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In vitro effects of tongue sole LPXRFa and kisspeptin on relative abundance of pituitary hormone mRNA and inhibitory action of LPXRFa on kisspeptin activation in the PKC pathway

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ABSTRACT

Results of previous studies indicated the existence of LPXRFa, the piscine ortholog of gonadotropin-inhibitory hormone (GnIH), and kisspeptin (Kiss2) in tongue sole (*Cynoglossus semilaevis*), and that LPXRFa exerts an inhibitory effect on Kiss2 activation in the protein kinase A (PKA) pathway. The functions in the control of reproduction and whether LPXRFa antagonizes the action of Kiss2 by inhibiting the protein kinase C (PKC) pathway, however, are still unknown. In the present study, there was an initial investigation of the direct effects of LPXRFa and Kiss2 on relative abundance of pituitary hormone mRNA transcripts using a whole pituitary culture system. Results indicated that LPXRFa-1 specifically functioned to increase relative abundance of *lhβ* mRNA when there were comparisons with the control, without any effect on relative abundance of *gh*, *gthα* and *fshβ* mRNA. Treatment with LPXRFa-2 resulted in a reduction in relative abundance of *gthα* and *lhβ* mRNA, and did not alter relative abundance of *fshβ* mRNA. Treatment of LPXRFa-2 resulted in a greater relative abundance of *gh* mRNA. Treatment with Kiss2, however, resulted in an increase in relative abundance of *gthα* and *fshβ* mRNA transcripts, without altering relative abundances of *gh* and *lhβ* mRNA. Subsequently, there was valuation of the potential interaction between LPXRFa and kisspeptin in COS-7 cells transfected with the cognate receptors. Both LPXRFa-1 and LPXRFa-2 suppressed serum responsive element-dependent luciferase (SRE-luc) activity when compared to stimulation with Kiss2 alone, indicating an inhibitory effect of LPXRFa on kisspeptin activation on the PKC pathway. Overall, data from the present study provide novel evidence for differential actions of LPXRFa and kisspeptin on pituitary hormone synthesis as well as for the interaction between LPXRFa and kisspeptin systems in teleosts.

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1. Introduction

Gonadotropin-inhibitory hormone (GnIH) was originally discovered as a novel hypothalamic neuropeptide inhibiting gonadotropin release in quail (Tsutsui et al., 2000). Subsequently, GnIH homologs have been detected to be present in the brain of a variety of vertebrates (Munoz-Cueto et al., 2017; Ubuka et al., 2016). Depending on species, GnIH precursor polypeptide is possibly cleaved into two to four mature peptides which possess a characteristic -LPXRFa (X = L or Q) motif at the C-terminus and are designated as LPXRFa peptides (Tsutsui et al., 2018). Accumulating evidence suggests LPXRFa has important functions in regulating reproduction in birds and mammals through its inhibitory actions on release of gonadotropin-releasing hormone (GnRH) and gonadotropins (GTHs) from the hypothalamus and anterior pituitary, respectively. The precise physiological role of LPXRFa in the hypothalamic–pituitary–gonadal (HPG) axis is still not known for fish (Munoz-Cueto et al., 2017; Ubuka et al., 2016). For example, treatment with goldfish LPXRFa peptides (gfLPXRFa-1, -2 and -3) resulted in stimulation of the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from cultured pituitary cells of sockeye salmon (Amano et al., 2006). Intraperitoneal injection of zebrafish LPXRFa-3 reduced the plasma LH concentrations in goldfish (Zhang et al., 2010). Interestingly, gfLPXRFa-3 exerted both stimulatory and inhibitory effects on pituitary LH release and relative abundance of gonadotropin subunit mRNA depending on maturational status of the fish and administration route of gfLPXRFa-3 (Moussavi et al., 2012, 2013). Considering these previous research findings, the functional role of LPXRFa in reproduction remains unclear in fish. The inconsistent actions of LPXRFa in various studies could occur because of differences among studies in species where assessments have occurred, sex of experimental animals, reproductive stage of experimental animals, type of peptides used in the research, dose of the peptides, route of administration of the peptides and/or the elapsed time after treatment before responses to treatments were evaluated (Munoz-Cueto et al., 2017).

