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Gonadotropin inhibitory-hormone modulates neurosteroids-synthesizing enzymes expression and aggressive behavior in male sea bass, *Dicentrarchus labrax*

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Neurosteroids are involved in the regulation of multiple behavioral and physiological processes and metabolic activities in the vertebrate brain. However, central mechanisms of how neurosteroid synthesis is regulated is far to be understood. Gonadotropin-inhibitory hormone (GNIH) is a hypothalamic neuropeptide that negatively regulates gonadotropin secretion but also inhibits sexual and aggressive behaviors in birds and mammals by modulating aromatase enzyme and neuroestrogen synthesis. In a previous study performed in male sea bass, we reported that Gnih inhibited the reproductive axis by acting at the three levels of the brain-pituitary-gonad axis. Moreover, the presence of Gnih cells and fibers in the telencephalon, mesencephalon and rhombencephalon suggests a role of Gnih in regulating other important brain functions in sea bass, including behavior. In this study, we have analyzed the effects of the intracerebroventricular (icv) injection of sbGnih-2 on the brain and pituitary expression of the main neurosteroids-synthesizing enzymes (*stAR*, *cyp17*, *3 β -hsd*, *17 β -hsd*, *cyp19b*, *cyp7b*), as well as on estrogen and androgen receptors (*era*, *er β 1*, *er β 2*, *ar*). A combination of immunohistochemistry and *in situ* hybridization was also used to identify putative interaction of Gnih- and aromatase-positive cells. We also performed a mirror test study as a proxy to measure aggression levels and agonistic behavior after icv injection of sbGnih-2. Central administration of sbGnih-2 at different doses reduced the transcript levels of *3 β -hsd* and *17 β -hsd*, and increased the expression of *cyp19b* (brain aromatase) in the sea bass brain. Neuroanatomical results suggest that paracrine and neuroendocrine actions could mediate Gnih effects on aromatase expression. Central administration of sbGnih-2 also decreased the pituitary expression of *17 β -hsd* and estrogen receptors (*er β 2*). The mirror test analysis

showed that sbGnih-2 affected the agonistic/aggressive behavior of sea bass as revealed by the decreased interaction with the mirror, lower time spent in the mirror zone, increased latency to establish contact with the mirror and higher mean distance to the mirror zone. In contrast, locomotor activity parameters measured were not affected by sbGnih-2 injection. Taken together, our results showed for the first time in fish that Gnih inhibits social-aggressive behavior and affects the gene expression of neurosteroid-synthesizing enzymes giving rise to neuroandrogens and neuroestrogens in the sea bass brain.

KEYWORDS

gonadotropin-inhibitory hormone, aggressive behavior, aromatase, neuroestrogens, neuroandrogens, fish, brain

Introduction

The brain is not only a target for steroid hormones produced in peripheral tissues such as the gonads, the interrenal/adrenal glands and the mammalian placenta but also has its local production by converting cholesterol and circulating steroids into neuroactive steroids known as neurosteroids (Ubuka and Tsutsui, 2022). Neurosteroids have been involved in the regulation of multiple physiological processes, metabolic activities and behaviors, including food intake, sexual activity, aggressiveness, anxiety, depression, body temperature, blood pressure, brain plasticity and neurogenesis in tetrapod vertebrates (Lapchak and Araujo, 2001; Belelli et al., 2006; Strous et al., 2006; Do Rego et al., 2009; Azcoitia et al., 2018; He et al., 2019). In teleost fish, neurosteroids also appear implicated on sex differentiation, reproduction, stress, neurogenesis, cell migration and social-aggressive behavioral responses (Diotel et al., 2013; Huffman et al., 2013; Haraguchi et al., 2015; Pellegrini et al., 2016; Ramallo et al., 2017). Although it has been established that aggressive behavior of male fish is largely dependent of testicular androgens (Oliveira et al., 2002; O'Connor et al., 2011; Teles and Oliveira, 2016), in some cases, plasma circulating androgen concentration was not related to the aggressive rating of teleosts (Vullioud et al., 2013; Almeida et al., 2014). It should be noted that the brain of fish is well known for its capacity to synthesize androgens and to convert testicular and locally-synthesized androgens (notably, testosterone) into estrogens (estradiol), whose actions are mediated by brain androgen and estrogen receptors (Callard et al., 1990; Diotel et al., 2011a). The transformation of C19 androgens, such as testosterone (T), into C18 estrogens, such as estradiol (E2), is elicited by the brain aromatase (cytochrome P450) enzyme, indicating that estrogens signaling could also play an important role in the brain of males (Perkins and Roselli, 2007; Ramallo et al., 2017; Azcoitia et al., 2018). Brain aromatase, encoded by the *cyp19b* gene, has been involved in the inhibition of aggressive behavior in different teleost species studied to date. For example, in a social species like the African cichlid fish *Astatotilapia burtoni*, subordinate males expressed higher concentration levels of aromatase than dominant males (Huffman et al., 2013). In the blue-banded goby (*Lythrypnus dalli*), a sex-

changing fish, it was also observed that socially induced decreases in brain aromatase levels produced an increase in aggressive behavior, suggesting that aromatase activity may modulate changes in teleost social behavior (Black et al., 2005). Despite its functional significance, little is known about how aromatase synthesis and activity are regulated in relation to behavior in fish.

In 2000, a novel neuropeptide belonging to the RFamide family was identified in the hypothalamus of the quail (*Coturnix japonica*) and it was reported that it actively inhibited the gonadotropin secretion on the pituitary gland (Tsutsui et al., 2000). As a result, it was termed as gonadotropin-inhibitory hormone or GNIH. Within the mammalian brain, GNIH neurons were present in the dorsomedial (DMH), paraventricular (PVN) and ventromedial (VMH) nuclei of the hypothalamus (Kriegsfeld et al., 2006; Clarke et al., 2008; Dardente et al., 2008; Soga et al., 2014), whereas they were restricted to PVN neurons in birds (Tsutsui et al., 2000; Bentley et al., 2003; Ukena et al., 2003; Osugi et al., 2004). However, teleosts seem to exhibit a much wider distribution of Gnih cells, at least in some of the species analyzed so far (Muñoz-Cueto et al., 2017; Di Yorio et al., 2019).

On the other hand, increasing evidences are indicating that neuropeptides and neurosteroids interactions can play an important role in the regulation of brain functions (Do Rego et al., 2009). Recently, Ubuka and colleagues demonstrated that GNIH regulates neuroestrogen synthesis by stimulating the activity of cytochrome P450 aromatase in the quail brain, and proposed that GNIH could inhibit male socio-sexual behavior throughout its actions on aromatase-producing cells located in the preoptic area of male birds (Ubuka et al., 2014). So far, in teleost fish, which represent more than half of vertebrates, most of the reported effects of Gnih have been focused on the reproductive axis, i.e., regulation of gonadotropin synthesis and release, seasonal reproduction and steroidogenesis (Muñoz-Cueto et al., 2017; Di Yorio et al., 2019). However, the role that Gnih plays in the regulation of neurosteroids synthesis and behavior remains unknown in fish.

The European sea bass (*Dicentrarchus labrax*) is one of the most important species for European aquaculture and has been commonly used in our laboratory as model species for neuroanatomical, neuroendocrinological, physiological, cellular,

molecular and behavioral studies (Cerdá-Reverter et al., 2001a; Cerdá-Reverter et al., 2001b; González-Martínez et al., 2001; González-Martínez et al., 2002; Cerdá-Reverter et al., 2008; Servili et al., 2009; Servili et al., 2010; Sánchez-Vázquez and Muñoz-Cueto, 2014; Paullada-Salmerón et al., 2016a; Paullada-Salmerón et al., 2016b; Paullada-Salmerón et al., 2016c; Cowan et al., 2017; Paullada-Salmerón et al., 2017; Paullada-Salmerón et al., 2019; Wang et al., 2022; Paullada-Salmerón et al., 2023). Recently, we identified a sea bass *gnih* gene encoding a precursor with two different RFamide peptides, named as sbGnih-1 and sbGnih-2, and elucidated the immunohistochemical localization of Gnih neurons and their projections in the brain and pituitary by using specific antibodies against both peptides (Paullada-Salmerón et al., 2016a). In agreement to other fish species (Biswas et al., 2015; Di Yorico et al., 2016; Ogawa et al., 2016; Aliaga-Guerrero et al., 2018), our results evidenced a profuse innervation of Gnih neurons entering the neurohypophysis to control gonadotropin secretion and targeting neuroendocrine brain regions such as the ventral telencephalon, preoptic area and hypothalamus but also reaching non-hypophysiotrophic regions as the habenula and midbrain/hindbrain areas, suggesting that Gnih might be also involved in the regulation of behavior in fish (Paullada-Salmerón et al., 2016a). Moreover, both sbGnih-1 and sbGnih-2 decreased androgen (T and 11-ketotestosterone, 11-KT) plasma levels at early- and mid-spermatogenesis stages, affecting the progression testicular gametogenesis (Paullada-Salmerón et al., 2016c).

In order to better understand the role of Gnih system on neurosteroids synthesis and function, in the present study we investigated the *in vivo* effects of intracerebroventricular (icv) injection of sbGnih-2 peptide on brain and pituitary expression of neurosteroidogenic enzymes (steroidogenic acute regulatory protein, *star*; cytochrome P450 17 α -hydroxylase/C-17,20-lyase, *cyp17*; 3-beta-hydroxysteroid dehydrogenase, *3 β -hsd*; 17-beta-hydroxysteroid dehydrogenase, *17 β -hsd*; brain cytochrome P450 aromatase, *cyp19b*; cytochrome P450 7b, *cyp7b*), as well as estrogen and androgen receptors (*era*, *er β 1*, *er β 2*, *ar*). Moreover, we also tried to elucidate the existence of potential interactions between Gnih-immunoreactive and aromatase-expressing cells by using a combination of immunohistochemical and *in situ* hybridization methods. Finally, in order to investigate putative behavioral actions of Gnih in sea bass, we performed a mirror test analysis to determine the sbGnih-2 effects on agonistic behavior and aggression levels in this species.

Materials and methods

Animals

For gene expression, immunohistochemical and *in situ* hybridization analyses, two-years-old adult spermiating male specimens of European sea bass, *Dicentrarchus labrax* (459 \pm 103 g in weight), were obtained from CUPIMAR S.L (San Fernando, Spain) and housed in the “Laboratorio de Cultivos Marinos” (University of Cádiz, Puerto Real, Spain). All animals

were kept under natural winter photoperiod conditions and constant temperature and salinity (19 \pm 1°C and 39 ‰, respectively) and fed once per day with commercial dry pellets (1% body weight, Skretting España S.A, Burgos, Spain) using automatic feeders (EHEIM GmbH & Co. KG, Deizisau, Germany) 2 h after light onset.

