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Gonadotropin Inhibitory Hormone Down-Regulates the Brain-Pituitary Reproductive Axis of Male European Sea Bass (*Dicentrarchus labrax*)[1]

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ABSTRACT

Gonadotropin-inhibitory hormone (GnIH) inhibits gonadotropin synthesis and release from the pituitary of birds and mammals. However, the physiological role of orthologous GnIH peptides on the reproductive axis of fish is still uncertain, and their actions on the main neuroendocrine systems controlling reproduction (i.e., GnRHs, kisspeptins) have received little attention. In a recent study performed in the European sea bass, we cloned a cDNA encoding a precursor polypeptide that contained C-terminal MPMRFamide (sbGnIH-1) and MPQRFamide (sbGnIH-2) peptide sequences, developed a specific antiserum against sbGnIH-2, and characterized its central and pituitary GnIH projections in this species. In this study, we analyzed the effects of intracerebroventricular injection of sbGnIH-1 and sbGnIH-2 on brain and pituitary expression of reproductive hormone genes (*gnrh1, gnrh2, gnrh3, kiss1, kiss2, gnih, lhbeta, fshbeta*), and their receptors (*gnrhr II-1a, gnrhr II-2b, kiss1r, kiss2r,* and *gnihr*) as well as on plasma Fsh and Lh levels. In addition, we determined the effects of GnIH on pituitary somatotropin (Gh) expression. The results obtained revealed the inhibitory role of sbGnIH-2 on brain *gnrh2, kiss1, kiss2, kiss1r, gnih,* and *gnihr* transcripts and on pituitary *fshbeta, lhbeta, gh,* and *gnrhr-II-1a* expression, whereas sbGnIH-1 only down-regulated brain *gnrh1* expression. However, at different doses, central administration of both sbGnIH-1 and sbGnIH-2 decreased Lh plasma levels. Our work represents the first study reporting the effects of centrally administered GnIH in fish and provides evidence of the differential actions of sbGnIH-1 and sbGnIH-2 on the reproductive axis of sea bass, the main inhibitory role being exerted by the sbGnIH-2 peptide.

fish, gonadotropin-releasing hormone (GnRH), gonadotropins, kisspeptins, LPXRFamide, perciforms, reproduction, sea bass

INTRODUCTION

As in other vertebrates, gonadotropin-releasing hormone (GnRH) constitutes the main neuroendocrine factor stimulating the secretion of gonadotropins in fish, its functional antagonist being represented by dopamine, which inhibits the secretion of these adenohypophyseal hormones and blocks the reproductive process [1, 2]. This dopaminergic inhibition has been demonstrated in representative species of some teleost orders such as Cypriniformes, Salmoniformes, Siluriformes, Cichliformes, and Mugiliformes (for a review, see [2]). However, no dopaminergic inhibition has been demonstrated at all in perciform species studied up to date, including the European sea bass (*Dicentrarchus labrax*) [3–6]. Whether neuroendocrine factors inhibiting reproduction are lacking in these teleosts or they remain to be identified should be deciphered.

Gonadotropin inhibitory hormone (GnIH) is a hypothalamic neuropeptide that belongs to the RFamide peptide family and was first discovered in birds [7]. In the past 15 yr, GnIH orthologs have been identified not only in other vertebrates, from lampreys to mammals, but also in protochordates [8–19]. These GnIH genes encode a precursor polypeptide that may produce two to four C-terminal LPXRFamide peptides [19]. Although most of the reported effects of these GnIH orthologs are related to reproduction (e.g., regulation of gonadotropin synthesis and release, reproductive development, seasonal reproduction, estrous/menstrual cycle, steroidogenesis and germ cell maturation, sex behavior), GnIH also appears to be involved in the regulation of feeding, growth, stress response, and cardiac contractile function [19]. These actions are mediated via G protein-coupled GnIH receptors (GnIHR), of which two different subtypes, GPR147 and GPR74, have been described up to date [20–22].

Following on from pioneer research in avian species [7, 23, 24], subsequent in vivo and in vitro studies performed in mammals demonstrated that GnIH could also inhibit the reproductive process in this group of vertebrates [25–28]. RFRP-3, a mammalian GnIH ortholog, reduces gonadotropin synthesis and release through its inhibitory actions on GnRH neurons and/or pituitary gonadotropes [26, 27, 29]. Furthermore, immunoreactive (ir) GnIH fibers were found in close proximity to GnRH cells in the hypothalamus of different mammalian species [25, 30, 31], and GnIH receptor mRNA or protein were present in GnRH neurons [28], suggesting that GnIH may indirectly regulate gonadotropin secretion from the pituitary via this neuropeptidergic system. In this sense, RFRP-

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3 peptide was also found to inhibit the synthesis and release of GnRH in pigs [32]. However, in the Siberian hamster, GnIH peptides (RPFRP-1 and RPFRP-3) inhibited or stimulated Lh release depending on the photoperiod regimes, suggesting that GnIH peptides are fine tuning Lh levels in an opposing fashion across the seasons [28].

In contrast to tetrapods, in which the inhibitory effect of GnIH on reproduction appears rather conserved, much more functional diversity was observed between fish species analyzed up to date. Goldfish GnIHs stimulated the release of Lh, Fsh, and Gh in sockeye salmon [33]. In tilapia, LPXRFa-2 peptide increased the release of Lh, Fsh, and Gh both in vivo and in vitro [15]. In addition, the goldfish LPXRFa-1 form increased the expression of gonadotropin mRNA from cultured pituitary of grass puffer [34]. However, intraperitoneal injection of the zebrafish LPXRFa-3 form decreased serum Lh levels in goldfish in vivo [35]. Additionally, the administration of goldfish LPXRFa-2 and LPXRF-3 peptides suppressed the *gnrh3*, *lhβ,* and *fshβ* mRNA levels in the same species [36].

The European sea bass is an important species for marine aquaculture in Europe and has also represented an interesting fish model for the study of environmental and neuroendocrine control of reproduction [5, 37–49]. Recently, we cloned a GnIH ortholog precursor containing two putative GnIH peptides (sbGnIH-1 and sbGnIH-2) in the European sea bass, analyzed its expression in central nervous system and peripheral tissues, and elucidated the immunohistochemical localization of GnIH cells and their projections in the brain and pituitary by using an antibody against the endogenous amino-acid sequence of a teleost GnIH peptide [17]. In this previous work, we reported the presence of GnIH fibers in neuroendocrine areas where GnRH and kisspeptin cells are found as well as in close proximity to Fsh, Lh, and Gh cells [17]. Therefore, in order to elucidate the functional role of GnIH in the regulation of the reproductive axis of the European sea bass, in the present study we investigated the in vivo effects of intracerebroventricular (icv) injection of sbGnIH-1 and sbGnIH-2 on brain and pituitary expression of reproductive hormones genes as well as the physiological action of both GnIHs on Fsh and Lh plasma levels in this species.