Kisspeptin (Kiss) is also recognized as a key regulator in the development and upregulation of the reproductive system in mammals (Tsutsui, 2009; Tsutsui et al., 2010). There have been different findings with regard to presence of *kiss*1 and *kiss*2 genes in different fish species. Only the *kiss2* gene is present in some species. In contrast, both *kiss1* and *kiss2* genes have been characterized in some other species (Mechaly et al., 2013; Pasquier et al., 2014; Tena-Sempere et al., 2012). It is noteworthy that the functional significance of the kisspeptin system in teleost reproduction remains unclear. Treatments with goldfish Kiss1 resulted in a greater relative abundance of *lhβ* mRNA *in vitro* (Yang et al., 2010), and treatment with zebrafish Kiss2, but not Kiss1, resulted in a greater relative abundance of *lhβ* and *fshβ in vivo* (Kitahashi et al., 2009). In contrast, there was a specific reduction in relative abundance of *lhβ* mRNA transcripts induced by Kiss1 and Kiss2 in primary cultures of European eel pituitary cells (Pasquier et al., 2018). Interestingly, treatment with neither Kiss1 nor Kiss2 affected the relative abundances of *lhβ* and *fshβ* mRNA transcripts of sea bass pituitary cells (Espigares et al., 2015). When results of these previous studies are considered, the mode of action of Kiss1 and Kiss2 on the control of reproduction may be divergent among fish species.

In a preliminary study, the cDNA sequence of LPXRFa encoding two putative LPXRFa peptides was obtained from tongue sole, and the impact of LPXRFa on the central control of reproduction at the hypothalamus was evaluated (Liu et al., 2017; Wang et al., 2018a). The tongue sole LPXRFa receptor (LPXRFa-R) has been characterized and the molecular mechanisms of LPXRFa actions were investigated using a mammalian cell line combined with a pharmacological approach (Wang et al., 2018b). Likewise, there has been identification of a functional Kiss2/Kiss2 receptor (Kiss2R) system in the same species and there was examination of the functions of Kiss2 in the central control of reproduction (Wang et al., 2017a,b). The functional role of tongue sole LPXRFa and kisspeptin in the hypophysiotropic functions, however, is still unclear. In addition, there are results that indicate Kiss2 functions in both the PKA and PKC pathways to exert activations of biochemical processes, and LPXRFa had an inhibitory effect on Kiss2 activation in the PKA pathway (Wang et al., 2017c). Because LPXRFa can also function in activation of the PKC pathway (Wang et al., 2018b), further investigation is warranted to clarify whether a synergistic functions can be detected for LPXRFa and kisspeptin combined *in vitro* or whether LPXRFa interferes with these functions in the PKC pathway when there is activity induction by kisspeptin and *vice versa*. The aims of the present study, therefore, were (1) to investigate the direct effects of LPXRFa and kisspeptin in regulating the synthesis of pituitary gonadotropin and growth hormone, and (2) to further examine the possible interaction between the LPXRFa and kisspeptin cell regulatory systems.

2. Materials and methods

2.1. Animals

All of the animal experiments were approved by the Animal Care and Use Committee of the Chinese Academy of Fishery Sciences. Approximately 2-year-old female tongue sole were purchased from a local fishery in Qingdao, China. The fish were reared in an indoor concrete tank with recirculating seawater at room temperature with imposition of a cyclical light–dark photoperiod (12 h:12 h). The fish were fed to satiation twice daily with a commercially available dry diet (Shengsuo Aquafeed Co., Ltd., Yantai, China) as described in detail previously (Wang et al., 2017a).

2.2. Reagents

Synthetic peptides corresponding to tongue sole Kiss2 decapeptide (FNFNPFGLRF-NH2) (Wang et al., 2017b), LPXRFa-1 (SLDLERLNMRVTPTASKSSLPTIIKLYPPTVNPHIHANMPMRF-NH2), and LPXRFa-2 (EVEPEDDQSHNTPNMPQRF-NH2) (Wang et al., 2018a) were provided by ChinaPeptides Co., Ltd. (Shanghai, China) with a purity of 99.72%, 95.68% and 98.28%, respectively, as

determined by HPLC. All of the three peptides were dissolved with distilled water and aliquots of concentrated stock solutions were stored at −80 °C until use. These stock peptides were previously used for pharmacological analysis and there was a biological potency with *in vitro* conditions (Liu et al., 2017; Wang et al., 2017a,c, 2018b).

2.3. Whole pituitary culture

The whole pituitary culture experiments were performed as previously described (Shahjahan et al., 2011; Wang et al., 2016, 2014) with some modifications. Briefly, the fish (BW = 749.2 ± 14.1 g and GSI = 0.95 ± 0.14%, n = 72) were anesthetized with 0.05% MS222 (Sigma) and sacrificed *via* decapitation. Subsequently, pituitaries were immediately harvested and placed in ice-cold culture medium (Leibovitz's L-15 medium (Gibco) supplemented with 1.19 g/L HEPES, 2.66 g/L sodium chloride, 1 g/L bovine serum albumin, 100,000 U/L penicillin, and 100 mg/L streptomycin, pH 7.2). Pituitaries were washed twice with ice-cold L-15 medium and then transferred to 24-well culture plates individually. After pre-incubation at 25 °C for 1 h, the medium was aspirated and replaced with fresh medium containing various concentrations (0.1, 10 and 1000 nM) of Kiss2 and LPXRFa peptides, respectively (n = 6 pituitaries per concentration of the respective test substances). Control wells were treated with similar dilutions of distilled water (n = 6). Following incubation at 25 °C for 24 h, pituitaries were immediately immersed in RNAiso Plus reagent (Takara) and stored at −80 °C until RNA extraction.