For behavioral analysis, two-years-old adult spermiating male specimens of European sea bass, *D. labrax* (354 \pm 53 g in weight), were obtained from “Méditerranée Pisciculture” (Salses-le-Château, France) and kept in tanks of the Mutualized Aquariology Service of the Oceanological Observatory of Banyuls-sur-Mer (Sorbonne Université, CNRS, Banyuls-sur-Mer, France). All animals were acclimated during 3 weeks in 1 m³ indoor tanks (35 fish per tank) supplied with Mediterranean sea water (~37 ‰ salinity along the French Mediterranean coast) provided by an open circuit, at a flow rate of ~400 l/h, and maintained under natural temperature and simulated natural photoperiod (light provided by fluorescent bulbs). Fish were fed *ad libitum* with commercial dry pellets (Skretting France, Fontaine-lès-Vervins, France), administered once per day 2 h after light onset.

The animal experimental protocols were designed and approved in accordance with the Animal Care and Use Committee of the University of Cádiz (Spain) and of the Oceanological Observatory of Banyuls-sur-Mer (France) and in agreement with European Union Regulation (EC Directive 86/609/EEC) concerning the protection of experimental animals.

Hormones

The sea bass gonadotropin-inhibitory hormone-2 peptide (sbGnih-2, GenBank accession no. LN681205, SPNSTPNMPQRF-NH₂, Paullada-Salmerón et al., 2016a) was synthesized by Metabion International AG (Planegg, Germany). Synthetic peptide was amidated at the C-terminal end, purified by high-performance liquid chromatography (>98% purity) and dissolved in phosphate buffer saline (PBS) 1x sterilized according to the manufacturer's instructions.

Gnih administration

For the gene expression and mirror test analyses, animals were acclimatized for several weeks prior to the administration of the sbGnih-2 peptide in experimental tanks with identical photoperiod, temperature and salinity conditions to the housed tanks. Gnih peptide was injected to the fish intracerebroventricularly (icv) according to the procedure described by Paullada-Salmerón et al. (2016b). Briefly, sbGnih-2 was administrated in the third ventricle using a 25 μ l Hamilton microsyringe fitted with a 26-G needle (Hamilton, Reno, NV, USA) and driven by a micromanipulator. Each fish was anesthetized and immobilized and the needle was immediately inserted into a small hole drilled in the dorsal surface of the skull to dispense the peptide or the vehicle into the sea bass third ventricle. No mortality was observed after icv injection.

Gene expression analysis

At the beginning of the reproductive season, forty male sea bass were used to determine the effect of sbGnih-2 on brain and pituitary expression of neurosteroids-synthesizing enzymes and steroid receptors genes. These animals were divided into four different groups: three experimental groups and one control group of ten fish each. The three experimental groups were injected with sbGnih-2 at 1, 2 and 4 µg doses dissolved in 10 µl of PBS, respectively, while the control group received only PBS. Sampling was carried out at 6 and 12 h post-injection (hpi), according to our established method and previous results (Paullada-Salmerón et al., 2016b). At the end of the experiment, all fish were anesthetized by immersion in MS-222 and the brain and pituitary gland were dissected, frozen in liquid nitrogen, and stored at -80°C until assay.

For real-time quantitative PCR analysis (RT-qPCR), the procedure for RNA isolation and reverse transcription was performed as previously described by Paullada-Salmerón et al. (2016b). Total RNA was isolated from sea bass brain and pituitary by TRIsure reagent (Bioline, London, UK), according to the manufacturer's protocol. Tissues were homogenized in a mixer mill MM400 (Retsch GmbH, Haan, Germany) and total RNA concentration and quality were measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1µg) was retro-transcribed and DNA

removed using a Primer Script™ RT Reagent Kit with gDNA Eraser (Takara Bio Inc., Shiga, Japan). To analyze brain and pituitary expression of neurosteroidogenic enzymes (*star*, *cyp17*, *3β-hsd*, *17β-hsd*, *cyp19b*, *cyp7b*), estrogen and androgen receptors (*erα*, *erβ1*, *erβ2*, *ar*), RT-qPCR was conducted by using Bio-Rad CFX96 Touch detection system (Bio-Rad, Richmond, CA, USA) with the SYBR Premix ExTaq™ (Tli RNase H Plus; Takara Bio Inc.). Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA) and sequences, amplicon size and PCR conditions are shown in Table 1. Experiments were done by duplicates 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. As internal standard housekeeping genes *l17* and *elfα* were used (Table 1). The relative expression of genes analyzed was calculated by the $\Delta\Delta$ -Ct method (Livak and Schmittgen, 2001).

Immunohistochemical analysis

For the immunohistochemical localization of Gnih, we used immunofluorescence and a specific rabbit polyclonal antibody against sbGnih-2 generated and previously validated in our

TABLE 1 Primers used for quantitative real-time PCR.

Gene.	GenBank Accession No.	5' to 3' sequence	Annealing Temperature (°C)	Amplicon size (pb)
<i>star</i>	EF409994.1	fw: GGTGTGGTCAGAGCGGAGAA rv: GAGGTGGTTGGCAAAGTCCA	60	174
<i>cyp17</i>	JQ861953.1	fw: GCGGCTGCTGAACTGTCCT rv: AGTGTCACGATGCCCTGGC	63	150
<i>3βhsd</i>	JQ861952.1	fw: AGGAGGACAACTGGTGGAG rv: GGGATTCGGTGCCAAACGTC	58	196
<i>17βhsd</i>	XM_051378943.1	fw: AATCCGCCGACCTACGCTGG rv: CGTGGGAAGGTAGCCGTTG	60	104
<i>cyp19b</i>	AY138522.1	fw: CTCGTTCTGCGTGGGCAA rv: GCTGCTGCGACAGGTTGTTG	60	141
<i>cyp7b</i>	KF857306.1	fw: TCTGCCCTCCCTAATAGAGA rv: CGAACAGAGTCAGGTAGCCA	60	155
<i>erα</i>	AJ505009.1	fw: CGTGGCTGGAGGTGCTGATG rv: GGCAGACAACTCCTCGGGT	61	198
<i>erβ1</i>	AJ489523.1	fw: CCACTCCCAGGCTCGGTCTC rv: CGCCAGATGACACCACGCC	65	140
<i>erβ2</i>	AJ489524.1	fw: TGCCCTCAGCCTCTCGTCTA rv: ATGTCGGCTTCCCTGCTGC	62	182
<i>ar</i>	AY647256.1	fw: GCATCACCTCACCTCCACAT rv: TTCTCTGTCTCTCTCGCCTG	60	183
<i>l17</i>	AF139590	fw: CAGGAGTGGGTGACATGGTC rv: GACTTCCGTGCGCCGATCAC	61	97
<i>elfα</i>	AJ866727	fw: CTGTGCTGATCGTTGCTGCTGGTGT rv: CGTGCTCGCGGGTCTGTCC	61	75

fw, forward primers; rv, reverse primers.

laboratory (Paullada-Salmerón et al., 2016a). Before immunostaining, sections were transferred for 15 min to Coons buffer pH 7.4 (0.01 M Veronal, 0.15 M NaCl) containing 0.1–0.2% Triton X-100 (CBT) and then saturated in CBT containing 0.5% bovine serum albumin for 30 min. Sections were incubated overnight in a moist chamber at room temperature with sbGnih-2 antibody (1:1500 dilution in CBT 0.5% bovine albumin serum). Sections were washed in CBT (3 x 10 min) and incubated for 2 h in a dark chamber at room temperature with goat anti-rabbit Alexa Fluor 594 or 488 (1:500 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After washing in CBT, slides were coverslipped with Vectashield mounting medium with DAPI (Vector laboratories, Burlingame, CA, USA). To confirm the specificity of the immunostaining, controls were performed by preabsorption of primary sbGnih-2 antiserum with synthetic sbGnih-1 (NH₂-PLHLHANMPMRF-CONH₂) and sbGnih-2 (NH₂-SPNSTPNMPQRF-CONH₂) peptides (100 ng/μl), replacement of primary antiserum with the corresponding preimmunized serum, and omission of primary or secondary antisera.

Double immunohistochemical and *in situ* hybridization detection of sbGnih-2 and aromatase-expressing cells in sea bass brain

In order to identify putative colocalization and/or interactions of sbGnih-2 and aromatase-expressing cells, we used a combination of immunohistochemical and *in situ* hybridization procedures. Three male sea bass specimens were anesthetized and transcardially perfused with 0.65% NaCl followed by 4% paraformaldehyde with 0.2% picric acid in PBS buffer pH 7.5 at 4°C. Then, brains were dissected, postfixed overnight at 4°C, cryoprotected in 15% sucrose solution and embedded in Tissue-Tek. Serial coronal brain sections obtained with a cryomicrotome were first processed for *in situ* hybridization and, subsequently, for sbGnih-2 immunostaining as indicated above.

For *in situ* hybridization, messenger RNA used for the synthesis of specific riboprobes corresponded to the ORF-coding region of the European sea bass brain aromatase (Blázquez and Piferrer, 2004). The specific riboprobes, of 584 nucleotides in length, were synthesized using a pSpark II vector (Canvax Biotech, Córdoba, Spain) containing DNA sequence of sea bass brain aromatase synthesized by Bio Basic Inc. (Markham, Canada). Antisense and sense single-stranded mRNA probes were obtained by transcription with T7 and SP6 RNA polymerases (Promega Biotech Ibérica S.L., Alcobendas, Spain) on plasmids linearized with SacI and SacII restriction enzymes using DIG RNA labelling MIX (Sigma Aldrich, Madrid, Spain). The size and integrity of the DIG-labelled antisense and sense probes were checked by 1% agarose gel electrophoresis under denaturing conditions, and their concentration was evaluated by spectrophotometric analysis.

In situ hybridization was performed as described previously (Frau et al., 2022). Cryostat sections were rinsed in 1x PBS pH 7.4

and post-fixed in PAF 4% diluted in PBS. After postfixation, the slides were washed two times in PBS and deproteinized with proteinase K at 37°C for 5 minutes in PBS, washed, and postfixed again in 4% PAF. Next, sections were rinsed at room temperature in 2x SSC buffer for 10 min. Before hybridization, the antisense and sense DIG-probes were preheated at 75°C for 5 minutes. The slides were then hybridized at 60°C overnight in a hybridization buffer (2 × SSC; 2.5% dextran sulphate; 50% deionized formamide; 5 × Denhardt's solution; 50 μg/ml of yeast tRNA, pH 8; 4 mM EDTA) with riboprobes. Subsequently, the slides were rinsed in SSC at 60°C, followed by two rinses at 60 °C for 30 min in SSC/50% deionized formamide. The sections were rinsed in 0.2 and 0.1 x SSC at room temperature and blocked for 1 h in a solution of 100 mM Tris-HCl buffer, 150 mM NaCl (pH 7.5) with 0.5% BSA, and 0.2% Triton X-100. Afterwards, the slides were incubated overnight with an alkaline phosphatase-conjugated anti-DIG antibody (1:500; Sigma Aldrich). The third day, the sections were revealed with NBT/BCIP (stock solution; Sigma Aldrich) for 0.5–1 h. After washing with PBS, immunohistochemistry of Gnih was performed following the protocol described above, and sections were coverslipped with Vectashield mounting medium with DAPI (Vector Laboratories) and observed under a fluorescence photomicroscope as indicated below.

