Signaling pathways activated by sea bass gonadotropin-inhibitory hormone peptides in COS-7 cells transfected with their cognate receptor

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Results of previous studies provided evidence for the existence of a functional gonadotropin-inhibitory hormone (GnIH) system in the European sea bass, Dicentrarchus labrax, which exerted an inhibitory action on the brain-pituitary-gonadal axis of this species. Herein, we further elucidated the intracellular signaling pathways mediating in sea bass GnIH actions and the potential interactions with sea bass kisspeptin (Kiss) signaling. Although GnIH1 and GnIH2 had no effect on basal CRE-luc activity, they significantly decreased forskolin-elicited CRE-luc activity in COS-7 cells transfected with their cognate receptor. Moreover, an evident increase in SRE-luc activity was noticed when COS-7 cells expressing GnIHR were challenged with both GnIH peptides, and this stimulatory action was significantly reduced by two inhibitors of the PKC pathway. Notably, GnIH2 antagonized Kiss2-evoked CRE-luc activity in COS-7 cells expressing GnIHR and Kiss2 receptor (Kiss2R). However, GnIH peptides did not alter NFAT-RE-luc activity and ERK phosphorylation levels. These data indicate that sea bass GniHR signals can be transduced through the PKA and PKC pathways, and GniH can interfere with kisspeptin actions by reducing its signaling. Our results provide additional evidence for the understanding of signaling pathways activated by GniH peptides in teleosts, and represent a starting point for the study of interactions with multiple neuroendocrine factors on cell signaling.

KEYWORDS
GnIH, GnIH receptor, kisspeptin, kisspeptin receptor, signaling pathway
Introduction

Since the first discovery of gonadotropin-inhibitory hormone (GnIH) in the quail, the presence of GnIH orthologs has been reported in a variety of vertebrate species, including fish (1, 2). Phylogenetic, synteny and functional analysis revealed that the GnIH and NPFF genes, both of which belong to the family of the RFamide peptides, may have diverged from a common ancestral gene by whole-genome duplication during vertebrate evolution (2, 3). Two paralogous G protein-coupled receptors (GPCRs), GPR147 and GPR74, have been identified as the common receptors for GnIH (GnIHRs) and NPFF (NPFFRs) (2). However, GPR147 is regarded as the primary receptor for GnIH based on the higher binding affinity of GnIH to GPR147 compared to GPR74 (4, 5). In turn, the NPFF precursor encodes NPFF and NPAF mature peptides, and these two peptides preferentially activate GPR74 (2). Multiple lines of evidence indicated that GnIH not only suppresses reproduction in vertebrates through its inhibitory actions on the brain-pituitary-gonadal axis, but also participates in stress response, feeding and reproductive behaviors (1, 2, 6). Despite its involvement in the pituitary-gonadal axis, but also participates in stress response, GnIH has a significant effect on the brain-pituitary-gonadal axis.

Materials and methods

Peptides

Synthetic peptides (23, 24, 27) corresponding to European sea bass GnIH1 (PLHLHANMPMRF-NH$_2$), GnIH2 (SPNSTPNMPQRF-NH$_2$), NPFF (NSVLHQPPQR-NH$_2$), NPAF (DWEAAPQIJWSMAYQPQR-NH$_2$), Kiss1 ([pGLU]
DVSSYNLSFGLRY-NH₂ and Kiss2 (SKFNFNPGLRF-NH₂) were purchased from ChinaPeptides Co., Ltd. (Shanghai, China) with a purity of 98.09%, 96.18%, 96.18%, 96.54%, 96.10% and 96.12%, respectively, as determined by HPLC. All peptides were amidated at the C-termini, and Kiss1 contained a pyroglutamylated N-terminus. These neuropeptides were prepared with distilled water and aliquots were stored at -20°C.