2.4. RNA extraction, reverse-transcription, and real-time quantitative PCR

All experiments were performed as described in detail previously (Wang et al., 2017a, b). Briefly, total RNA was extracted using the RNAiso Plus reagent according to the manufacturer's instructions. The purity and yield of RNA were assessed by a NanoDrop 2000C spectrophotometer (Thermo Scientific) with 260:280 ratios between 1.8 and 2.0. The integrity of RNA was determined using a 1% agarose electrophoresis gel with ethidium bromide staining, which indicated that RNA was not degraded and, therefore, the RNA was used for subsequent cDNA synthesis. The first strand cDNA was synthesized using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Code No. RR047A) as follows: 1 μg of total RNA was incubated with gDNA Eraser at 42 °C for 2 min to eliminate contaminating genomic DNA. Reverse transcription (RT) was performed in a volume of 20 μL of the reaction mixture containing 10 μL of RNA solution, 1 μL of PrimeScript RT Enzyme Mix I (including RNase inhibitor), 1 μL of RT Primer Mix (Oligo dT primer and random hexamers), 4 μL of 5 × PrimeScript Buffer, and 4 μL of RNase-free water. Reverse transcription was performed for 15 min at 37 °C, followed by 85 °C for 5 s, and the RT products were stored at −20 °C.

Relative abundances of mRNA transcripts were determined with real-time quantitative PCR and the primers used in this study are listed in Table 1. A total of 20 μL of the PCR reaction volume contained 10 μL of 2 × SYBR® *Premix Ex Taq II* (Takara, Code No. RR820A), 0.8 μL of forward and reverse primers (10 μM each), 2 μL of diluted cDNA templates and 7.2 μL of RNase-free water. The amplification of samples was carried out with the Mastercycler® ep *realplex* Real-time PCR System (Eppendorf) using the following thermal cycling profiles: 95 °C for 30 s, and 40 cycles of 95 °C for 5 s and 60 °C for 20 s. Each sample was analyzed in duplicate. At the end of the amplification, a melting curve analysis was performed to confirm the presence of a single PCR product. The 18S gene was used as the internal reference and remained stable during the present study. Standard curves were generated for each gene with 10-fold serial dilutions of cDNA. Efficiencies and R^2 for standard curve are shown in Table 1. Relative abundances of mRNA transcripts were normalized to the abundance of 18S and were quantified using the comparative Ct method.

2.5. Cell culture, transfection, and signal transduction analysis

To evaluate the possible interaction between LPXRFa and kisspeptin signaling involved in the PKC pathway, there was use of SRE-luc as a reporter gene for PKC activation. The entire open reading frames of tongue sole LPXRFa-R (Wang et al., 2018b) and Kiss2R (Wang et al., 2017a) cDNAs were subcloned into the *XbaI* and *HindIII* sites of pcDNA3.1 expression vector (Invitrogen) and all constructs were verified by sequencing. Cell culture and transfection experiments were generally performed as previously described (Wang et al., 2017c). In brief, COS-7 cells were seeded in 24-well tissue culture plates at a density of 1×10^5 cells/well in 1 mL of

Table 1
Information of primers used for real-time quantitative PCR.

Name	Primer sequence(5'-3')	Amplicon size(bp)	PCR efficiency	R^2	GenBank accession no.
gh-F	TTATAGACCAGCGGCGTTTC	179	94%	0.998	HQ334196
gh-R	ATGCTTGTTGTCGGGGATG				
gthα-F	TTCCCCACTCCTCTAACGACA	116	108%	0.993	JQ364953
gthα-R	ACCACAATACCAGCCACCACTAC				
lhβ-F	TCCACCTGACACTAACGCTG	191	90%	0.995	JQ277934
lhβ-R	GTTTGGTTCCTTTGTTCTGC				
fshβ-F	TGATGGGTGTCCAGAGGAAG	95	110%	0.988	JQ277933
fshβ-R	CAACAAACCGTCCACAGTCC				
18S-F	GGTCTGTGATGCCCTTAGATGTC	107	100%	0.988	GQ426786
18S-R	AGTGGGGTTCAGCGGGTTAC				