Analysis of images and figure production

Immunohistochemical and *in situ* hybridization sections were observed under a Zeiss Imager D2 photomicroscope (Zeiss, Oberkochen, Germany) and images were acquired with an Axiocam 503 camera (Zeiss) and processed using the ZEN software (Zeiss). Plates were assembled using Photoshop CS6 (Adobe Systems, San Jose, CA, USA) without alteration except for light or contrast adjustments. For the precise localization of immunoreactive sbGnih-2 and aromatase-expressing cells, a sea bass brain atlas was used (Cerdá-Reverter et al., 2001a; Cerdá-Reverter et al., 2001b; Cerdá-Reverter et al., 2008), with some changes in the nomenclature to adapt it to the current neuromeric/prosomic models of brain organization (Puelles, 2019).

Behavioral analysis

During the reproductive period, thirty pairs of sea bass males were used to test the effect of sbGnih-2 administration on agonistic behavior and aggression levels by using the mirror test. Animals were anesthetized and sbGnih-2 was administered in the third ventricle between 8.30 and 11:00 h with a single dose of 2 μg dissolved in 10 μl of PBS, which was revealed as the most effective dose after icv injection in our previous study performed in sea bass (Paullada-Salmerón et al., 2016b), while the control group was injected with PBS alone. Subsequently, animals were placed in the experimental tank fifteen minutes before the behavioral test for recovery. For mirror test and behavioral data analysis, the experimental procedure described by Manciooco and coworkers

(Manciooco et al., 2015) was used with slight modification. Fifteen minutes after administration of sbGnih-2 or vehicle, the behavior of each pair of male sea bass was recorded for 30 minutes using a digital camera (Supplemental Material 1). The position of the camera was defined to allow video coverage of the whole surface of the experimental tank (110 x 70 x 50 cm). The camera was turned on avoiding sudden movements and no evidence of stress behavior (e.g., no change in swimming movements or change in pigmentation) was observed. All experimental fish pairs were tested out of their home tanks, isolated from the social group, and the behavior of both fishes from each pair was recorded and analyzed (15 pairs, $n = 30$ for control fish; 15 pairs, $n = 30$ for sbGnih-2 injected fish). Behavioral analysis was performed using Noldus EthoVision XT software and some parameters defined by Noldus system (proximity, relative movement, mobility, acceleration state, velocity and distance moved, latency to the first event, C-shape bending of the body in front of the mirror) were analyzed. All parameters measured are shown in Table 2. In each pair of animals, the first animal that moved was defined as the focal subject and its recognition was carried out based on slight body differences (e.g., the size, color, shape of the caudal fin), while the second individual was defined as the companion. There were no statistical differences in the behavioral parameters analyzed between the focal and the companion subjects of each pair of fish and, therefore, both individuals were used for the statistical analysis presented in the results section.

Data analysis

All results are expressed as mean \pm standard error of the mean (SEM). Statistical differences in relative gene expression levels, hormonal levels and behavioral response were examined by one-way ANOVA followed by Tukey *post hoc* test using Statgraphics Centurion XVI software (Statpoint Technologies, Warrenton, VA, USA) and GraphPad Prims 9 Software (San Diego, USA). Before the analysis, datasets were tested for normality and homoscedasticity of variance, and if necessary, were log- or square root-transformed. When data did not fulfil the requirements of the ANOVA, data were analyzed using the Kruskal Wallis non-parametric test. In all cases, statistical significance was established at $p < 0.05$.

Results

Physiological effects of sbGnih-2 on the expression of brain and pituitary neurosteroids-synthesizing enzymes and steroid receptors

In the brain, icv injection of sbGnih-2 provoked a reduction of *17 β -hsd* mRNA levels at 6 hpi with the higher doses (2 and 4 μ g; $p < 0.0088$ and 0.0452 , respectively; Figure 1D) and a significant decrease on *3 β -hsd* expression at 12 hpi with the three doses tested (1, 2 and 4 μ g; $p < 0.0073$, 0.0056 and 0.0222 , respectively; Figure 2C), as well as an increased expression of *cyp19b* at 6 hpi with the lower dose (1 μ g; $p < 0.0002$; Figure 1E). No significant effects on brain mRNAs levels of *star* (Figures 1A, 2A), *cyp17* (Figures 1B, 2B), *3 β -hsd* (at 6 hpi, Figure 1C), *17 β -hsd* (at 12 hpi, Figure 2D), *cyp19b* (at 12 hpi, Figure 2E), *cyp7b* (Figures 1F, 2F), *er α* (Figures 1G, 2G), *er β 1* (Figures 1H, 2H), *er β 2* (Figures 1I, 2I) and *ar* (Figures 1J, 2J) were observed in fish treated with sbGnih-2 peptide.

The effect of sbGnih-2 at pituitary level was less evident than in the brain. Both *17 β -hsd* mRNA levels ($p < 0.0216$; Figure 3B) and *er β 2* expression ($p < 0.0053$; Figure 3F) were significantly decreased in fish treated with the lower dose of sbGnih-2 (1 μ g) at 6 hpi. The expression of *star*, *cyp17* and *cyp7b* enzymes was not detected in sea bass pituitary and no significant effect of sbGnih-2 was detected for the remaining neurosteroids-synthesizing enzymes and steroid receptors analyzed (Figures 3, 4).

Overlapping distribution of Gnih-immunoreactivity and aromatase expression in the sea bass forebrain

In addition to that reported in our previous immunohistochemical study (Paullada-Salmerón et al., 2016a), the immunohistochemical analysis using the anti-sbGnih-2 antiserum revealed the presence of immunostained cells adopting a peripheral distribution along the telencephalic, preoptic and prethalamic/thalamic ventricles (Figure 5). These Gnih-immunoreactive (ir) cells were particularly evident in the caudo-medial region of the dorsal (Dm4) and ventral

TABLE 2 Behaviors recorded in the mirror test, including parameters related to the mirror and locomotor activity of male sea bass specimens.

Test	Behavior	Description
Mirror	Latency to first contact with the mirror	Time recorded between the beginning of the test and the first contact of the subject with the mirror
Mirror	Time spent in mirror zone	Time spent by the subject in the mirror zone defined as closer zone to the mirror
Mirror	Mean distance to mirror zone	Distance maintained with the mirror
Mirror	Contacts with the mirror	Touching and/or knocking the mirror by the mouth.
Locomotor activity	Distance moved	Distance swam during the test
Locomotor activity	Velocity max	Maximal velocity measured during the test
Locomotor activity	Acceleration max	Maximal acceleration measured during the test
Locomotor activity	Mean distance between subjects	Distance maintained between both subjects

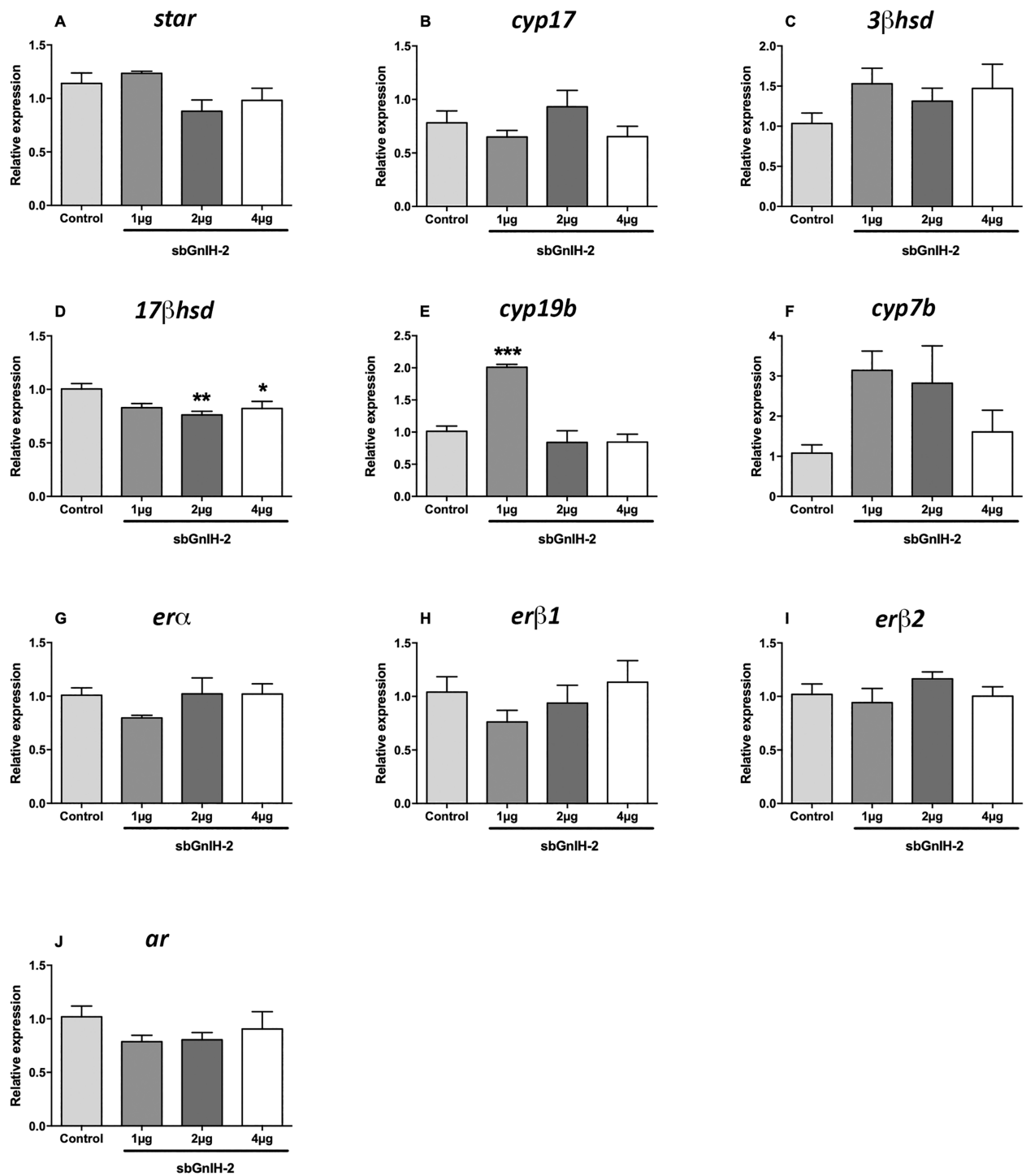


FIGURE 1
 Effects of *in vivo* intracerebroventricular (icv) injection of different doses (1µg, 2µg and 4µg) of sbGnIH-2 on the relative expression of neurosteroids-synthesizing enzymes and steroid receptors in the brain of male sea bass at 6 hours post-injection. (A). Steroidogenic acute regulatory protein (*star*); (B). Cytochrome P450 17alpha-hydroxylase/17,20-lyase (*cyp17*); (C). 3β-hydroxysteroid dehydrogenase (*3β-hsd*); (D). 17β-hydroxysteroid dehydrogenase (*17β-hsd*); (E). Brain cytochrome P450 aromatase (*cyp19b*); (F). Cytochrome P450 7α-hydroxylase (*cyp7b*); (G). Estrogen receptor alpha (*era*); (H). Estrogen receptor beta 1 (*erβ1*); (I). Estrogen receptor beta 2 (*erβ2*). (J). Androgen receptor (*ar*). Results are expressed as mean ± SEM (n=5). Asterisks denote significant differences between mean values of GnIH-treated animals and controls by one-way ANOVA followed by Tukey *post hoc* test (*, p<0.05; **, p<0.01; ***, p<0.001).