Plasmids

Both CRE-luc and SRE-luc plasmids (BD Biosciences Clontech, CA, USA) contained the firefly luciferase gene and have been validated in a previous study (25). The NFAT-RE-luc plasmid also included the firefly luciferase gene and was purchased from Promega (Madison, WI, USA). The pRL-TK plasmid, which constitutively expresses the Renilla reniformis luciferase gene, was provided by Promega and used for normalization of the transfection efficiency. The entire open reading frames of sea bass *gnihr* (GPR147-type), *kiss1r* and *kiss2r* genes were obtained by PCR amplification using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and the specific primers (Table 1), and then subcloned into the HindIII and EcoRI sites of the expression vector pCDNA3.1/Zeo(+) (Invitrogen, Waltham, MA, USA), respectively. All receptor constructs (pCDNA3.1-GnIHR, pCDNA3.1-Kiss1R and pCDNA3.1-Kiss2R) were extracted with Endo-Free Plasmid DNA Mini Kit (Omega Bio-tek, Norcross, GA, USA) and verified by sequencing.

Reagents for cell culture, transfection and signaling pathways

COS-7 cells (ATCC, Manassas, VA, USA), Dulbecco’s Modified Eagle Medium (DMEM) containing high glucose (4.5 g/L, Gibco, Waltham, MA, USA), fetal bovine serum (FBS, Gibco), 100×penicillin/streptomycin antibiotics (Gibco), OptiMEM (Gibco), Lipofectamine 3000 (Invitrogen), 5×Passive Lysis Buffer (Promega), Dual-Glo® Luciferase Assay System (Promega), forskolin (FSK, Calbiochem), and GF109203X (Calbiochem) were purchased from Promega (Madison, WI, USA) and veriﬁed. Fetal bovine serum (FBS, Gibco), 100×penicillin/streptomycin antibiotics (Gibco), Opti-MEM (Gibco), Lipofectamine 3000 (Invitrogen), 5×Passive Lysis Buffer (Promega), Dual-Glo® Luciferase Assay System (Promega), forskolin (FSK, Calbiochem), and GF109203X (Calbiochem) were purchased from Promega (Madison, WI, USA) and veriﬁed. Fetal bovine serum (FBS, Gibco), 100×penicillin/streptomycin antibiotics (Gibco), Opti-MEM (Gibco), Lipofectamine 3000 (Invitrogen), 5×Passive Lysis Buffer (Promega), Dual-Glo® Luciferase Assay System (Promega), forskolin (FSK, Calbiochem), and GF109203X (Calbiochem) were purchased from Promega (Madison, WI, USA) and veriﬁed.

All experimental protocols were followed as described previously (15, 29) with some modiﬁcations. One day before transfection, COS-7 cells were seeded in 24-well plates at a density of 1×10⁵ cells/well/mL of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and maintained in a humidified 5% CO₂ atmosphere at 37°C. For each well, cells were co-transfected with CRE-luc/SRE-luc/NFAT-RE-luc (200 ng), pCDNA3.1-GnIHR (200 ng), and pRL-TK (20 ng) using Lipofectamine 3000 in 500 μL Opti-MEM. After starvation overnight, (1) cells were then treated with GnIH peptides (10, 100, 1000 nM), NPFF (1000 nM), and NPAF (1000 nM) for 6 h; (2) cells were challenged for 6 h with 10 μM FSK alone or co-treated with 1000 nM GnIH1, GnIH2, NPFF, and NPAF; (3) cells were incubated for 6 h with 1000 nM GnIH peptides alone or in the presence of U73122 (phospholipase C [PLC] inhibitor, 10 μM) and GF109203X (PKC inhibitor, 10 μM). Finally, cells were harvested using 1×Passive Lysis Buffer (100 μL/well) and luminescence was determined with Dual-Glo® Luciferase Assay System on the LB963 luminometer (Berthold Technologies GmbH & Co.KG, Bad Wildbad, Germany). Luciferase activity values were calculated by dividing the firefly luciferase units by the Renilla luciferase values for each sample. The values obtained for the controls were set as 1 for each experiment, and the experimental values which were divided by those of the controls are presented as fold increase. Each transfection experiment was performed in triplicate and repeated at least twice. A parallel control transfection experiment was performed with the empty pCDNA3.1 vector, CRE-luc, SRE-luc, or NFAT-RE-luc and the internal reference pRL-TK.