DMEM containing 10% FBS and 1% penicillin/streptomycin (Gibco) at 37 °C under an atmosphere of 5% CO2 for 20 h. To determine whether LPXRFa peptides are capable of activating Kiss2R through the SRE pathway and *vice versa*, 100 ng pcDNA3.1-Kiss2R or 300 ng pcDNA3.1-LPXRFa-R, 500 ng SRE-luc reporter plasmid, and 50 ng pRL-TK (for normalization of transfection efficiency) containing the *Renilla* luciferase reporter gene were co-transfected into the cells using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions. At 6 h post-transfection, culture medium was replaced and cells were treated with vehicle or a high dose (1 μM) of LPXRFa and Kiss2 for 24 h. In addition, to study the possible interaction of LPXRFa and Kiss2 signaling involved in the PKC pathway, cells were co-transfected with 500 ng SRE-luc reporter plasmid, 100 ng pcDNA3.1-Kiss2R, 300 ng pcDNA3.1-LPXRFa-R and 50 ng pRL-TK, and were treated for 24-h with a submaximal dose (100 nM) of Kiss2 (Wang et al., 2017c) and 1 μM of LPXRFa (Wang et al., 2018b) alone or stimulated simultaneously with Kiss2 and LPXRFa peptides. At the end of each incubation period, cells were harvested and luminescence was measured by Dual Luciferase Kit (Promega). Each experiment was repeated at least twice, and all treatments included six replicate wells in each experiment. A parallel control transfection experiment was performed with empty pcDNA3.1, SRE-luc and the internal control pRL-TK.

2.6. Statistical analysis

Pooled data are presented reported as the mean ± SEM and were analyzed using a one-way ANOVA followed by use of Duncan's multiple range test using SPSS17.0 software (Chicago, IL, USA). Normality and homoscedasticity assumptions were tested prior to the analysis, and the values were log- or square root-transformed when required. When data did not satisfy the requirements for conducting the parametric ANOVA, the data were analyzed using the nonparametric Kruskal-Wallis ANOVA on ranks followed by Bonferroni's test. Differences between groups with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of LPXRFa and Kiss2 peptides on pituitary function

Although there were no significant changes in *gh* (Fig. 1A1), *gthα* (Fig. 1B1) and *fshβ* (Fig. 1D1) relative abundances of mRNA in response to various doses of LPXRFa-1, relative abundance of *lhβ* mRNA transcripts was greater as a result of treatment with 0.1 nM of LPXRFa-1 (Fig. 1C1). Furthermore, treatment with 1000 nM of LPXRFa-2 resulted in a markedly greater relative abundance of *gh* mRNA (Fig. 1A2), while relative abundances of both *gthα* (Fig. 1B2) and *lhβ* (Fig. 1C2) were less as a result of treatment with 0.1 nM of LPXRFa-2. The relative abundances of *fshβ* transcripts were unaffected by the LPXRFa-2 treatment (Fig. 1D2). Treatment with 10 nM of Kiss2 resulted in a greater relative abundance of *gthα* and *fshβ* mRNAs (Fig. 1B3 and D3), without affecting the relative abundances of *gh* and *lhβ* mRNA (Fig. 1A3 and C3).

3.2. LPXRFa reduces Kiss2-induced PKC pathway

No response in SRE-luc activity was observed when cells transfected with empty pcDNA3.1 were stimulated with a large dose (1 μM) of Kiss2 or LPXRFa peptides (Fig. 2A), indicating that COS-7 cells do not naturally express endogenous kisspeptin and LPXRFa receptor genes. Subsequently there was determination of whether LPXRFa could induce activation of Kiss2R through the PKC pathway and *vice versa*. As depicted in Fig. 2B, there was greater SRE-luc activity when cells transfected with LPXRFa-R were treated with 1 μM of LPXRFa peptides, while there was no activation of LPXRFa-R as a result of treatment with 1 μM of Kiss2. Similarly, treatment with Kiss2 markedly stimulated SRE-luc activity in COS-7 cells expressing the cognate receptor, whereas treatment with neither LPXRFa-1 nor LPXRFa-2 affected the SRE-luc activity compared to the control values (Fig. 2C). Thus, it is clear that each peptide functions *via* its respective receptor.

There was subsequent determination of the possible interaction between LPXRFa system and kisspeptin system. As depicted in Fig. 3A, treatment with LPXRFa-1 or Kiss2 alone resulted in stimulation of SRE-luc activity in COS-7 cells transfected with the cognate receptors. There, however, was no synergistic activity of these two peptides combined and co-stimulation with both ligands resulted in a reduction of SRE-luc activity when compared to stimulation with Kiss2 alone (Fig. 3A). Similarly, treatment with LPXRFa-2 counteracted the stimulatory effect of Kiss2 on SRE-luc activity (Fig. 3B).

