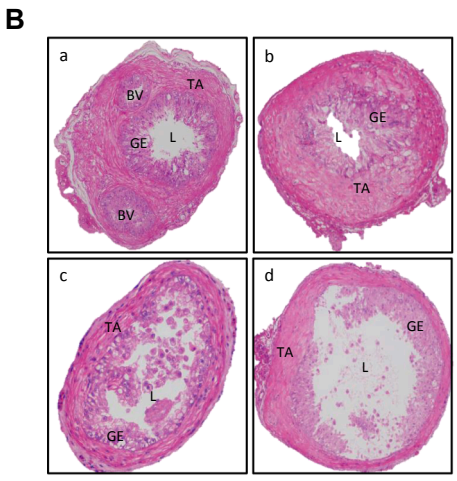

Fig. 6. Expression profile of GnIH in the brain of male seahorses (n = 8) during different reproductive stages. A. The mRNA expression levels identified by real-time PCR normalized against β-actin transcript and presented as mean ± SEM. Asterisks denote significant differences between different stages ($P < 0.05$). B. Histology of gonads at different reproductive stages in male seahorses. a. Juvenile (JUV), continuous gonadal epithelium (GE) within the blood vessel (BV) surrounded by tunica albuginea (TA) and no cells in the lumen (L); b. Early Puberty (EP), continuous gonadal epithelium containing spermatogonia and primary spermatocytes; c. Advanced Puberty (AP), discontinuous gonadal epithelium with some spermatogonia and several free spermatids in the lumen; d. Mature (MAT), discontinuous gonadal epithelium with many mature spermatozoa secreted into the tubule lumen.

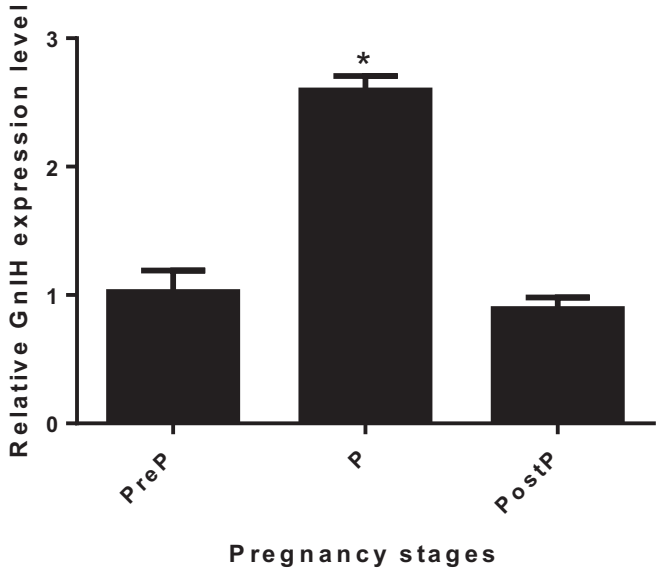

Fig. 7. Expression profile of the GnIH precursor gene in the brain of male lined seahorses (n = 8) during different pregnancy stages. PreP, pre-pregnant stage; P, pregnant stage; PostP, post-parturition stage. mRNA expression levels were determined by real-time PCR, normalized against the β-actin transcript, and presented as mean ± SEM. Asterisks denote significant differences between different stages ($P < 0.05$).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.04.018.

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