MATERIALS AND METHODS

Animals

Four-year-old male European sea bass, *D. labrax* (body length and weight of 48.65 ± 0.46 cm and 1636 ± 59 g, respectively) were obtained from CUPIMAR S.L (San Fernando, Spain), housed in the Laboratorio de Cultivos Marinos (University of Cádiz, Puerto Real, Spain, 36° 31′ 51.55″ N, 6° 12′ 38.78″ W), and maintained under natural conditions at a salinity of 39 parts per thousand. Fish were fed twice daily with commercial dry pellets using automatic feeders (1% body weight; L6 Obtibass Skretting España S.A, Burgos Spain). Spermiating animals were anesthetized at the end of November by immersion in MS-222 (100–200mg/L of sea water; Sigma, St. Louis, MO). All animals were treated in agreement with the European Union Regulation (EC. Directive 86/609/EEC) concerning the protection of experimental animals and in accordance with the Society for Study of Reproduction's specific guidelines and standards. Animal experimental protocols were approved by the Animal Care and Use Committee of the University of Cádiz. Measures were taken to avoid suffering of the animals.

Peptide Synthesis

The sbGnIH-1 (PLHLHANMPMRF-NH_2) and sbGnIH-2 (SPNSTPNMPQRF-NH_2) peptides (GenBank accession no. LN681205) were synthesized by Thermo Fisher Scientific GmbH (Ulm, Germany). Synthetic peptides were amidated at the C terminal end and purified by high-performance liquid chromatography (>95% purity). The peptides were dissolved in PBS 1× according to the manufacturer's instructions and stored at −20°C until use.

Administration Procedure

Peptides were icv administered to the fish according to the procedure described by Espigares et al. [49]. The sbGnIH-1 and sbGnIH-2 peptides were injected using a 10-μl Hamilton microsyringe fitted with a 26-gauge needle (Hamilton, Reno, NV) and driven by a micromanipulator. Each fish was anesthetized and immobilized with its dorsal side upward and a small hole was made in the midline, at the caudal apex of the pineal window, with a tiny drill bit. The needle was then immediately inserted into the drill hole to a depth of 14 mm, to dispense the peptide or the vehicle into the third ventricle. To achieve a suitable administration, the solution was injected slowly, and the needle was extracted after 10 sec. The adequate depth and position of the injection were determined in a preliminary test by injecting a blue dye into a practice subject and then dissecting the brain to observe its distribution pattern and the reliability and accuracy of the administration procedure.

Experimental Design and Sampling

The experiment was performed at the beginning of the reproductive period (end of November). For the analysis of sbGnIH-1 and sbGnIH-2 effects, 40 sexually mature 4-yr-old males were divided into two groups of 20 animals each. In turn, these animals were divided into four different groups, consisting of three experimental and one control group of five fish each. The experimental groups were administered sbGnIH-1 or sbGnIH-2 at 09:00 with doses of 1, 2, or 4 μg dissolved in 8 μl of vehicle (PBS) per fish, while the control group received 8 μl of vehicle solution alone per animal.

Based on preliminary time-course analysis, fish from each group ($n = 5$) were sampled 6 h postinjection (hpi). The fish were anesthetized by immersion in MS-222 and euthanized, and the brain and pituitary gland were quickly dissected, frozen in liquid nitrogen, and stored at −80°C for subsequent RNA extraction. Blood samples were withdrawn from the caudal vein (1 ml of blood), and the plasma obtained after centrifugation (3000 rpm, 15 min, 4°C) was stored at −80°C until use.

RNA Extraction and Reverse Transcription for Real-Time Quantitative PCR Analysis

Total RNA from sea bass brain and pituitary was extracted with TRIsure reagent (Bioline, London, United Kingdom), according to the manufacturer's protocol. Tissues were homogenized in a mixer mill MM400 (Retsch, Haan, Germany) using four to five stainless steel beads. Total RNA concentration was quantified on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Total RNA (1 μg) was retrotranscribed and DNA removed using a QuantiTec Reverse Transcription Kit (Qiagen, Hilden, Germany). Primers for the quantitative PCR assays and their amplicon sizes are shown in the Table 1. Real-time PCR analysis was performed in a PCR Bio-Rad CFX96 Touch detection system (Bio-Rad, Richmond, CA), using a SensiFAST SYBR No-ROX Kit (Bioline). PCR conditions were as follows: initial denaturation 2 min at 95°C and 40 cycles of 15 sec at 95°C and 25 sec at the optimal temperature for each primer pair (details provided in Table 1) for annealing-extension. Duplicates of each sample were analyzed in the same test. Standard curves were generated for each gene with 10-fold serial dilutions of cDNA, and all calibration curves exhibited slopes close to −3.32 and efficiencies around 100%. Melting curves were performed for each sample in order to confirm that a single product was amplified. The expression of the target genes was normalized against three different reference genes (*18s, L17,* and *ef1-alpha*). The relative expression of genes analyzed was calculated by the −ΔΔCt method [50].

Hormone Analysis

Plasmatic levels of Lh and Fsh were determined using two respective homologous enzyme-linked immunosorbent assays developed for sea bass [51, 52]. The sensitivity for the plasma measurements was 0.10 $ng \cdot ml^{-1}$ for Lh and 0.33 $ng \cdot ml^{-1}$ for Fsh.

Statistics

All results are presented as a mean ± the standard error of the mean (SEM). One-way ANOVA tests were used to compare gene expression mean values followed by Student-Newman-Keuls post hoc tests, using Statgraphic Plus 5.1 software (Statpoint Technologies, Warrenton, VA). Prior to analysis, data were checked for normality and homogeneity of variance, and the values were log- or square root-transformed when required. When data did not accomplish the requirements of the parametric ANOVA, they were analyzed using the nonparametric Kruskal-Wallis ANOVA on ranks followed by Bonferroni test.

TABLE 1. Primers used for quantitative real-time PCR.