4. Discussion

Both LPXRFa and kisspeptin are members of the RFamide peptide family, and functional properties of these two peptides in various species indicate that LPXRFa and kisspeptin function as key neuropeptides controlling reproductive activity (Ohga et al., 2018; Pasquier et al., 2014; Tsutsui et al., 2018). In the present study, there was investigation of the functional effects of tongue sole LPXRFa and kisspeptin on relative abundance of pituitary mRNA transcripts. This transcript abundance was assumed to be an indicator of rates of gene expression *in vitro*. The hypothesis, therefore, was that LPXRFa interferes with the PKC pathway induced by kisspeptin in COS-7 cells transfected with the cognate receptors.

Although orthologous LPXRFa peptides have been detected in a variety of teleosts, the physiological effects on reproduction and other physiological processes have only been investigated in a few fish species, and functional diversity was observed compared to mammals and birds (Munoz-Cueto et al., 2017; Ogawa and Parhar, 2014; Ubuka et al., 2016). In the current study, treatment with tongue sole LPXRFa-1 specifically resulted in greater relative abundances of *lhβ* mRNA, while treatment with LPXRFa-2 resulted in

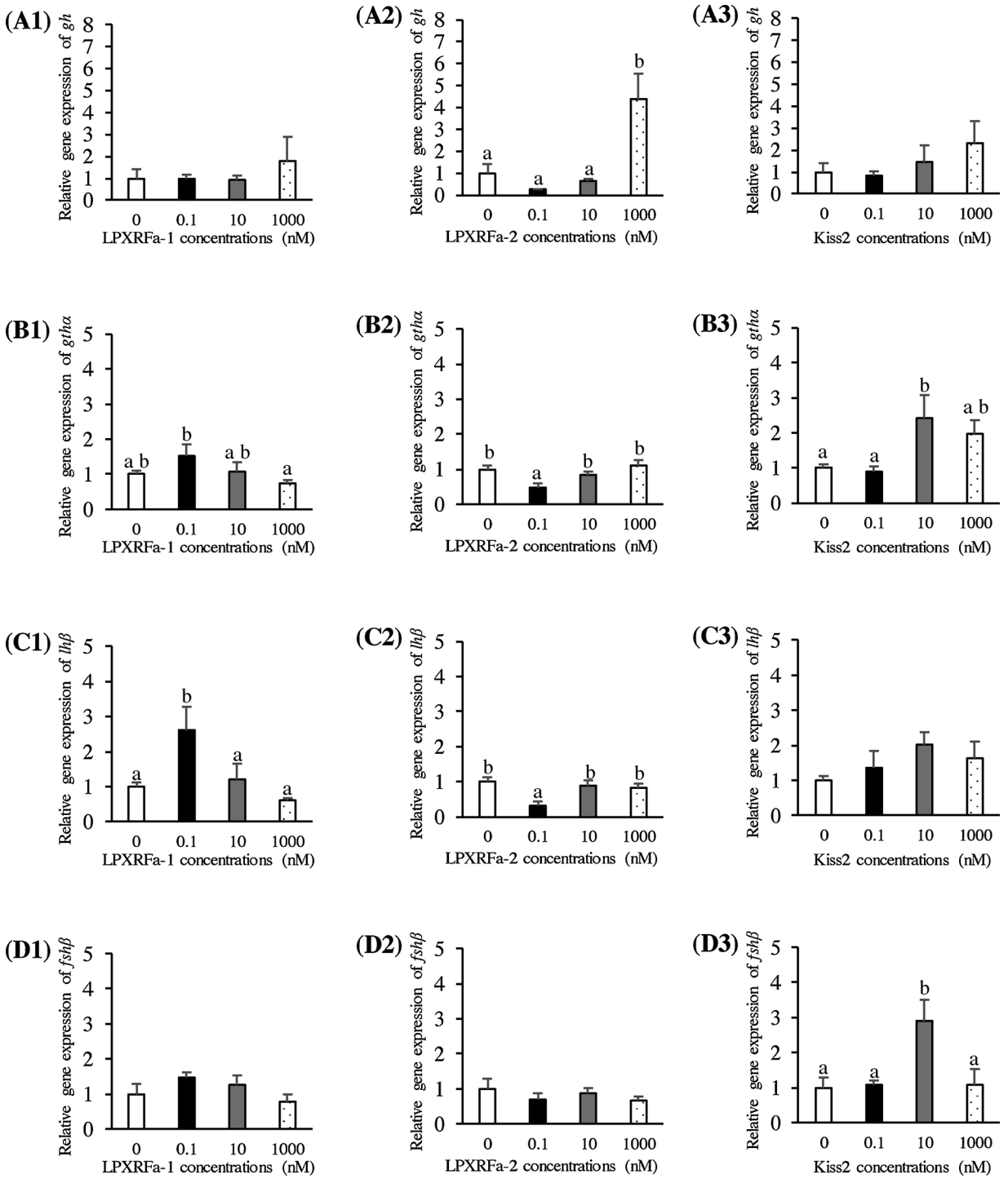

Fig. 1. *In vitro* effects of tongue sole LPXRFa (A1-D2) and kisspeptin (A3-D3) peptides on relative abundances of pituitary *gh* (A1-A3), *gthα* (B1-B3), *lhβ* (C1-C3) and *fshβ* (D1-D3) mRNA transcripts; Pituitaries were treated with various concentrations (0.1, 10 and 1000 nM) of tongue sole LPXRFa-1, LPXRFa-2 and Kiss2 peptides for 24 h and then collected for the analysis of relative abundances