In addition, we further evaluated the possible interactions between sea bass GnIH and kisspeptin signaling involved in the PKA pathway. First, to determine if GnIH peptides are capable of activating Kiss1R and Kiss2R through the CRE-luc pathway and vice versa, cells were co-transfected with pCDNA3.1-GnIHR/pcDNA3.1-Kiss1R/pcDNA3.1-Kiss2R (200 ng/well), CRE-luc (200 ng/well), and pRL-TK (20 ng/well). After

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**TABLE 1 Primer list for construction of pCDNA3.1-receptors.**

| Gene   | Primer sequence (5'→3')                                                                 | GenBank accession no. |
|--------|----------------------------------------------------------------------------------------|-----------------------|
| gnihr  | Forward: CCCAACGCTATGAGGTACTAGACAAAAC, Reverse: CGGAATTCCTAGTATTCCCGCCGGCTG            | LN681206              |
| kiss1r | Forward: CCCAACGCTATGAGGTACTAGACAAAAC, Reverse: CGGAATTCCTAGTATTCCCGCCGGCTG            | JN202446              |
| kiss2r | Forward: CCCAACGCTATGAGGTACTAGACAAAAC, Reverse: CGGAATTCCTAGTATTCCCGCCGGCTG            | JN202447              |
starvation overnight, cells were treated with GnIH and kisspeptin peptides (1 μM) for 6 h, and luciferase activity in cell extracts was measured. Second, to obtain the potential interactions among GnIHR, Kiss1R and Kiss2R signaling, cells were co-transfected with pcDNA3.1-GnIHR, pcDNA3.1-Kiss1R/polDNA3.1-Kiss2R, CRE-luc, and pRL-TK, challenged with GnIH and kisspeptin alone or a combination of the two peptides for 6 h, and then harvested for assays.

Western blot analysis

Whether the ERK pathway is activated by GnIH peptides was investigated by Western blot analysis (30). As mentioned above, COS-7 cells were seeded in 24-well plates (2×10^5 cells/well of DMEM), transfected with pcDNA3.1-GnIHR (200 ng/well), starved overnight, and then challenged with 1 μM GnIH1, GnIH2, NPFF, and NPAF for 10 min. The dose and treatment time were chosen based on previous reports (20, 31). Cells were harvested using 1× Cell Lysis Buffer (100 μL/well, Cell Signaling Technology, Danvers, MA, USA) supplemented with Pierce Protease and Phosphatase Inhibitor Mini Tablets (ThermoFisher Scientific, Waltham, MA, USA), and protein concentrations were measured with Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). Equal amounts of total proteins (14 μg/lane) were separated by 12% SDS-PAGE, and then electrotransferred onto nitrocellulose membranes, which was blocked with 5% bovine serum albumin in TBST at room temperature for 1 h. The membranes were washed three times (10 min each time) with TBST and incubated with Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (1:1000, Cell Signaling Technology) overnight at 4°C. After another three washes, the membranes were incubated with HRP-linked anti-rabbit IgG antibody (1:2000, Cell Signaling Technology) at room temperature for 1 h, washed, and visualized with Pierce™ ECL Plus Western Blotting Substrate (ThermoFisher Scientific). The protein bands were quantified using a densitometry software (Bio-Rad, Hercules, CA, USA). Subsequently, the membranes were incubated with Restore™ Western Blot Stripping Buffer (ThermoFisher Scientific) and reused for another immunodetection with p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technology) to normalize the blots.