Gene	GenBank accession no.	Sequences (5′ to 3′)[a]	Annealing temperature (°C)	Amplicon size (bp)
gnrh1	AF224279	F: GGTCCTATGGACTGAGTCCAGG R: TGATTCCTCTGCACAACCTAA	61	131
gnrh2	AF224281	F: GTGTGAGGCAGGAGAATGCA R: CTGGCTAAGGCATCCAGAATG	61	81
gnrh3	AF224280	F: TGTGGGAGAGCTAGAGGCAAC R: GTTTGGGCACTCGCCTCTT	60	81
gnrhr-II-2b	AJ606685	F: AGACTCTGAAGATGACGGTGGT R: AGTGAAGCGTCTCTTCTCATCC	60	250
gnrhr-II-1a	AJ419594	F: CTCTGGCTATCAATAAGGC R: CTCGGGATGGATGATGGT	60	125
kiss1	FJ008914	F: GCATCAATACTGGCATCAGCAAAGA R: TCAACCATTCTGACCTGGGAAACTT	63	94
kiss2	FJ008915	F: GGGAGGATTCCAGCCCGTGTTTCT R: GAGGCCGAACGGGTTGAAGTTGAA	61	104
kiss1r	JN202446	F: TGGTGGCTCTGTTCCTCATCT R: CGTAACTGCGTAGGCCAAAAG	63	78
kiss2r	JN202447	F: CGTCACAGTCTACCCCCTGAA R: CAGATGCTGACAATCATGGCTACT	63	69
lhβ	AF543315	F: TTGAGCTTCCTGACTGTCCA R: GCAGGCTCTCGAAGGTACAG	60	177
fshβ	AF543314	F: ACCAACATCAGCATCCAAGTG R: TTCTCTGTTCAGGCCTCTCATAGT	63	127
gh	X65716	F: GACAAGCACGAGACACAACG R: CTGTCAGGGAACATCTCTGC	60	206
sbgnih	LN681205	F: CCCACCACCAGCAAAATCAGCC R: TCCCAAGACCTTCCGAACCTC	61	176
sbgnihr	LN681208	F: GTACGGAAGCATCGGAGTCAAAC R: CCAGGACAGCATGAAAAGCAAAG	60	178
l17	AF139590	F: CAGGAGTGGGTGACATGGTC R: GACTTCCGCTGCCGTATCAC	60,5	97
18s	AY831388	F: TCAGACCCAAAACCCATGCG R: ACCCTGATTCCCCGTTACCC	60	182
elfα	AJ866727	F: CTGTGCTGATCGTTGCTGCTGGTGTT R: CGTGCTCGCGGGTCTGTCC	61	75

[a] Forward (F) and reverse (R) primers were obtained from Integrated DNA Techonologies.

Statistical significance was established as $P < 0.05$. All graphics were created using PRISM 6 software.

RESULTS

Effects of sbGnIH-1 and sbGnIH-2 Peptides on Brain Gene Expression

As a first step, we examined the effect of icv injection of sbGnIH-1 and sbGnIH-2 peptides (1, 2, and 4 μg doses per fish) on the expression levels of the three sea bass GnRH genes (*gnrh1, gnrh2,* and *gnrh3*) and the main GnRH receptor (*gnrhr-II-2b*) expressed in the brain (Fig. 1). The administration of sbGnIH-1 induced a significant decrease in brain *gnrh1* gene expression with the three doses tested when compared to the control group ($P < 0.0045$; Fig. 1A). However, no significant effect was observed on *gnrh2* and *gnrh3* expression (Fig. 1, B and C). Treatment with sbGnIH-2 significantly decreased the expression of *gnrh2* at all doses tested ($P < 0.0045$; Fig. 1F), but no differences versus the control levels were observed for both *gnrh1* and *gnrh3* (Fig. 1, E and G). Neither sbGnIH-1 nor sbGnIH-2 had significant effects on brain *gnrhr-II-2b* expression (Fig. 1, D and H).

In order to elucidate whether the kisspeptin system is a target for the central action of GnIH in sea bass, the mRNA levels of kiss1 and kiss2 were also analyzed. No variation was observed in *kiss1* and *kiss2* mRNA levels in fish injected with sbGnIH-1 (Fig. 2, A and B). Nevertheless, injection of sbGnIH-2 decreased *kiss1* mRNA level at a dose of 2 μg ($P < 0.0006$; Fig. 2E) and *kiss2* expression at doses of 2 and 4 μg ($P < 0.0345$; Fig. 2F). We also analyzed the action of sbGnIH-1 and sbGnIH-2 on the expression levels of the two sea bass kisspeptin receptor genes (*kiss1r and kiss2r*). No significant differences were observed in *kiss1r* and *kiss2r* expression in fish treated with sbGnIH-1 (Fig. 2, C and D). However, the administration of sbGnIH-2 resulted in a significant decrease of *kiss1r* expression at a dose of 2 μg ($P < 0.0345$), but had no effect on *kiss2r* transcript levels when compared to the control group (Fig. 2, G and H).

To investigate the autoregulation of the GnIH system, we also analyzed the brain expression levels of the *sbgnih* and sbGnIH receptor (*sbgnihr*) genes after icv injections of sbGnIH-1 and sbGnIH-2 (Fig. 3). Only the fish treated with sbGnIH-2 at doses of 1 and 2 μg showed a significant decrease in brain *sbgnih* ($P < 0.0003$) and *sbgnihr* ($P < 0.0042$) mRNA levels (Fig. 3, A–D).

Effects of sbGnIH-1 and sbGnIH-2 Peptides on Pituitary Gene Expression

Injection of sbGnIH-1 did not elicit significant changes in the expression of *lhβ, fshβ,* and *gh* (Fig. 4, A–C) nor in mRNA levels of the main GnRH receptor (*gnrhr-II-1a*) expressed in the pituitary (Fig. 4D). In contrast, the administration of sbGnIH-2 decreased the expression of *fshβ* ($P < 0.0017$) at doses of 2 and 4 μg (Fig. 4E), *lhβ* mRNA levels at all doses tested ($P < 0.0049$; Fig. 4F), and *gh* gene expression at the higher dose tested (4 μg, $P < 0.0088$; Fig. 4G). Moreover, sbGnIH-2 also significantly reduced *gnrhr-II-1a* transcript levels at doses of 2 and 4 μg ($P < 0.026$; Fig. 4H).