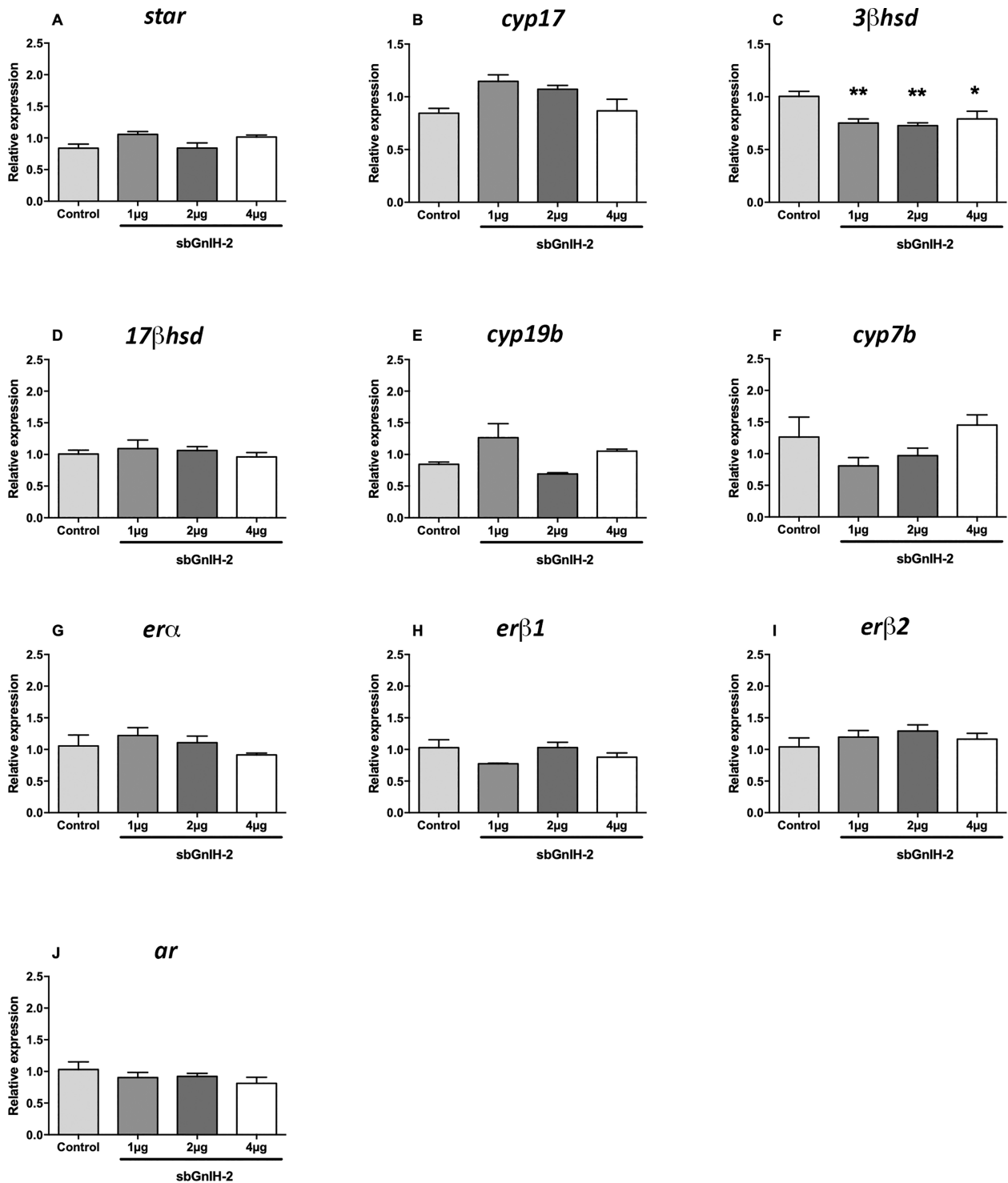
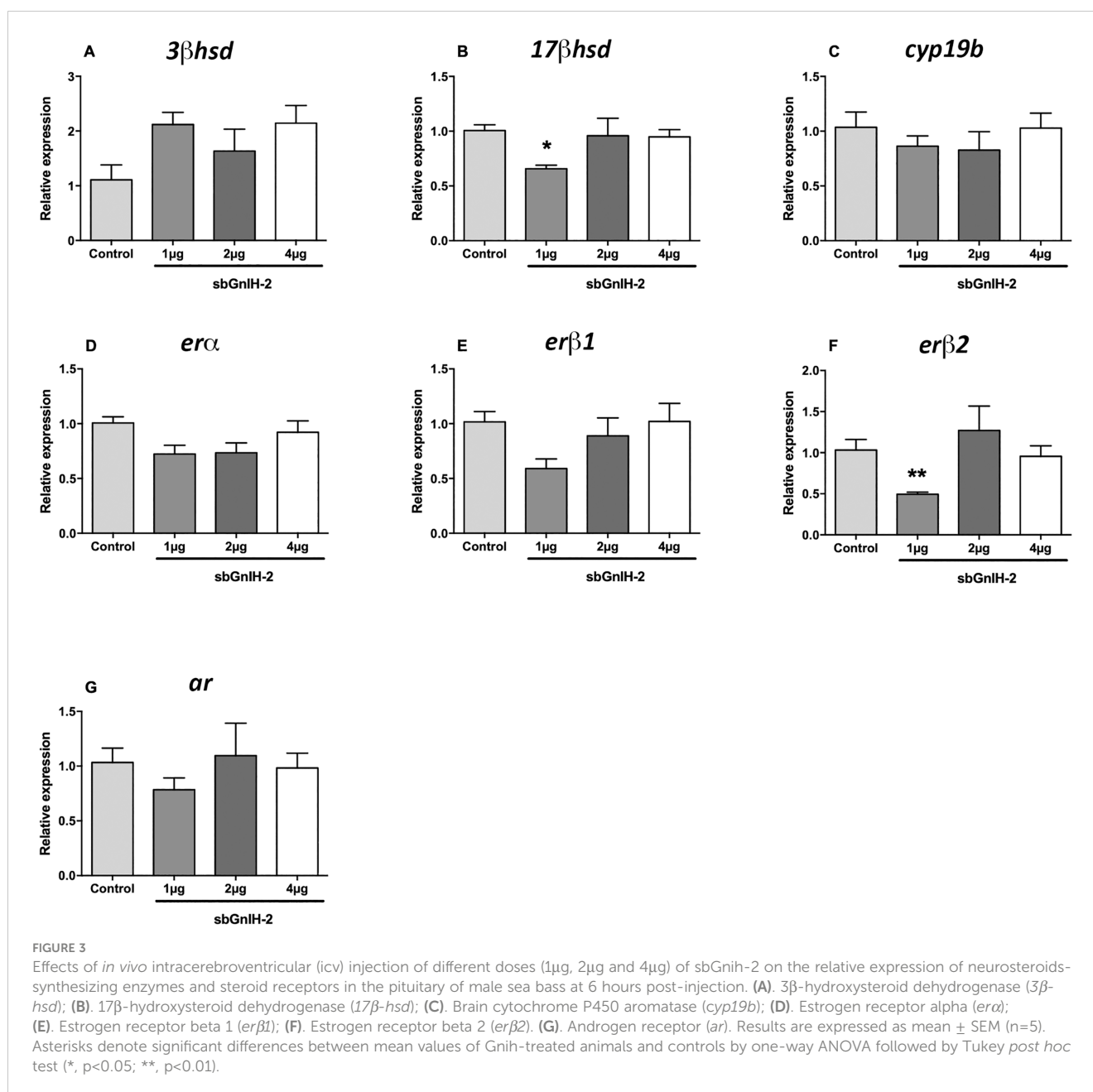