Statistical analysis

Data are presented as the mean ± SEM and were analyzed by one-way ANOVA followed by Duncan’s multiple range test using SPSS17.0 software. Normality and homoscedasticity assumptions were tested prior to the analysis. Differences were considered to be statistically significant when p < 0.05.

Results

Absence of sea bass GnIH receptor and NPFF receptor in COS-7 cells

As depicted in Figure 1A, there was no response in CRE-luc activity when COS-7 cells transfected with the empty expression vector pcDNA3.1 were challenged with 1 μM GnIH1, GnIH2, NPFF and NPAF peptides. Parallel treatment with 10 μM FSK acted as a positive control (Figure 1A). Similarly, neither SRE-luc activity nor NFAT-RE-luc activity were altered by the four peptides tested (1 μM, Figures 1B, C). These data indicated that COS-7 cells do not naturally express endogenous receptors for sea bass GnIH and NPFF peptides.

Coupling of sea bass GnIH receptor to G_i/o protein

As shown in Figure 2A, COS-7 cells transfected with sea bass GnIHR did not respond to GnIH1 and GnIH2 at doses ranging from 10 to 1000 nM in CRE-luc activity. As a comparative control, 1 μM NPFF and NPAF also did not modify CRE-luc activity (Figure 2A). However, these four peptides (1 μM) significantly reduced FSK-stimulated CRE-luc activity (Figure 2B), suggesting that sea bass GnIHR is coupled to G_i/o protein and can be activated by both GnIH and NPFF peptides.

Coupling of sea bass GnIH receptor to G_iaq protein

SRE-luc was employed as a reporter gene for activation of the PLC/PKC pathway. Both GnIH1 and GnIH2 increased SRE-luc activity in COS-7 cells transfected with sea bass GnIHR in a dose-dependent manner (Figure 3A). Similarly, a significant induction of SRE-luc activity was observed by 1 μM NPFF and NPAF (Figure 3A). These results indicated that sea bass GnIHR is coupled to G_iaq protein. To further confirm the involvement of the PLC/PKC pathway, two specific inhibitors (U73122 and GF109203X) were employed. As observed in Figure 3B, the stimulatory effects of GnIH peptides (1 μM) on SRE-luc activity were attenuated by 10 μM U73122 (PLC inhibitor) and totally abolished by 10 μM GF109203X (PKC inhibitor).

Absence of GnIH and NPFF effects on Ca^{2+} and ERK activation

NFAT-RE-luc was used to examine the possible participation of intracellular Ca^{2+} mobilization in activation of sea bass GnIHR. None of the peptides assayed (GnIH1, GnIH2,
NPFF, and NPAF) had any effect on NFAT-RE-luc activity (Figure 4A). On the other hand, ERK phosphorylation levels were also unaffected by these four peptides (1 μM), either (Figure 4B).

Activation of GnIH receptor reduces kisspeptin receptor signaling

Subsequently, we investigated the potential interactions between GnIH and kisspeptin on PKA pathway signaling. There was no response in CRE-luc activity when COS-7 cells expressing sea bass GnIHR were stimulated with 1 μM Kiss1 or Kiss2 (Figure 5A). Similarly, there was no activation of Kiss1R and Kiss2R after treatment with 1 μM GnIH1 and GnIH2 (Figures 5B, C). FSK (10 μM, Figure 5A), Kiss1 and Kiss2 (1 μM, Figures 5B, C) acted as positive controls. These results evidenced that each peptide functions via its own receptor.

Both Kiss1 and Kiss2 induced a significant increase in CRE-luc activity in COS-7 cells co-transfected with sea bass GnIHR and Kiss1R, while neither GnIH1 nor GnIH2 affected the stimulatory effects evoked by kisspeptin peptides (Figure 5D). Similar results were observed in COS-7 cells expressing sea bass GnIHR and Kiss2R as a result of treatment with Kiss1 alone as well as co-administration of Kiss1 and GnIH1/GnIH2 (Figure 5E). However, GnIH2 elicited a significant reduction of CRE-luc activity when co-administered with Kiss2 compared to the stimulation provoked by Kiss2 alone (Figure 5E). Although not significant, there was also a slight reduction of
CRE-luc activity when cells were co-treated with Kiss2 and GnIH1 (Figure 5E).