of *gh* and *gth* subunit mRNA transcripts using real-time quantitative PCR; Data were normalized using the abundance of 18S RNA in the same sample and are presented as the mean ± SEM ($n = 6$ pituitaries/concentration/test substances); Groups with different letters are different from each other ($P < 0.05$; ANOVA followed by use of the Duncan's multiple range test).

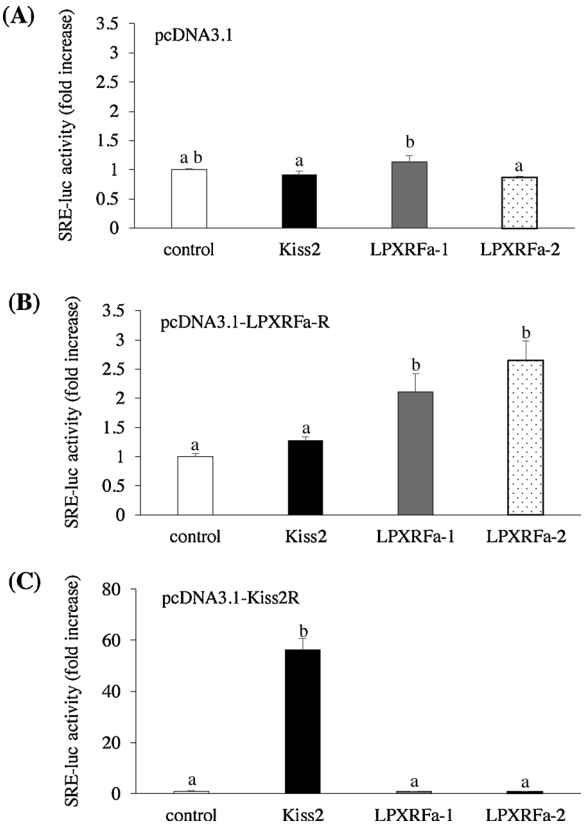

Fig. 2. LPXRFa and kisspeptin peptides function *via* their own receptors, respectively. (A) SRE-luc activity in COS-7 cells transfected with empty pcDNA3.1 expression vector and stimulated with 1 μM of Kiss2 or LPXRFa peptides. (B) SRE-luc activity in COS-7 cells transfected with pcDNA3.1-LPXRFa-R expression vector and stimulated with 1 μM of Kiss2 or LPXRFa peptides; (C) SRE-luc activity in COS-7 cells transfected with pcDNA3.1-Kiss2R expression vector and stimulated with 1 μM of Kiss2 or LPXRFa peptides; Data are presented as the mean ± SEM (n = 12 wells/treatment). Groups with different letters are different from each other ($P < 0.05$; Use of ANOVA followed by use of the Duncan's multiple range test).

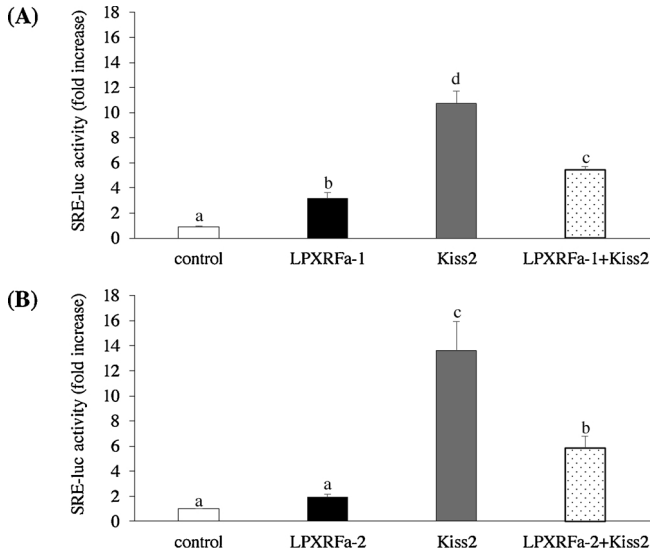

Fig. 3. LPXRFa can interfere with kisspeptin-induced SRE-luc activity; Analysis of SRE-luc activity in COS-7 cells transfected with both pcDNA3.1-Kiss2R and pcDNA3.1-LPXRFa-R expression vectors and stimulated with 100 nM of Kiss2 and 1 μM of LPXRFa peptides alone or co-treatment with both of these two peptides; Data are presented as the mean ± SEM (n = 12–18 wells/treatment); Groups with different letters are different from each other ($P < 0.05$; Use of ANOVA followed by use of the Duncan's multiple range test).