FIGURE 2

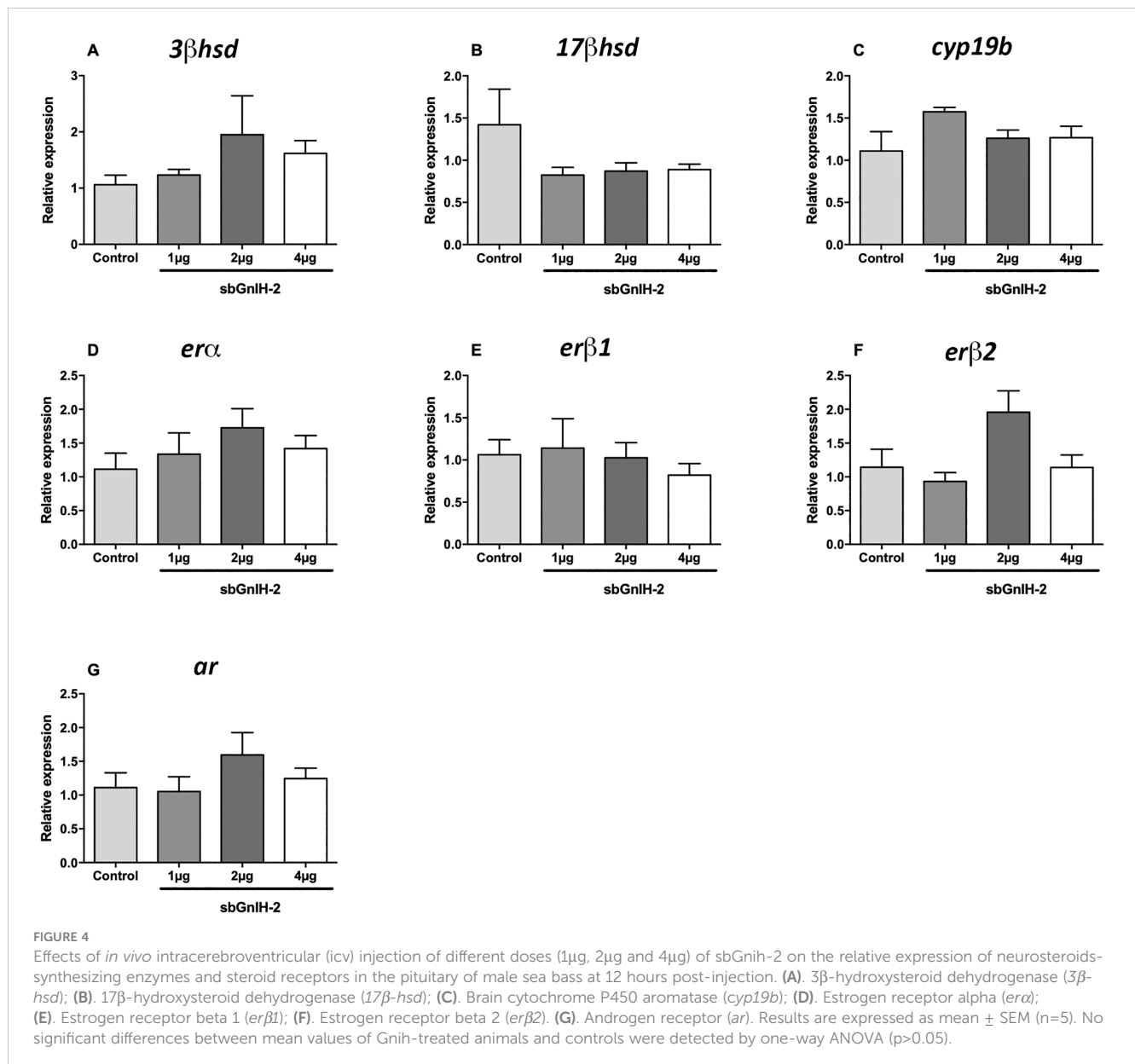
Effects of *in vivo* intracerebroventricular (icv) injection of different doses (1μg, 2μg and 4μg) of sbGnih-2 on the relative expression of neurosteroids-synthesizing enzymes and steroid receptors in the brain of male sea bass at 12 hours post-injection. (A). Steroidogenic acute regulatory protein (*star*); (B). Cytochrome P450 17alpha-hydroxylase/17,20-lyase (*cyp17*); (C). 3β-hydroxysteroid dehydrogenase (*3β-hsd*); (D). 17β-hydroxysteroid dehydrogenase (*17β-hsd*); (E). Brain cytochrome P450 aromatase (*cyp19b*); (F). Cytochrome P450 7α-hydroxylase (*cyp7b*); (G). Estrogen receptor alpha (*eraα*); (H). Estrogen receptor beta 1 (*erβ1*); (I). Estrogen receptor beta 2 (*erβ2*). (J). Androgen receptor (*ar*). Results are expressed as mean ± SEM (n=5). Asterisks denote significant differences between mean values of Gnih-treated animals and controls by one-way ANOVA followed by Tukey *post hoc* test (*, p<0.05; **, p<0.01).



(Vs, Vp) telencephalon (Figures 5A, B, E, F), the preoptic area (NPOpc, NPOav; Figures 5C, F) and prethalamus (VM; Figures 5D, G).

Double labelling using immunohistochemistry and *in situ* hybridization revealed the overlapping of Gnih immunoreactivity and brain cytochrome P450 aromatase (*cyp19b*) expression along the forebrain ventricle (Figures 6, 7). Gnih-ir cells were mostly found lining the subventricular zone, while aromatase-positive cells were located at the edge of the brain, lining the ventricular surface (see Figures 6C, F). In the telencephalon, the close apposition of Gnih-ir cells and aromatase-expressing cells was particularly evident in the caudo-medial subdivisions of the dorsal telencephalon (Dm4,

Figures 6A-C, G) and in the supracommissural (Vs, Figures 6A-C, G) and postcommissural (Vp, Figures 6D-F, H) nuclei of the ventral telencephalon. In the prethalamus, Gnih-ir cells were also in close proximity to aromatase-expressing cells along the ventricular surface of the ventromedial nucleus (VM, Figures 7A-D, G, black arrowheads in 7D). Moreover, Gnih-ir fiber terminals were evident in the vicinity of some aromatase-positive cells located in the most internal part of VM (Figure 7D, white arrowheads). No labelling was observed with sense riboprobes (Figure 7E) or when the primary sbGnih-2 antiserum was preabsorbed with the synthetic sbGnih-2 peptide (Figure 7F).



Gnih effects on agonistic/aggressive behavior and locomotor activity

We also tested the effect of central administration of sbGnih-2 on agonistic/aggressive behavior and locomotor activity of male sea bass (Figure 8, Supplementary Material). Icv injection of sbGnih-2 reduced the number of contacts with the mirror and the time spent in the mirror zone ($p < 0.0016$ and $p < 0.001$, respectively, Figures 8A, B). These results were consistent with the movement heat map analysis of control and sbGnih-2 treated animals (Figures 8I, J). In turn, sbGnih-2 administered fish showed an increase in time spent to establish the first contact with the mirror and in the mean distance to the mirror zone ($p < 0.0032$ and $p < 0.003$, respectively, Figures 8C, D). Concerning the locomotor activity, we did not observe any significant effect of sbGnih-2 on distance moved, maximal velocity of swimming, maximal acceleration nor mean distance between subjects (Figures 8E–H).

Discussion

Although the regulation of neurosteroid biosynthesis by neuropeptides and neurotransmitters has been extensively reported in tetrapod vertebrates (Do Rego et al., 2009; Ubuka and Tsutsui, 2022), studies in fish have mainly focused on the effects of temperature, social interactions and steroid hormones on aromatase activity and/or expression (Menuet et al., 2005; Piferrer and Blázquez, 2005; Diotel et al., 2010; Coumilleau et al., 2015). Gonadotropin-inhibitory hormone (GNIH/Gnih) is well known for playing a crucial role in the regulation of reproduction by acting on the brain and pituitary hormone synthesis and secretion (Kriegsfeld et al., 2006; Ubuka et al., 2014; Choi et al., 2016; Di Yorio et al., 2016; Ogawa et al., 2016; Paullada-Salmerón et al., 2016b; Aliaga-Guerrero et al., 2018). In light of the studies performed, this neuropeptide has also emerged as a key factor in regulating steroid hormone production both in the brain and

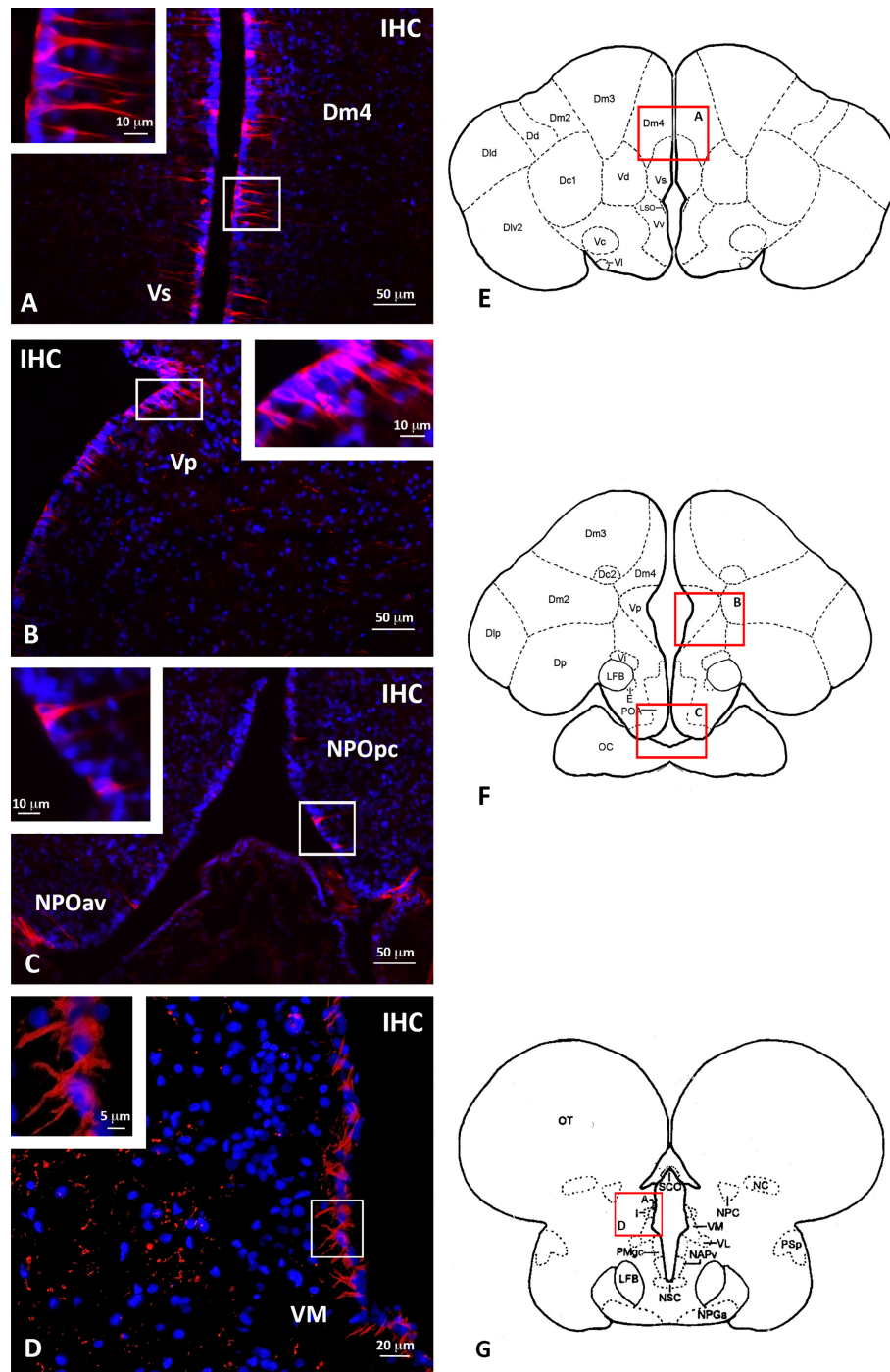


FIGURE 5

Localization of periventricular Gnih-immunoreactive (ir) cells in the telencephalon (A, B), preoptic area (C) and rostral prethalamus (D) of male sea bass. Schematic transverse sections through the brain of sea bass are shown in panels (E-G), indicating the areas with Gnih-ir cells magnified in panels (A-D, red rectangles). Insets in (A-D) show details of immunopositive Gnih perikarya present in white rectangles of these panels. Scale bars represent 50 μm in (A-C), 20 μm in (D), 10 μm in insets of panels (A-C), and 5 μm in inset of panel (D). Dm4, subdivision 4 of the medial part of the dorsal telencephalon; IHC, immunohistochemistry; NPOav, anteroventral part of the parvocellular preoptic nucleus; NPOpc, parvocellular part of the parvocellular preoptic nucleus; VM, ventromedial prethalamic nucleus; Vp, postcommissural nucleus of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon. For further abbreviations in panels (E-G) see [Cerdá-Reverter et al., 2001a](#); [Cerdá-Reverter et al., 2001b](#).