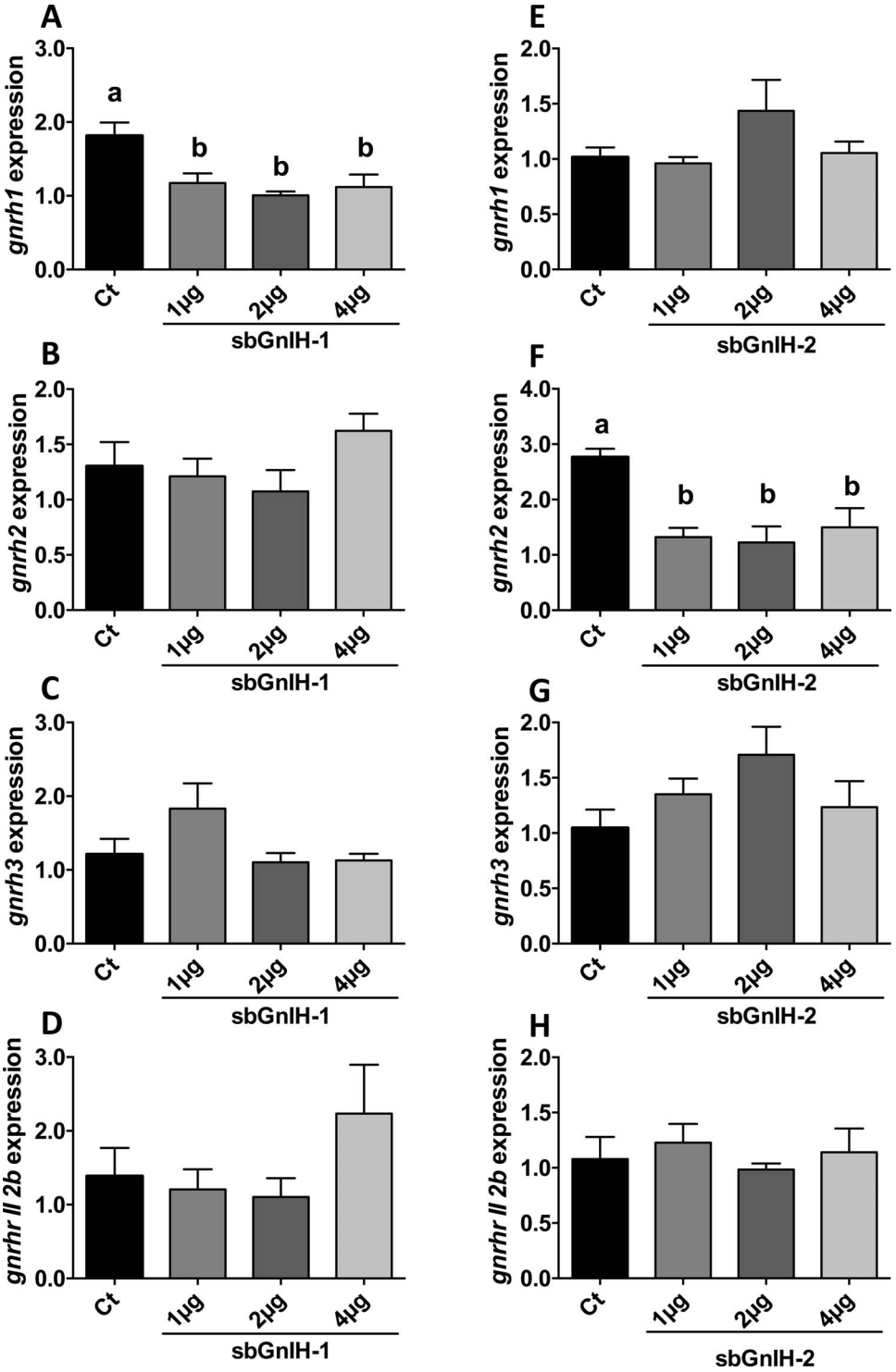

FIG. 1. Effect of in vivo intracerebroventricular (icv) injection of different doses (1, 2, and 4 µg) of sbGnIH-1 (**A**, **B**, **C**, **D**) and sbGnIH-2 (**E**, **F**, **G**, **H**) peptides on *gnrh1, gnrh2, gnrh3,* and *gnrhr-II-2b* relative expression at 6 hpi in the brain of male sea bass (Ct: control). Values are expressed as mean ± SEM (n = 5). Different lower case letters indicate significant differences between treatments (ANOVA, Student-Newman-Keuls test, $P < 0.05$).

Effects of sbGnIH-1 and sbGnIH-2 Peptides on Plasma Gonadotropin Levels

Finally, we analyzed the effects of icv injection of sbGnIH-1 and sbGnIH-2 on plasma Fsh and Lh levels. Neither of the two forms had a significant effect on plasma Fsh concentration at any of the doses tested (Fig. 5, A and C). A significant decrease in plasma Lh hormone levels was observed in animals injected with sbGnIH-1 at a dose of 4 µg ($P < 0.048$; Fig. 5B), while the effect of sbGnIH-2 in plasma Lh levels was evident at a dose of 1 µg ($P < 0.009$; Fig. 5D).

DISCUSSION

The pioneer studies performed in birds and subsequent work developed in mammals from the beginning of this