lesser relative abundances of *gthα* and *lhβ* mRNAs, indicating there were opposing effects of treatments with the two LPXRFa peptides on gonadotropin gene expression in the same species. Interestingly, there were opposite effects of what occurred with LH subunits in the present study as there was when there was treatment with the two LPXRFa peptides and there was quantification of FSH secretion in the cichlid fish *Cichlasoma dimerus* (Di Yorio et al., 2016). Furthermore, there are several other examples of differential regulation of gonadotropin gene expression by LPXRFa. *In vitro* treatment with gfLPXRFa-1 resulted in greater relative abundances of *lhβ* and *fshβ* mRNA transcripts in the grass puffer pituitary without affecting abundance of *gthα* transcripts (Shahjahan et al., 2011). Nevertheless, in zebrafish, treatment with LPXRFa-3 resulted in lesser relative abundances of *lhβ* and *gthα* mRNA transcripts *in vitro*, although there was no effect of this treatment on relative abundance of *fshβ* mRNA transcript (Spicer et al., 2017). Results of another *in vitro* study performed in goldfish indicated that treatment with neither gfLPXRFa-2 nor gfLPXRFa-3 affected relative abundances of *lhβ* and *fshβ* mRNA transcripts (Qi et al., 2013). Intraperitoneal injection of gfLPXRFa-2 and gfLPXRFa-3, however, resulted in a lesser relative abundance of *fshβ* mRNA, while treatment with only gfLPXRFa-2 resulted in a lesser relative abundance of *lhβ* (Qi et al., 2013). Similarly, inhibitory effects of LPXRFa peptides on abundances of *lhβ* and/or *fshβ* transcripts have been reported in other studies performed in orange-spotted grouper (Wang et al., 2015), common carp (Peng et al., 2016), sea bass (Paullada-Salmeron et al., 2016a, b), and Senegalese sole (Aliaga-Guerrero et al., 2018). Notably, there are opposing actions of gfLPXRFa-3 on relative abundance of gonadotropin subunits in goldfish depending on maturational status and administration route (Moussavi et al., 2012).

In addition to its effects on gonadotropins, LPXRFa affects the regulation of synthesis of growth hormone in fish. Treatment with tongue sole LPXRFa-2 resulted in a greater relative abundance of *gh* mRNA in the present study. Likewise, treatment with gfLPXRFa-1 resulted in a greater relative abundance of *gh* mRNA transcript in the grass puffer (Shahjahan et al., 2016). Furthermore, treatment with gfLPXRFa-3 resulted in greater relative abundances of goldfish pituitary *gh* mRNA at all three gonadal recrudescence stages examined *in vivo* and during early gonadal recrudescence *in vitro*. During mid- and late gonadal recrudescence, however, treatment with gfLPXRFa-3 resulted in a lesser relative abundance of *gh* mRNA *in vitro* (Moussavi et al., 2014). Intra-cerebroventricular injection of endogenous LPXRFa-2 also resulted in a lesser relative abundance of *gh* mRNA in male sea bass (Paullada-Salmeron et al., 2016b). Intramuscular injection of Senegalese sole LPXRFa-2 or LPXRFa-3, however, did not affect abundance of *gh* mRNA in sexually maturing sole males (Aliaga-Guerrero et al., 2018).When the present and previous results are considered, these LPXRFa peptides appear to regulate pituitary function in all vertebrates, but the hypophysiotropic functions are divergent among various species.