gonads ([Bentley et al., 2006](#); [Piekarski et al., 2013](#); [Paullada-Salmerón et al., 2016c](#); [Ubuka and Tsutsui, 2022](#)). Particularly, GnIH may act as a regulator of neuroestrogen synthesis in birds and mammals, suggesting a role of this neurohormone in the

control of social-sexual behaviors in these groups of vertebrates ([Ubuka et al., 2014](#); [Abdel-Aleem et al., 2019](#); [Ubuka and Tsutsui, 2022](#)). Despite remarkable advances in the knowledge of fish Gnih system in recent years, the role of this neuropeptide

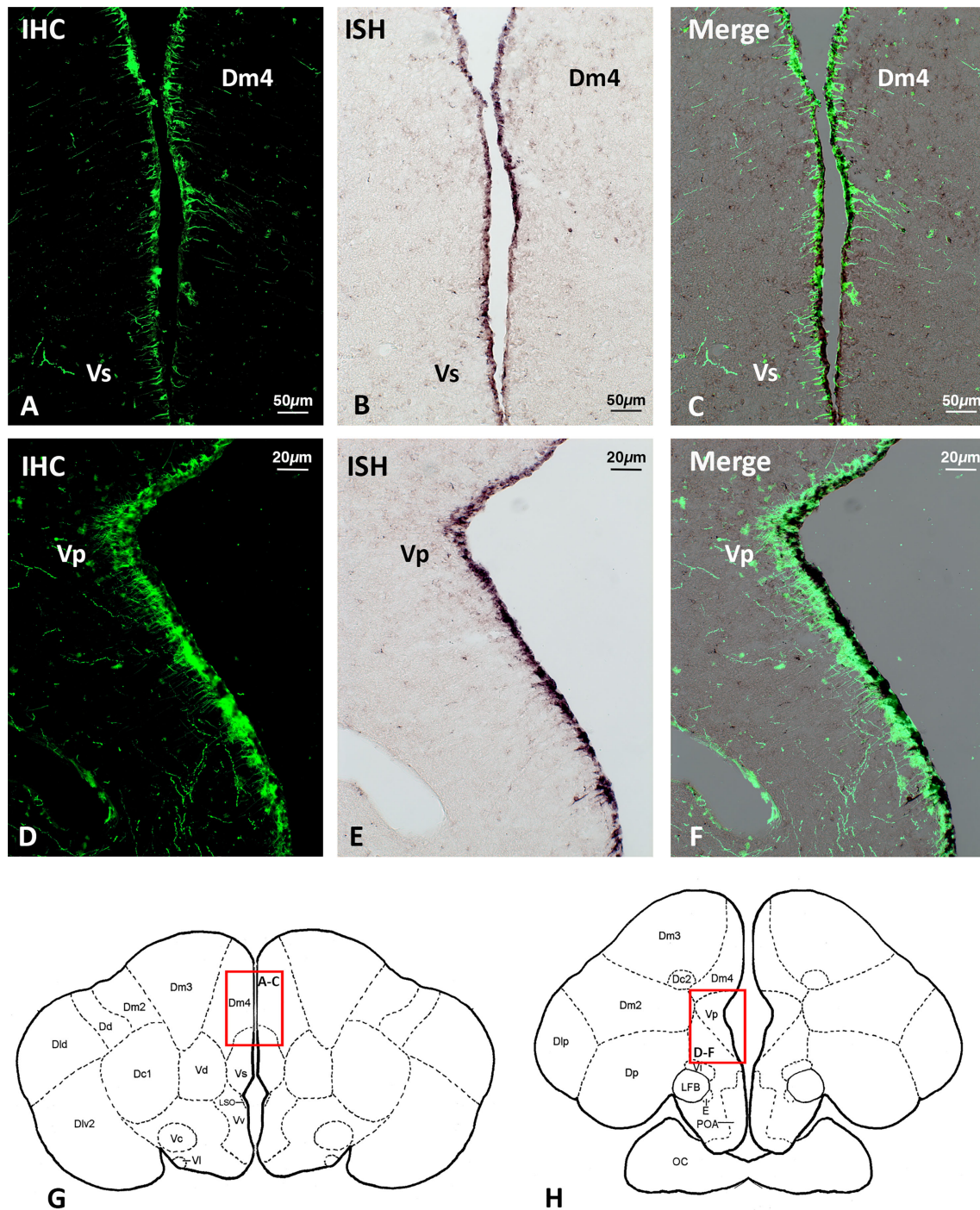
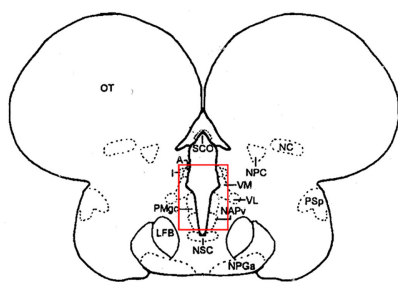
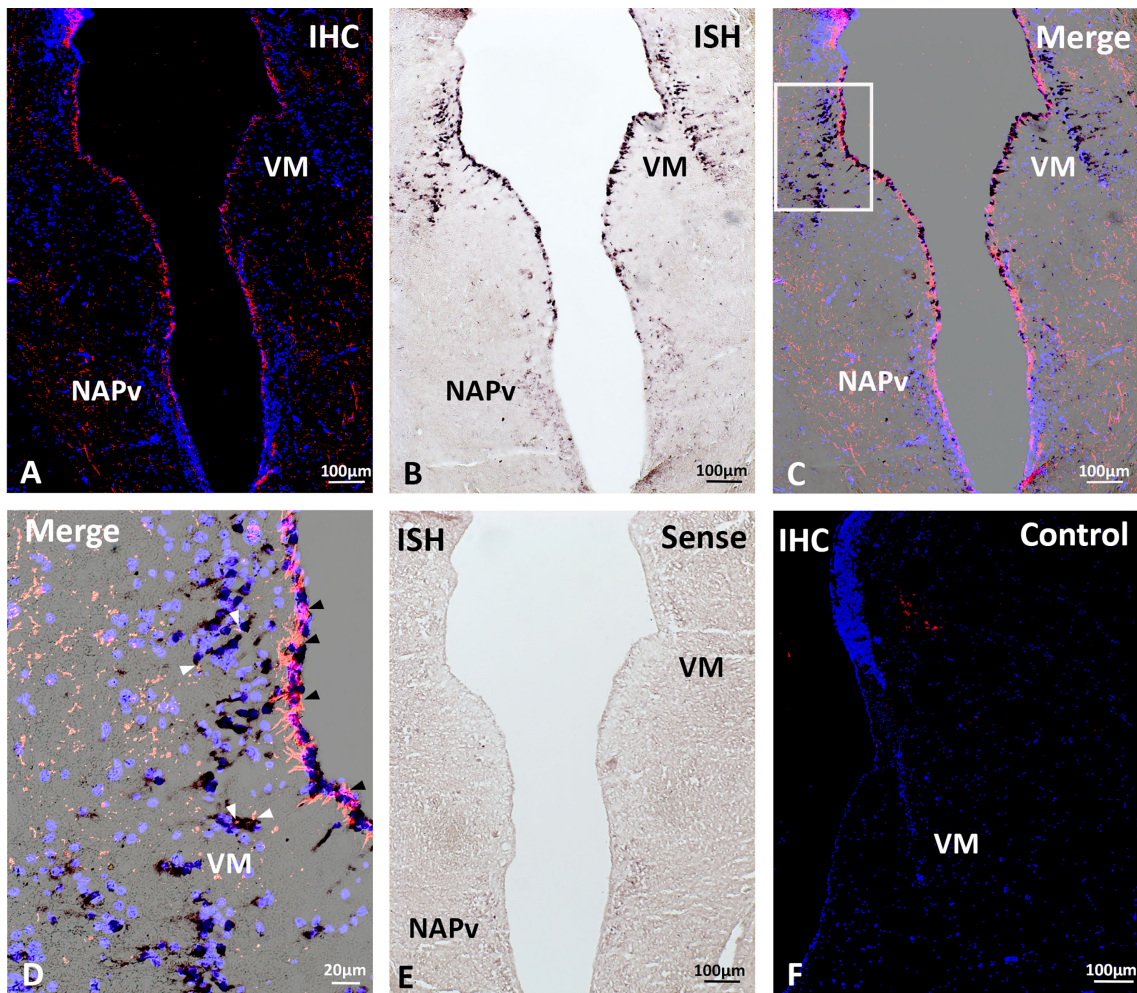


FIGURE 6

Localization of Gnih-immunoreactive (ir) cells (A, D) and brain cytochrome P450 aromatase (*cyp19b*)-expressing cells (B, E) in the periventricular telencephalon of male sea bass. Merge sections photomicrographs are provided in panels (C, F). Schematic transverse sections through the brain of sea bass are shown in panels (G, H), indicating the areas with Gnih-ir cells and aromatase-positive cells magnified in panels (A-C, G) and (D-F, H) (red rectangles). Note the overlapping distribution of subventricular Gnih-ir cells and ventricular aromatase-expressing cells along the telencephalic ventricle. Scale bars represent 50 μm in (A-C), and 20 μm in (D-F). Dm4, subdivision 4 of the medial part of the dorsal telencephalon; IHC, immunohistochemistry; ISH, *in situ* hybridization; Vp, postcommissural nucleus of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon. For further abbreviations in panels (E-G) see [Cerdá-Reverter et al., 2001a](#).

in the regulation of neurosteroid biosynthesis and its relationships in mediating social/aggressive behavior has not been addressed in fish to date. The current study revealed that intracerebroventricular administration of sbGnih-2 affected the

transcript levels of some of the main enzymes involved in neurosteroids biosynthesis (*3 β -hsd*, *17 β -hsd*, *cyp19b*), but also evoked changes in the expression levels of estrogen receptors (*er β 2*) at the brain and/or pituitary level. The present study also



G

FIGURE 7

Localization of periventricular GnRH-immunoreactive (ir) cells (A) and brain cytochrome P450 aromatase (*cyp19b*)-expressing cells (B) in the prethalamus and caudal preoptic area of male sea bass. Merge sections photomicrographs are provided in panels (C, D). Photomicrograph in panel (D) shows a detail of immunopositive GnRH cells and fibers and aromatase-expressing perikarya present in white rectangle of panel (C). Note the close apposition of GnRH immunoreactivity and aromatase-positive labelling in perikarya along the periventricular zone of the ventral prethalamic nucleus (black arrowheads). In turn, more internal aromatase-expressing cells of this nucleus appear innervated by immunopositive GnRH axon terminals (white arrowheads). Photomicrograph of panels (E, F) represent control sections of *in situ* hybridization and immunohistochemistry, respectively. Schematic transverse section through the brain of sea bass is shown in panel (G), indicating the area with GnRH-ir cells and aromatase expressing cells magnified in panels (A–D, red rectangle). Scale bars represent 100 μm in (A–C, E, F), and 20 μm in (D). IHC, immunohistochemistry; ISH, *in situ* hybridization; NAPv, anterior periventricular nucleus; VM, ventromedial prethalamic nucleus. For further abbreviations in panels (G), see [Cerdá-Reverter et al., 2001b](#).