Discussion

So far, physiological functions of the GnIH/GnIHR system have been investigated in different vertebrate groups, including fish, but the intricate web of intracellular signaling pathways mediating GnIH actions is still far from being fully understood (1, 8, 32). Results of our previous studies have revealed the existence of a functional GnIH system in sea bass, and provided evidence for the inhibitory role of GnIH in the reproductive axis of male sea bass, by acting at the brain, pituitary and gonadal levels (33). In the current study, the potential involvement of the PKA, PKC, Ca²⁺, and ERK pathways in the actions of sea bass GnIH peptides was evaluated using COS-7 cells expressing their cognate receptor. Neither GnIH1 nor GnIH2 had effects on basal CRE-luc activity in COS-7 cells expressing sea bass GnIHR, but efficiently reduced FSK-induced CRE-luc activity. These data indicate that sea bass GnIHR couples to Gᵢ protein, which is consistent with previous studies in orange-spotted grouper (14), half-smooth tongue sole (15), and chicken (11). On the contrary, tilapia GnIHR (12), chub mackerel GnIHR (13), and zebrafish GnIHR2 and GnIHR3 (16) are coupled to Gₛ protein. Interestingly, a switch between Gᵢ and Gₛ proteins is observed for medaka GnIHR (17). Taken together, these results show that GnIHRs in various species seem to couple to different heterotrimeric G proteins, which may underlie the functional diversity of the GnIH system reported in fish. For example, tilapia GnIH2 positively regulated both Lh and Fsh release in vivo and in vitro (12), whereas sea bass GnIH1 and GnIH2 down-regulated plasma Lh levels in vivo (22). It is worth mentioning that NPFF and NPAF can also suppress FSK-stimulated CRE-luc activity in COS-7 cells expressing sea bass GnIHR, indicating that GnIHR is a candidate receptor for these two peptides (5). Further studies are being directed in the laboratory to investigate NPFFR (GPR74) signaling pathways and how they are regulated by NPFF, NPAF and GnIH peptides, in order to determine the potency of each peptide in eliciting their responses through both paralogous receptors (GPR147 and GPR74).

In this study, both GnIH1 and GnIH2 increased SRE-luc activity in COS-7 cells expressing sea bass GnIHR, indicating
that this receptor may couple to G\textsubscript{\alpha \text{q}} protein and convey its signaling via the PKC pathway, which is in line with previous reports in tilapia (12), and tongue sole (15). However, orange-spotted grouper GnIH1 reduced SRE-luc activity in COS-7 cells transfected with its cognate receptor (14). No response in SRE-luc activity was observed by any of the three GnIH peptides with any of the three GnIHRs identified in zebra fish (16). Moreover, the stimulatory effect of sea bass GnIH on SRE-luc activity was inhibited by the PLC inhibitor U73122 and specially by the PKC inhibitor GF109203X, as observed in tongue sole (15), further confirming the involvement of the PLC/PKC pathway in sea bass GnIH actions.

Very limited information is available with respect to Ca\textsuperscript{2+} and ERK pathways mediating GnIH actions on target cells. Neither sea bass GnIH1 nor GnIH2 altered NFAT-RE-luc activity and ERK phosphorylation levels in the present study. Likewise, the three mouse GnIH peptides tested had no direct inhibitory effect on basal or kisspeptin-induced NFAT-RE-luc activity and ERK phosphorylation levels in GT1-7 cells (10). In contrast, sheep GnIH3 potently reduced GnRH-stimulated mobilization of intracellular calcium and phosphorylation of ERK in pituitary gonadotropes (18, 19). Previous results showed that goldfish Kiss1 can directly stimulate Lh and GtH release from primary cultures of pituitary cells in a Ca\textsuperscript{2+}-dependent manner (34), and zebrafish Kiss2 can also enhance the ERK and Akt phosphorylation levels in the female pituitary explants in vitro (35). Considering the opposite actions of GnIH and kisspeptin on gonadotropin secretion in sea bass (22, 24), we hypothesize that GnIH could antagonize kisspeptin signaling involved in Ca\textsuperscript{2+} and ERK routes, which is a promising topic of future research not only in sea bass but also in other fish species.