In the present study, treatment with tongue sole Kiss2 evidently induced an increase in relative abundances of *gthα* and *fshβ* mRNA transcript, without affecting relative abundance of *lhβ* mRNA transcript. Similarly, treatment of grouper with Kiss2 resulted in greater relative abundances of *fshβ* mRNA *in vivo* (Shi et al., 2010). Results of another two *in vivo* studies performed in zebrafish and chub mackerel indicated that treatment with Kiss2 resulted in increased relative abundances of both *lhβ* and *fshβ* mRNA (Kitahashi et al., 2009; Ohga et al., 2014). There was no effect of the Kiss2 treatment on relative abundances of *lhβ* and *fshβ* in yellowtail kingfish (Nocillado et al., 2013) and sea bass (Espigares et al., 2015). Treatment with Kiss1 and Kiss2 markedly suppressed the relative abundance of *lhβ* mRNA transcripts in primary cultures of European eel pituitary cells, without having any effect on relative abundance of *fshβ* mRNA (Pasquier et al., 2018), confirming the previous data with heterologous kisspeptins in the same species (Pasquier et al., 2011). Results of another study, in the striped bass, indicated that only Kiss1 had an inhibitory effect on *lhβ* gene expression, while both Kiss1 and Kiss2 upregulated *fshβ* gene expression (Zmora et al., 2015). With regard to *gh* gene expression, there were no significant changes after treatment with Kiss2 in tongue sole. Similarly, treatment with neither zebrafish Kiss1 nor Kiss2 affected the relative abundance of *gh* mRNA *in vivo* (Kitahashi et al., 2009). *In vitro* treatments with human/lamprey Kiss1 did not affect relative abundance of *gh* mRNA in the European eel (Pasquier et al., 2011). Treatment with Kiss1 resulted in greater *gh* gene expression in goldfish (Yang et al., 2010). Taken together these results indicate that kisspeptin can function at the pituitary in teleosts and have disparate results that could stem from differences in species, reproductive stages and administration routes of peptides. Furthermore, the two forms of kisspeptins may have different biological functions in fish species. It should be noted that studies of zebrafish mutant lines for the *kiss/kissr* genes have revealed that the reproductive capability was not impaired in knockouts of both sexes, suggesting that the Kiss/KissR systems are dispensable for reproduction in certain non-mammalian vertebrates (Tang et al., 2015). Thus, further investigation into the comparative physiology of kisspeptins and the relevant receptors will be necessary to understand the functionality of these paralog genes (Felip et al., 2015).

Multiple signals may underlie the functional diversity of the LPXRFa and kisspeptin systems, and the mechanisms through which LPXRFa and kisspeptin exert functions have been elucidated in several fish species (Munoz-Cueto et al., 2017; Ohga et al., 2018; Pasquier et al., 2014). Studies on the potential interaction of LPXRFa with kisspeptin signaling, however, are few in teleosts and even in other vertebrates. Tongue sole Kiss2 functions through the PKA and PKC pathways to exert its actions *via* its cognate receptor, and LPXRFa-2 could antagonize the action of Kiss2 by inhibiting PKA pathway activity (Wang et al., 2017c). In addition, tongue sole LPXRFa peptides can also activate the cognate receptors *via* the PKC pathway (Wang et al., 2018b). It, therefore, is important to investigate whether there is a synergistic activity for LPXRFa and kisspeptin combined *in vitro* or whether LPXRFa functions to interfere with functionality of the PKC pathway that is induced by kisspeptin and *vice versa*. In the current study, tongue sole LPXRFa-1 and LPXRFa-2 reduced Kiss2-induced SRE-luc activity, indicating that LPXRFa may also antagonize the action of Kiss2 by inhibiting the activity of the PKC pathway. Similarly, LPXRFa-2 and LPXRFa-3 suppressed Kiss2 activation of the PKC pathway in zebrafish (Spicer et al., 2017). In addition, LPXRFa-2, but not LPXRFa-3, also inhibited Kiss1 activation of the PKC pathway (Spicer et al., 2017). Mouse LPXRFa, however, had no inhibitory effect on kisspeptin activation of the PKC pathway in a mouse GnRH neuronal cell line (GT1-7) when there was overexpression of the cognate receptors (Son et al., 2016). Collectively, these results indicate differential involvement of signaling pathways in the action of LPXRFa and kisspeptin, and multiple signals may contribute to the functional divergence of these two peptides.

In summary, combined with results from previous studies, those of the present study provide further evidence that LPXRFa and

kisspeptin can exert direct effects on the reproductive axis by functioning not only at the hypothalamus (Liu et al., 2017; Wang et al., 2017a) but also at the pituitary. Furthermore, signal-transduction analysis revealed that LPXRFa may exert its effects *via* reducing the PKC activation of Kiss2R by Kiss2 in addition to inhibition of Kiss2-induced PKA pathway (Wang et al., 2017c). Nevertheless, it must be taken into account that the intricate pathways of intracellular signal transductions in response to LPXRFa and kisspeptin are still far from being fully understood in teleosts. Further studies, therefore, are warranted to investigate whether other signaling pathways, such as those for Ca^{2+} and MAPK, mediate the actions of fish LPXRFa or kisspeptin through similar mechanisms as occurs in mammals (Castano et al., 2009; Ubuka et al., 2013).

Conflict of interest

The authors declare no conflict of interest.

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