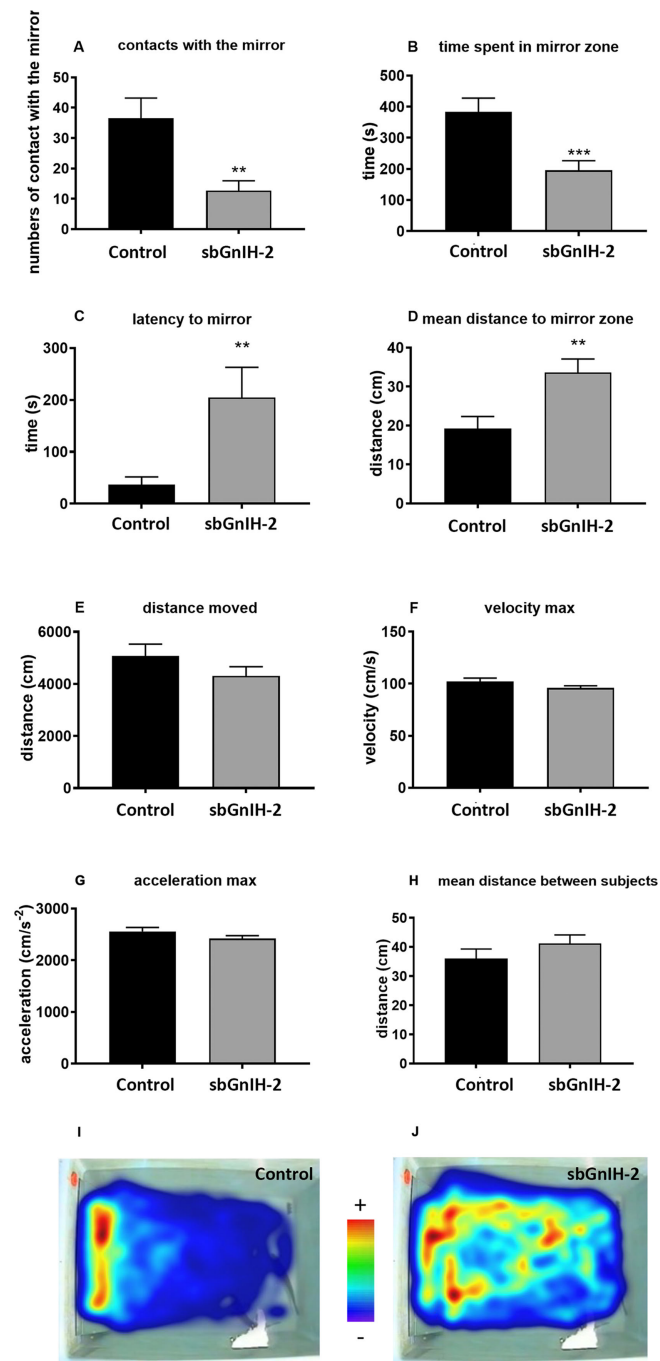


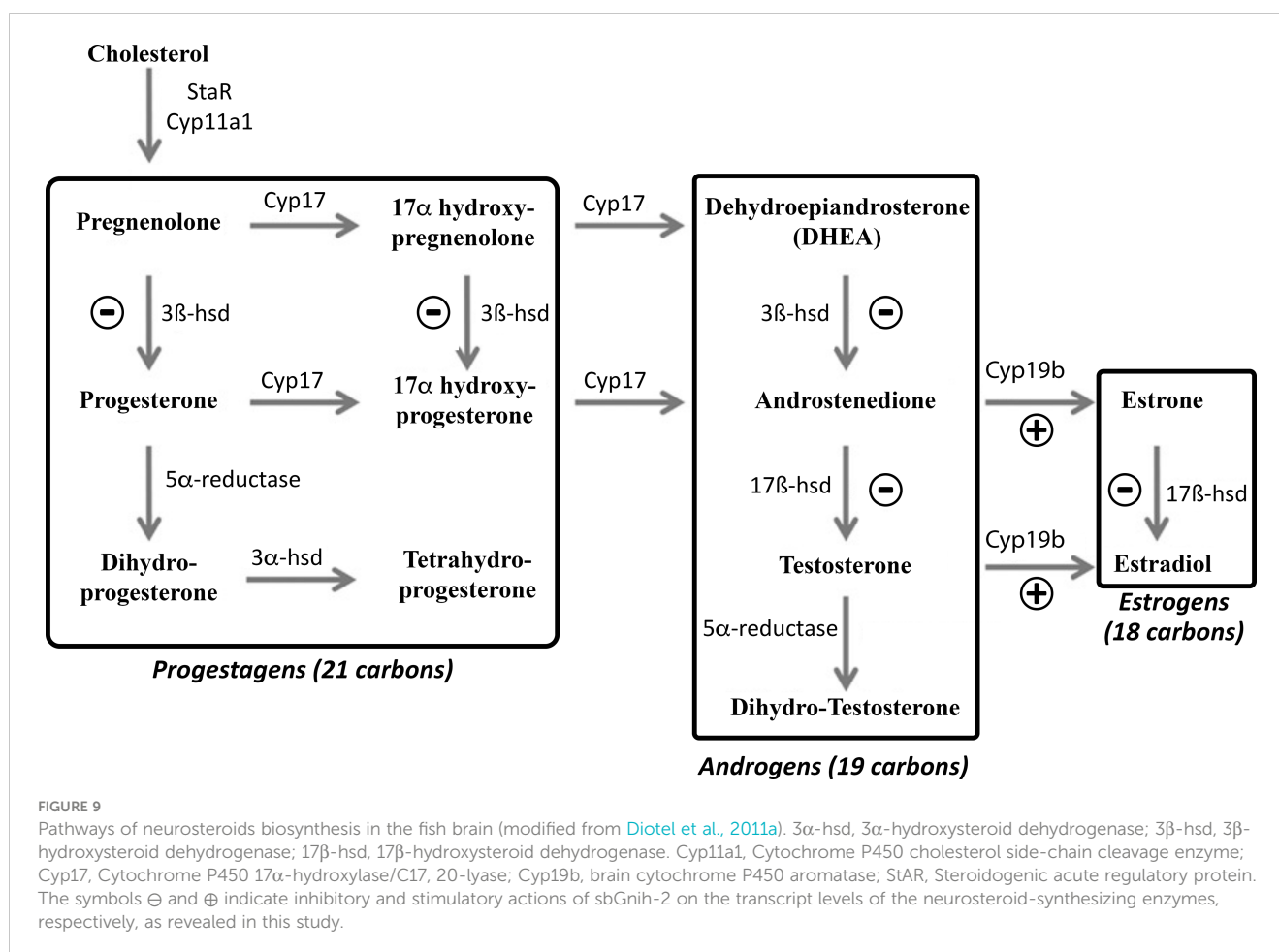
FIGURE 8

Effects of *in vivo* intracerebroventricular (icv) injection of sbGnih-2 (dose 2 μ g) on agonistic behavior and locomotor activity parameters of male sea bass as revealed by the mirror test. (A). Contacts with the mirror; (B). Time spent in the mirror zone; (C). Latency to the first contact with the mirror; (D). Mean distance to the mirror zone; (E). Distance moved during the test; (F). Maximal velocity measured during the test; (G). Maximal acceleration measured during the test; (H). Mean distance between subjects. (I). Movement heat map of control animals. (J). Movement heat map of sbGnih-2-treated animals. Results are expressed as mean \pm SEM of 15 couples per condition (n=30). Asterisks denote significant differences between mean values of Gnih-treated animals and controls by T-Student test (**, $p<0.01$; ***, $p<0.001$).

provides the first evidence of the inhibitory effect of Gnih on aggressive behavior in a teleost fish.

In the present study, we showed that central administration of sbGnih-2 peptide increased the transcripts levels of *cyp19b* (brain aromatase) in the brain of sea bass. Brain aromatase is responsible for converting androstenedione in estrone and T in E2 (Figure 9).

Therefore, this Gnih-induced increase in the expression of brain aromatase might lead to an elevation in the synthesis of neuroestrogens in the sea bass brain. In quail, GNIH stimulated in a dose-dependent manner the aromatase activity and increased E2 concentration in the preoptic area (POA), which was inhibited if GNIH was co-administered with the GNIH receptor antagonist,



RF9 or the aromatase inhibitor, fadrozole (Ubuka et al., 2014). Similar results have been obtained in mice, in which RFRP-1 stimulates *in vitro* aromatase activity in the POA (Ubuka and Tsutsui, 2022). It has been suggested that regulation of neuroestrogen synthesis by GNIH could be mediated by a decrease in the phosphorylation of the aromatase protein and the subsequent increase in its enzymatic activity (Balthazart et al., 2006; Ubuka et al., 2014). Therefore, the stimulatory actions of GNIH/Gnih on brain aromatase synthesis and/or activity appear to represent a conserved feature of vertebrate neurosteroidogenesis.

In fish, brain aromatase-expressing cells were detected mainly in the ventricular forebrain regions including the peripheral layer of the dorsal telencephalon, ventral telencephalon, preoptic area, prethalamus, thalamus, hypothalamus, and in the cerebellum (Menuet et al., 2003; Strobl-Mazzulla et al., 2005; Pellegrini et al., 2007; Okubo et al., 2011; Diotel et al., 2011a; Ramallo et al., 2017). In the sea bass, we found a similar distribution of aromatase-expressing cells in the periventricular layers of the anterior brain and particularly in the caudo-medial subdivisions of the dorsal and ventral telencephalon, preoptic area, prethalamus, thalamus and hypothalamus. In contrast to what is reported in birds and mammals, in which aromatase expression is mainly confined to neurons, brain aromatase is massively expressed in periventricular radial glial progenitor cells of teleost brain (Diotel et al., 2011a;

Coumailleau et al., 2015). Considering the distribution and appearance of aromatase-expressing cells detected in the present study by *in situ* hybridization, this seems to be true also in the sea bass brain, although some aromatase-positive cells, which adopt a more internal position in the prethalamic ventromedial nucleus, could correspond to neurons. Nevertheless, it has been shown that aromatase mRNA can be transported internally along radial processes in fish (Diotel et al., 2011b). In a previous study in the European sea bass, Gnih-ir fibers were detected in the periventricular preoptic area, but also in the dorsal and ventral telencephalon, prethalamus, thalamus and hypothalamus (Paullada-Salmerón et al., 2016a). Thus, a direct action of Gnih on aromatase-expressing cells could be envisaged in this species, as it was suggested in male quail, in which GNIH-ir neuronal fibers reach aromatase-ir cells present in the POA, which also expressed GNIH receptor mRNA (Ubuka et al., 2014). In fact, in the present study closely apposed Gnih-ir and aromatase-expressing cell bodies were detected along the periventricular surface of the dorsal (Dm4) and ventral (Vs, Vp) telencephalon and prethalamus (VM) of sea bass, whereas some aromatase-positive cells lying more internally in the VM were innervated by Gnih-ir fibers. These forebrain regions are also known for their high concentrations of estrogen receptor (ER) mRNAs or protein in sea bass and other teleost fish (Pellegrini et al., 2005; Strobl-Mazzulla et al., 2008; Muriach et al., 2008a;

Muriach et al., 2008b). Further morphofunctional studies seem necessary to characterize the presence of Gnih receptors in aromatase-expressing cells in fish.