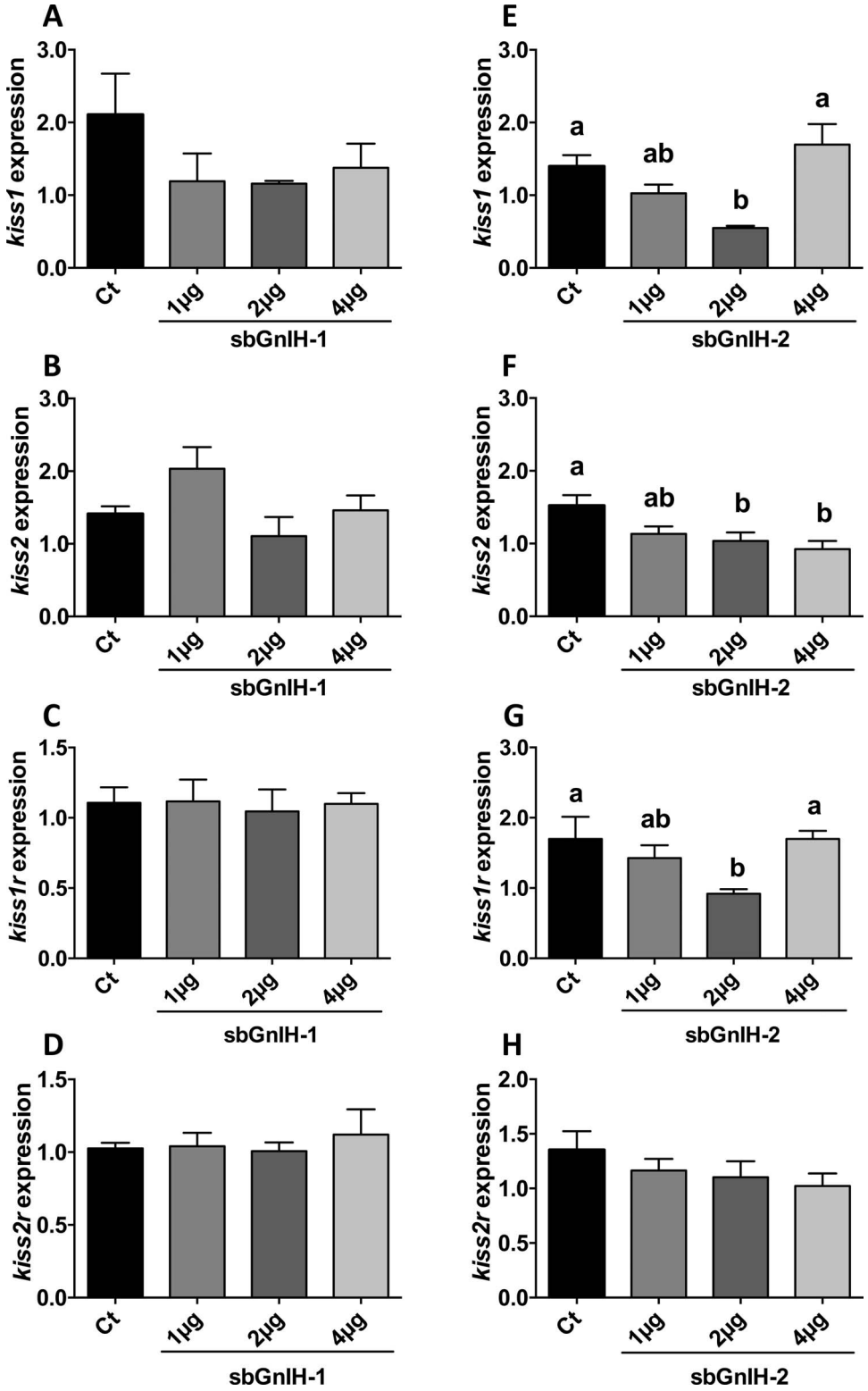

FIG. 2. Effect of in vivo icv injection of different doses (1, 2, and 4 µg) of sbGnIH-1 (**A**, **B**, **C**, **D**) and sbGnIH-2 (**E**, **F**, **G**, **H**) peptides on *kiss1, kiss2, kiss1r,* and *kiss2r* relative expression at 6 hpi in the brain of male sea bass (Ct: control). Values are expressed as mean ± SEM (n = 5). Different lower case letters indicate significant differences between treatments (ANOVA, Student-Newman-Keuls test, $P < 0.05$).

century have clearly established the role of GnIH in the reproductive axis of tetrapods through its inhibition of GnRH and gonadotropin secretion [7, 25, 29, 53, 54]. Until recently, this neurohormonal inhibitory role on fish reproduction was attributed to dopamine [1, 2, 55], but this dopaminergic inhibition does not seem to operate in some teleost groups such as perciforms [3–6].

From the discovery of GnIH, several GnIH orthologs have been identified in different teleost species, and their effects on the reproductive axis have been addressed by using pituitary

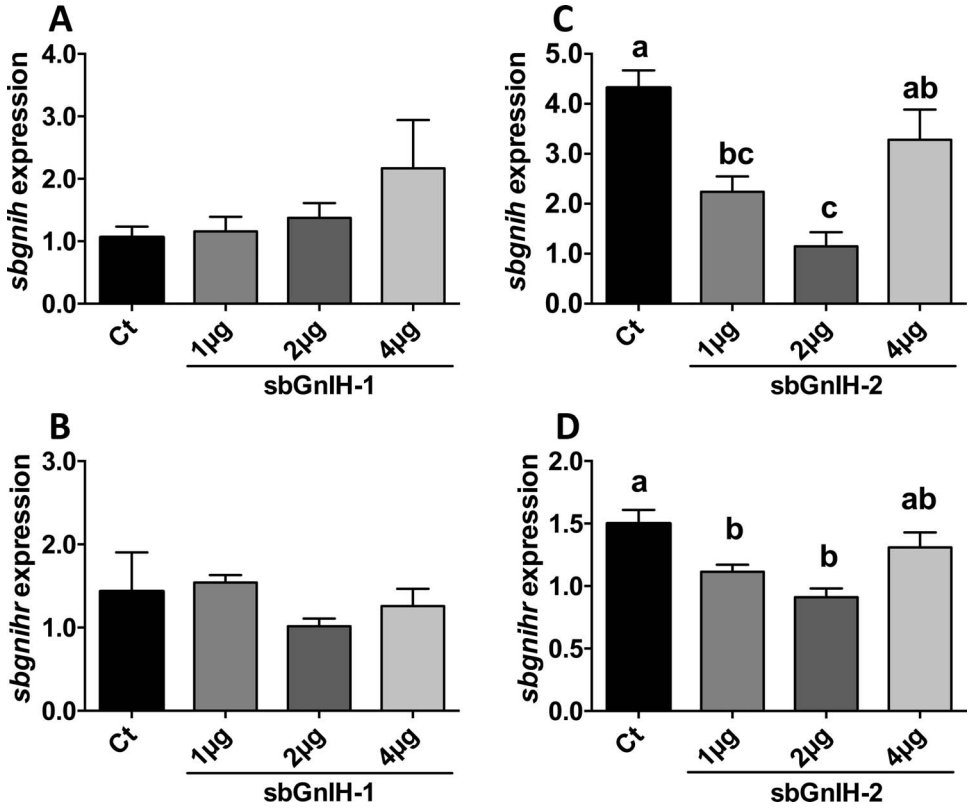

FIG. 3. Effect of in vivo icv injection of different doses (1, 2, and 4 µg) of sbGnIH-1 (**A**, **B**) and sbGnIH-2 (**C**, **D**) peptides on *sbgnih* and *sbgnihr* relative expression at 6 hpi in the brain of male sea bass (Ct: control). Values are expressed as mean ± SEM (n = 5). Different lower case letters indicate significant differences between treatments (ANOVA, Student-Newman-Keuls test, $P < 0.05$).

cell cultures and intraperitoneal injections [15, 16, 33, 35, 36, 56, 57]. To the best of our knowledge, this present paper represents the first study in which the effect of icv administration of GnIH has been reported in fish. Therefore, this work provides the first evidence of the inhibitory role of centrally administered GnIH in the reproductive axis of teleosts. Our findings revealed that icv administration of sbGnIH-1 and/or sbGnIH-2 altered the expression levels of *gnrh1, gnrh2, kiss1, kiss2,* and *kiss1r* in the brain as well as *lhβ, fshβ,* and *gnrhr-II-1a* mRNA levels in the pituitary, which results in decreased plasma levels of Lh, with sbGnIH-2 effects being much more evident. In addition, sbGnIH-2 but not sbGnIH-1 appears to regulate the expression of its own precursor and receptor genes. Previous studies performed in fish have shown that the GnIH system plays a significant role in the regulation of gonadotropin secretion [15, 16, 33, 35, 36]. However, both stimulatory and inhibitory effects have been reported, and the nature of these effects seems to vary considerably depending on the species, the physiological status, and the route of administration of the GnIH peptide. Considering that GnIH is a neuropeptide, our results suggest that the central administration could represent the most accurate way to elucidate the physiological role of cerebral GnIH on reproductive function.