As mentioned above, sea bass GnIHR is coupled to G\textsubscript{\alpha i} protein, while sea bass Kiss1R and Kiss2R are coupled to G\textsubscript{\alpha \text{q}} protein (25). This implies that activation of GnIHR could interfere with signaling of Kiss1R and Kiss2R in this species, as reported in half-smooth tongue sole, in which GnIH2 reduced Kiss2-elicited CRE-luc activity in a dose-dependent manner when COS-7 cells were co-transfected with half-smooth tongue sole GnIHR and Kiss2R and co-stimulated with both Kiss2 and GnIH2 (29). Indeed, in the present study, an
inhibitory action of sea bass GnIH2 on Kiss2-induced CRE-luc activity was observed in COS-7 cells expressing both GnIHR and Kiss2R, which is in accordance with the fact that GnIH2 and Kiss2 are more potent regulators in the control of sea bass reproduction than GnIH1 and Kiss1, respectively (22, 24). It should be noted that GnIH2 (but not GnIH1) inhibited the synthesis of Kiss1, Kiss1R, and notably Kiss2, in sea bass (22). Reasons for the lack of effects of GnIH peptides on Kiss1R signaling are not known, but could perhaps be due to a low ratio of GnIHR to Kiss1R (1:1), which may cause less responsiveness to the ligand. For instance, chicken GnIH inhibited GnRH receptor (GnRHR) signaling more effectively as the ratio of GnIHR to GnRHR increased (11). Thus, it seems necessary to further investigate the temporal expression patterns of gnihr, kiss1r and kiss2r mRNAs along the reproductive axis of sea bass during a reproductive cycle. Another possibility is that GnIH may exert more potent inhibitory actions partially through GPR74 which also couples to G protein (36). Further investigation is warranted to clarify whether a synergistic effect can be detected for GPR147 and GPR74 combined.

To the best of our knowledge, neuroanatomical co-localisation of GnIHR with Kiss1R or Kiss2R in the same cell has never been shown in sea bass or other fish species. However, the presence of GnIHR (12, 37, 38) and/or kisspeptin receptors (39–42) has been reported in the pituitary of several teleost species, including sea bass, suggesting that some endocrine cells of the adenohypophysis (e.g., gonadotropes, corticotropes, melanotropes) could exhibit both receptor types. Interestingly, the distribution of GnIH-immunoreactive fibres (21) overlaps with Kiss2 projections and Kiss1R- and Kiss2R-expressing cells (39) in many central areas of the sea bass, suggesting that GnIH and Kiss receptors could also co-localise in brain cells of this species. Therefore, future studies should also be directed to elucidate which pituitary and brain cells co-express GnIHR and Kiss1R/Kiss2R in sea bass.

In summary, we have investigated the possible signaling pathways involved in the actions of sea bass GnIH peptides, and revealed that sea bass GnIH signals can be transduced via both PKA and PKC pathways. In addition, our results support the consideration that sea bass GnIH can interfere with kisspeptin...
signaling involving the PKA pathway. The results obtained in the present study enlarge our knowledge on GnIH signaling pathways in teleosts and represent a starting point to further examine the interactions of GnIH with other neuroendocrine factors (e.g., GnRH, Npy, Spexin) on cell signaling.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

BW and JAM-C designed research. BW, JAP-S, and AV-C performed experiments. BW analyzed data and wrote the paper. AG and JAM-C edited the manuscript. AG provided some plasmids, and JAM-C provided funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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