Our research showed that treatments with sbGnih-2 resulted in a decrease in *3β-hsd* expression mRNA levels in the sea bass brain. The 3β-HSD protein is an important steroidogenic enzyme that is essential for the synthesis of progesterone from pregnenolone and 17α-hydroxyprogesterone from 17α-hydroxypregnenolone, but also converts dehydroepiandrosterone (DHEA) to androstenedione, which is, in turn, a relevant precursor for the production of testosterone (Figure 9; Sakamoto et al., 2001; Schlinger et al., 2008; Diotel et al., 2011a). Studies developed in birds have reported that 3β-HSD, working in concert with aromatase, could be involved in neurobehavioral functions through its actions on DHEA for the synthesis of estrogens (Schlinger et al., 2008; Pradhan et al., 2010). In zebrafish, *3β-hsd* mRNA expression and/or protein activity were characterized in numerous brain regions including the olfactory bulbs, ventral and dorsomedial telencephalon, preoptic area, hypothalamus, optic tectum, cerebellum and lateral recess, demonstrating that the teleost brain can synthesize neurosteroids (Sakamoto et al., 2001; Diotel et al., 2011a; Diotel et al., 2011b). It should be noted that sea bass Gnih innervation reaches the same neural areas where *3β-hsd* steroidogenic enzyme-expressing cells have been reported in the fish brain (Paullada-Salmerón et al., 2016a), which could suggest the existence of putative interactions of Gnih system with 3β-Hsd in the brain of sea bass. Further studies should be directed to identifying *3β-hsd*-expressing cells in the brain of sea bass and its tentative colocalization with Gnih cells and projections.

On the other hand, our study also evidenced a significant reduction of *17β-hsd* transcript levels in the brain and pituitary of sea bass after sbGnih-2 administration. Together with 3β-HSD, the 17β-HSD enzyme is also essential for androgen biosynthesis, by promoting the conversion of androstenedione to T, but also converts estrone into E2 in the brain of mammalian and non-mammalian vertebrates (Figure 9; Steckelbroeck et al., 1999; Mindnich et al., 2004; Hojo et al., 2004; Diotel et al., 2011a). Previous studies in fish have demonstrated that androgens arising from gonads, such as T and/or 11-KT, are involved in the regulation of aggressive and sexual behavior, possibly through their actions on brain areas related to motivational or motor pattern regulation (Remage-Healey and Bass, 2006; Remage-Healey and Bass, 2007; Lord et al., 2009; Chang et al., 2012). It is not known whether the effects of Gnih on aggressive behavior observed in the present study are mediated by peripheral E2 or, alternatively, by peripheral T converted locally to E2 by Cyp19, which could act on androgen and estrogen receptors present in the sea bass brain. However, reproductive but not aggressive behavior is affected in castrated tilapia, suggesting the involvement of independent central mechanisms in aggressive behavior (Almeida et al., 2014), which could be, at least in part, due to local production of androgens from DHEA into the fish brain (Soma et al., 2008; Wingfield et al., 2018). It is plausible to think that DHEA may also be converted to active neuroandrogens in sea bass brain through the enzymatic activities

of 3β-Hsd and 17β-Hsd and exert an influence over aggressive behavior. Therefore, Gnih might be involved in the neuroandrogen-mediated regulation of different behavioral traits by inhibiting *3β-hsd/17β-hsd* expression in sea bass brain. The concomitant Gnih-induced inhibition of *3β-hsd* and *17β-hsd* gene transcription without a reduction on *cyp17* and *cyp7b* transcript levels could determine the accumulation of DHEA. Whether alternative pathways from DHEA leading to the production of DHEA sulphate and/or 7α-hydroxy DHEA also operate in the fish brain, as it has been described in mammals (Maninger et al., 2009), needs further investigation.

Our work also showed that central administration of sbGnih-2 elicited a decrease of *17β-hsd* and *erβ2* expression at the pituitary level. The pituitary gland contains a high density of estrogen and androgen receptors in fish, including sea bass (Piferrer and Blázquez, 2005; Harbott et al., 2007; Strobl-Mazzulla et al., 2008; Muriach et al., 2008b), and neurosteroids-synthesizing enzymes such as brain aromatase (Cyp19b) have also been detected in the pituitary of rainbow trout (Menuet et al., 2003), zebrafish (Goto-Kazeto et al., 2004), pejerrey (Strobl-Mazzulla et al., 2005), sea bass (Alvarado et al., 2016) and the weakly electric fish, *Apteronotus leptorhynchus* (Shaw and Krahe, 2018). As 17β-Hsd transforms androstenedione in T and estrone in E2 (Figure 9), Gnih actions on *17β-hsd* expression could reduce T and E2 availability in the pituitary gland. In a previous study, we reported the presence of Gnih-ir fibers reaching the proximal *pars distalis* (PPD) of the sea bass pituitary gland (Paullada-Salmerón et al., 2016a). Moreover, we showed that peripherally-administered Gnih decreased T and 11-KT plasma levels at early- and mid-spermatogenesis stages, affecting the testicular gametogenesis of sea bass (Paullada-Salmerón et al., 2016c). Interestingly, a study performed in sea bass showed that castration also reduced neurosteroid-synthesizing enzymes (*cyp19b*) and estrogen receptor (*erα*) expression in the pituitary while T replacement after gonadectomy stimulated its expression to a level that was comparable to that of the control fish (Alvarado et al., 2016). Whether the Gnih actions on the pituitary reported in the present study represent direct or indirect (through gonadal feedback) effects should be elucidated. In any case, our results suggest that Gnih might be regulating the synthesis and actions of steroids not only at the brain level but also in the pituitary.

Finally, we have shown early inhibitory actions of sbGnih-2 in agonistic/aggressive behavior of sea bass using the mirror test (decreased interaction with the mirror, lower time spent in the mirror zone, increased latency to establish contact with the mirror and greater mean distance to the mirror zone) without affecting the locomotor activity (no difference in distance moved, maximum velocity and acceleration and mean distance between subjects). The short-term effects of Gnih in agonistic/aggressive behavior (15 to 45 min after Gnih administration) could be due to rapid actions on brain aromatase activity, as it has been previously reported in birds (Ubuka et al., 2014), while the subsequent effects on the transcription of *3β-hsd*, *17β-hsd* and *cyp19b* genes (6- or 12-hours post administration) could sustain long-lasting actions of Gnih on

brain neurosteroids repertoire. Several studies have reported a possible effect of the social environment on aromatase mRNA expression and/or activity within the brain of teleosts and there is strong evidence that brain aromatase is involved in the regulation of socio-sexual and aggressive behavior in vertebrates, including fish (Black et al., 2005; Black et al., 2011; Iwata et al., 2012; Huffman et al., 2013; Ubuka and Tsutsui, 2014; Ubuka et al., 2014; Ramallo et al., 2017; Quintana et al., 2021). In the weakly electric banded knifefish (*Gymnotus omarorum*), brain aromatase is upregulated in dominant but not subordinate fish (Quintana et al., 2021). Treatment with fadrozole (an aromatase inhibitor) in dominant males of the African cichlid fish *Astatotilapia burtoni*, increased aromatase expression in the preoptic area and decreased aggressive but not reproductive behavior (Huffman et al., 2013). Moreover, high aromatase mRNA expression was found in the magnocellular and gigantocellular regions of the preoptic area of subordinate male of the African cichlid fish compared to dominant fish (Huffman et al., 2013). In turn, in the simultaneous hermaphroditic bluebanded goby *Lythrypnus dalli*, a low brain aromatase activity is related to increased aggression (Black et al., 2005). As mentioned above, GNIH seems to represent a key factor in the regulation of social and reproductive behaviors in birds and mammals (Kriegsfeld et al., 2006; Ubuka et al., 2014; Abdel-Aleem et al., 2019). Thus, as reported in sea bass in the current study, icv injection of GNIH suppressed the male aggressive behavior in male quail as quickly as 30 min after its administration (Ubuka et al., 2014). As mentioned above, centrally administered GNIH also activates short term aromatase activity (probably by dephosphorylation of phosphorylated aromatase) and increases neuroestrogen synthesis in the POA and the administration of GNIH antagonist RF9 inhibited the action of GNIH on aromatase and neuroestrogen synthesis in the brain of quail. Furthermore, RNA interference of *GNIH* gene decreased diencephalic *GNIH* mRNA expression and concentration, as well as the density of GNIH-immunoreactive fibers in the ventral tegmental area, increasing the locomotor activity and aggressive-sexual behavior, which suggests that GNIH may play a role in the social interactions controlling these behavioral responses in birds (Ubuka et al., 2013; Ubuka et al., 2014).

In conclusion, our molecular results provide the first evidence of the Gnih actions on the gene expression of neurosteroid-synthesizing enzymes (*3 β -hsd*, *17 β -hsd* and *cyp19b*) in a fish species, the European sea bass, which could inhibit the production of aromatizable neuroandrogens (i.e., androstenedione and testosterone) and promote their conversion into neuroestrogens (i.e., estrone and estradiol). In turn, neuroanatomical data suggest that paracrine but also neuroendocrine actions could mediate the Gnih effects on brain aromatase expression in this species. The elucidation of the mechanisms underlying the short-term behavioral effects of Gnih, the subsequent actions of this neuropeptide on the gene expression of neurosteroidogenic enzymes, and whether Gnih actions on the transcription of neurosteroid-synthesizing genes and/or neurosteroids levels are also involved in other processes (e.g., neurogenesis, sex differentiation, puberty and/or seasonal reproduction) in sea bass will require further investigation in future studies.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Materials](#), further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by Ethics Committee of the University of Cádiz (Spain), Ethics Committee of the Oceanological Observatory of Banyuls-sur-Mer (France), and the General Directorate for Agricultural and Livestock Production of the Regional Government of Andalusia (approval number 15/12/2020/143 and 15/12/2020/144).

Author contributions

JAM-C contributed to the conception of the study, with the help of TU and ELM. JAP-S and GHL performed the icv Gnih injections and gene expression analysis. JAP-S performed the immunohistochemical and *in situ* hybridization analyses. GHL, MF and LB planned and performed the behavioral study. JAP-S and JAM-C wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author TU is employed by International Cancer Laboratory Co., Ltd.

The remaining 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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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1185652/full#supplementary-material>

SUPPLEMENTARY MATERIAL

Behavioral video recording of control and sbGnih-2 intracerebroventricularly-injected male sea bass.

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