Our results show that sbGnIH-1 induces a decrease in *gnrh1* mRNA levels in the sea bass brain. It is worth mentioning that GnRH-1 represents the main hypophysiotropic form of GnRH in sea bass, and its fibers innervate profusely the gonadotropic and somototropic cells in the pituitary [38, 39]. Our recent study performed in sea bass has demonstrated that sbGnIH-ir cell projections reach the ventral telencephalon, the parvocellular preoptic nucleus, and the lateral tuberal nucleus of the hypothalamus [17], where sea bass GnRH-1 cells are located [37, 38]. In addition, morphological evidence of GnIH-GnRH interactions has been reported in rodents [30] and birds [58]. Furthermore, the expression of the GnIH receptor has been shown in GnRH-1 neurons [28, 59]. It seems therefore plausible that some of the actions of GnIH on the reproductive axis to inhibit gonadotropin release might be centrally mediated through the indirect action of sbGnIH-1 at the level of the preoptic/hypothalamic GnRH-1 neurons. In accordance with our results, the intraperitoneal administration of gLPXRFa1 decreased *gnrh1* mRNA levels in the hypothalamus of the grouper [16]. As in the case of icv injection of Kiss1 and Kiss2 [49], no effect of centrally administered sbGnIH was observed on *gnrh3* expression, which suggests that this GnRH form is not relevant in the mediation of Kiss and GnIH actions on the reproductive axis of sea bass.

On the other hand, the icv injection of sbGnIH-2 suppressed *gnrh2* mRNA levels in the brain but had no effect on *gnrh1* or *gnrh3* transcript levels. In monkeys and birds, GnIH neurons send projections to midbrain GnRH-2 neurons, which express the GnIH receptor GPR147 [58, 60, 61]. There are indications showing the role of GnRH-2 in the modulation of sexual behavior [62, 63]. It is interesting to note that GnIH participates not only in neuroendocrine functions but also in behavioral control in birds and mammals [64]. GnIH inhibits sexual and aggressive behavior, by acting within the brains of birds and mammals, and some of these effects seem to be related to GnIH-induced changes in the biosynthesis of neuroestrogens in the preoptic area [65–67]. In sea bass, both GnIH and GnRH-2 are expressed in tegmental midbrain cells and profusely innervate sensory-motor areas and the spinal cord [17, 38],

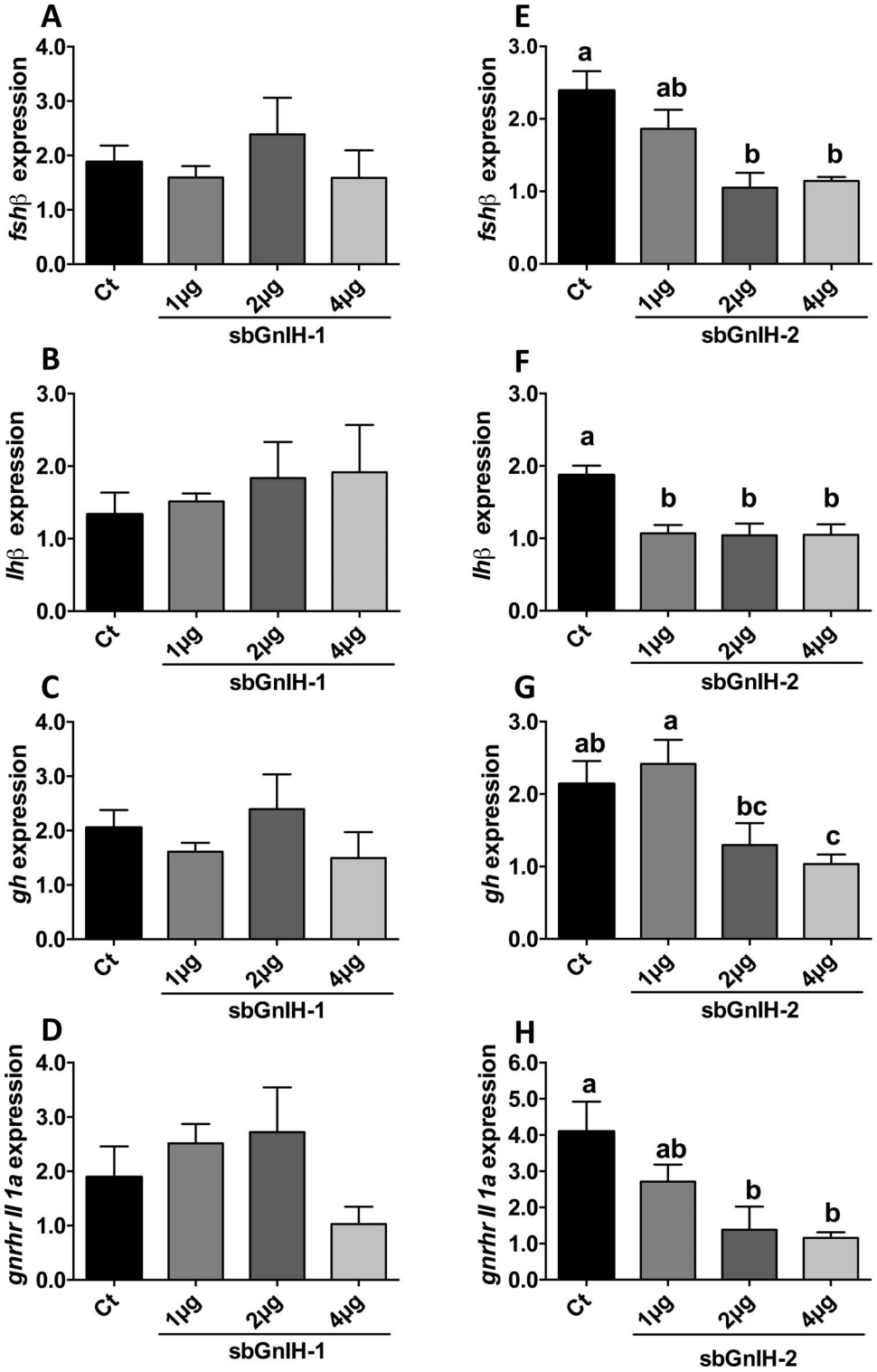

FIG. 4. Effect of in vivo icv injection of different doses (1, 2, and 4 µg) of sbGnIH-1 (**A**, **B**, **C**, **D**) and sbGnIH-2 (**E**, **F**, **G**, **H**) peptides on *lhβ*, *fshβ*, *gh*, and *gnrhr-II-1a* relative expression at 6 hpi in the pituitary of male sea bass (Ct: control). Values are expressed as mean ± SEM (n = 5). Different lower case letters indicate significant differences between treatments (ANOVA, Student-Newman-Keuls test for **A** to **G**; Kruskal-Wallis, Bonferroni test for **H**, $P < 0.05$).

suggesting that both neuropeptides could also modulate sensory-motor activity and behavior in this species. In addition, both GnRH-2 and GnIH innervate the pineal organ of the European sea bass [17, 44]. It should be noted that fish pineal is a light-sensitive organ that transduces daily and seasonal photoperiod information into the neuroendocrine signal melatonin [68]. Previous studies have demonstrated that GnIH is part of the mechanism driving photoperiodic information and melatonin-induced seasonal changes to the reproductive axis of birds and mammals [28, 64, 69–71]. Whether the inhibitory effects of sbGnIH-2 on *gnrh2* transcript levels reported here are affecting the sexual

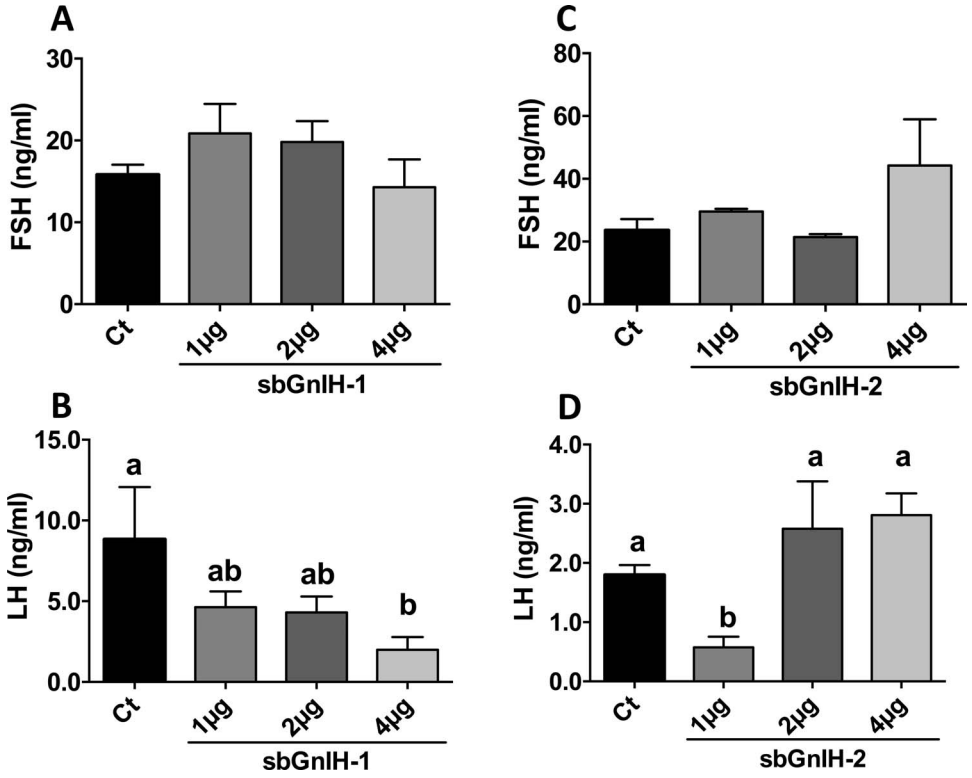

FIG. 5. Effect of in vivo icv injection of different doses (1, 2, and 4 µg) of sbGnIH-1 (**A**, **B**) and sbGnIH-2 (**C**, **D**) peptides on plasma Fsh (**A**, **C**) and plasma Lh (**B**, **D**) levels at 6 hpi (Ct: control). Values are expressed as mean ± SEM (n = 5). Different lower case letters indicate significant differences between treatments (ANOVA, Student-Newman-Keuls test, $P < 0.05$).

behavior and/or mediating the photoperiodic control of reproduction in the European sea bass remains to be elucidated.

Our results provide, for the first time in teleosts, physiological evidence of the action of GnIH on the kisspeptin system. We found that central administration of sbGnIH-2, but not sbGnIH-1, significantly decreased *kiss1, kiss2,* and *kiss1* receptor mRNA levels. In contrast, no significant alteration in either *kiss1* or *kiss2* mRNA levels was observed after intraperitoneal injection with any of the three GnIH peptides present in the grouper *Epinephelus coioides* [16]. In sea bass, both *kiss1* and *kiss2* mRNAs were detected by RT-PCR in the brain and gonads, and their stimulatory role in the control of reproduction has recently been demonstrated [48, 49, 72]. Moreover, recent studies in sea bass have revealed the presence of a conspicuous population of Kiss2 cells surrounding the dorsal, ventral, and lateral extents of the hypothalamic nucleus of the lateral recess [46]. GnIH fibers are in close proximity to kisspeptin neurons from the hypothalamic arcuate nucleus of mice, which also contain GnIH receptors [73]. Interestingly, earlier investigation from our laboratory by using immunohistochemical techniques reported that sbGnIH-ir fibers profusely innervated the different subdivisions of the nucleus of lateral recess of sea bass, where kiss2 cells are located [17, 46]. These results suggest that sbGnIH might be modulating the reproductive axis of sea bass through its direct central action not only on GnRH but also on kiss2 neurons. On the other hand, the habenula of sea bass contains kiss1 neurons [46] as well as pinealofugal fibers and pinealopetal neurons [74]. This epithalamic structure linked to the pineal organ also receives GnIH-ir projections in sea bass [17], which could directly target habenular kiss1 neurons and might be responsible of the decrease in *kiss1* and *kiss1r* transcript levels reported in the present study. Recent studies revealed a strong expression of *kiss1r* on habenular *kiss1*-expressing neurons from sea bass [47], and an autocrine regulation of Kiss1 through the Kiss1r in the habenula of zebrafish and other teleost species [75–77]. Whether the observed inhibition of *kiss1r* expression is the result of the direct action of sbGnIH-2 on the *kiss1r* gene or an indirect consequence of the decrease of *kiss1* expression is still an open question. Interestingly, sea bass specimens exposed to different photoperiods present significant expression differences in some clock and brain-pituitary-gonadal axis related genes well before the first detectable endocrine and morphological responses of the reproductive axis [78]. In this direction, further research in progress in our laboratories is being directed toward elucidating if sbGnIH/Kiss1/pineal interactions could participate in the process of sea bass sexual maturation in relation to photoperiod.

In contrast to that reported in birds and mammals, the physiological action of GnIH in the regulation of gonadotrophin synthesis and release in fish is still a matter of controversy [19, 64]. Our data show that central administration of sbGnIH-2 elicited a decrease in *lhβ* and *fshβ* mRNA levels in the pituitary and diminished Lh but not Fsh plasma levels. In a previous study, we identified the presence of sbGnIH-2-ir fibers in the proximal pars distalis of the sea bass pituitary, in close proximity to Fsh and Lh cells [17], suggesting that sbGnIH-2 could regulate gonadotropin release by acting directly on these adenohypophyseal cells. In addition, the administration of sbGnIH-2 peptide also decreased *gnrhr-II-1a* mRNA levels in the pituitary of sea bass. We have shown previously that GnRHR-II-1a is the only GnRH receptor exhibiting remarkable affinity for hypophysiotropic GnRHs [44], being highly expressed in the pituitary of sea bass, where it colocalizes with all Lh and some Fsh cells [40]. Therefore, it

is possible that the action of sbGnIH-2 on gonadotropin synthesis and/or release could be mediated, at least in part, by its modulation of pituitary GnRH signaling and effects. In turn, sbGnIH-1 provoked a significant decrease in plasma Lh levels but not in pituitary *lhβ, fshβ,* and GnRH receptor expression or Fsh plasma levels. This reduction in plasma Lh levels could be correlated with the inhibition of brain *gnrh1* expression observed in fish treated with sbGnIH-1. The inhibitory effect of both sbGnIH peptides on Lh but not on Fsh plasma levels reinforce the consideration that Lh is the most relevant gonadotropin in spermiating male sea bass, as previously proposed in this species [79]. According to our results, intraperitoneal administration of GnIH-2 peptide suppressed the mRNA levels of both *lhβ* and *fshβ* in late vitellogenic goldfish [36], and the administration of zebrafish LPXRFa3 also decreased plasma Lh levels in the same species [35]. Furthermore, a recent study performed in grouper showed that treatment with endogenous GnIH-2 peptide also decreased *lhβ* mRNA levels in the pituitary gland [16]. However, in vitro treatment of cultured pituitary cells with goldfish LPXRFa peptides increased the release of Lh and Fsh in sockeye salmon and the expression of gonadotropins in grass puffer [33, 34]. In goldfish, in vivo administration of LPXRFa modulated *lhβ, fshβ,* and *lpxrfa*-R mRNA levels and serum Lh concentration in a reproductive stage-dependent manner [56, 57]. Taken together, these results suggest that inhibitory or stimulatory roles of GnIH on gonadotropin synthesis and release in teleosts could be dependent on the species, the reproductive stage of the animals, and/or the route of administration of the peptide.

In the present study, we also reported the inhibitory effects of sbGnIH-2 on sea bass *gh* transcript levels. This result is consistent with the presence of sbGnIH-2-ir fibers in contact with Gh cells in the sea bass pituitary [17]. A hypothalamic LPXRFamide peptide with both in vitro and in vivo Gh-releasing activities was identified in bullfrog [80], and icv administration of a GnIH ortholog increased plasma levels of Gh in rats [30]. In fish, the picture is again much more uncertain. While stimulatory effects of goldfish GnIH on Gh release was observed in sockeye salmon using cultured pituitary cells [33], GnIH exerted complex stimulatory or inhibitory effects on basal and GnRH-stimulated Gh cell functions in a seasonal reproductive stage-dependent manner in goldfish [19, 81]. It should be noted that our experiment was done in mature males at the beginning of the reproductive season. Further experiments at other reproductive stages appear necessary to obtain a clearer picture on putative seasonal effects of sbGnIH on sea bass Gh cell function.

Finally, our results also showed that sbGnIH-2, but not sbGnIH-1, down-regulated brain *gnih* and *gnihr* mRNA levels, indicating that the sbGnIH-2 form exerts the main autocrine regulation (through a negative feedback) on the brain GnIH system. The C-terminal LPXRFamide (X = L or Q) motif appears critical for the binding of GnIH orthologs to GnIH receptors [19, 20]. In sea bass, sbGnIH-1 and sbGnIH-2 peptides exhibit MPMRFamide and MPQRFamide C-terminal motifs [17], that is, two and one amino acid substitutions in relation to the canonical LPXRFamide motif, respectively. In the grouper, the GnIH receptor bound with different affinity to the three GnIH peptides present in the GnIH precursor from this species, and the authors suggested that this variation of activity might be due to their different C-terminal amino acid sequences [16]. A similar basis of explanation could be used to explain the differential response to sbGnIH-1 and sbGnIH-2 observed in the present study, the latter being much more active than the former in the reproductive axis of sea bass.

In summary, in this study performed in male European sea bass, we report for the first time in fish that centrally administered GnIH peptides play an inhibitory role in the reproductive axis, by acting at both brain (on GnRH and kisspeptin expression) and pituitary (on GnRH receptors and gonadotropin synthesis and release) levels. As dopaminergic inhibition does not seem to operate in the reproductive axis of sea bass [5], GnIH is set to become the leading candidate to exert this neurohormonal inhibitory role in this species. However, GnIH-dopamine relationships should be explored because, at least in mammals, it has been shown that GnIH interacts with hypothalamic dopaminergic neurons [60, 82]. We also provide evidence of the differential actions of sbGnIH-1 and sbGnIH-2 on the reproductive axis of male sea bass, the main inhibitory role being exerted by sbGnIH-2, which suggests that this peptide is the main biologically active ligand for the GnIH receptor. The effects of sbGnIH-2 on *gnrh2* and *kiss1*/*kiss1r* expression, together with the pattern of innervation of sbGnIH-2 cells reported in the brain of sea bass [17] suggest that GnIH might participate not only in neuroendocrine functions but also in behavioral and photoperiodic control of reproduction in this species. Whether the actions of GnIH are similar in female sea bass or in animals at different reproductive stages remains to be elucidated. Further studies in female specimens and along the reproductive cycle will contribute toward a more complete picture of this neuropeptidergic system in this species.

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