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Cloning and Expression of Gonadotropin-Releasing Hormone Receptor in the Brain and Pituitary of the European Sea Bass: An In Situ Hybridization Study[1]

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ABSTRACT

A full-length cDNA encoding a GnRH receptor (GnRH-R) has been obtained from the pituitary of the European sea bass, *Dicentrarchus labrax*. The complete cDNA is 1814 base pairs (bp) in length and encodes a protein of 416 amino acids. The 5′ UTR and 3′ UTR are 239 bp and 324 bp in size, respectively. The expression sites of this GnRH-R were studied in the brain and pituitary of sea bass by means of in situ hybridization. A quantitative analysis of the expression of the GnRH-R gene along the reproductive cycle was also performed. The GnRH-R brain expression was especially relevant in the ventral telencephalon and rostral preoptic area. Some GnRH-R messenger-expressing cells were also evident in the dorsal telencephalon, caudal preoptic area, ventral thalamus, and periventricular hypothalamus. A conspicuous and specific GnRH-R expression was detected in the pineal gland. The highest expression of the GnRH-R gene was observed in the proximal pars distalis of the pituitary. This expression was evident in all LH cells and some FSH cells but not in somatotrophs. In the pituitary, the quantitative analysis revealed a higher expression of GnRH-R gene during late vitellogenesis in comparison with maturation, spawning, and postspawning/resting periods. However, in the brain, the highest GnRH-R expression was evident at spawning or postspawning/resting periods. These results suggest that the expression of this GnRH-R is regulated in a different manner in the brain and the pituitary of sea bass.

central nervous system, gonadotropin-releasing hormone receptor, neuroendocrinology, pituitary, seasonal reproduction

INTRODUCTION

The decapeptide GnRH plays a major role in controlling the release of gonadotroph [1], but has also been implicated in the stimulation of growth hormone [2] and prolactin [3] secretion. However, increasing evidence suggests that GnRH serves important autocrine/paracrine actions in extrapituitary tissues such as gonad, liver, kidney, placenta, breast, and prostate [4, 5]. In addition, GnRH may also act in the brain to modulate nesting [6] and sexual behavior [7], but the precise sites of GnRH actions in the central nervous system are unknown.

In perciforms, three different GnRH forms are expressed, seabream GnRH (sbGnRH), salmon GnRH (sGnRH) and chicken II GnRH (cGnRH-II) [8]. It is clearly stated that the sbGnRH form represents the main hypophysiotrophic hormone [8, 9], whereas the roles of the two other GnRH forms expressed, sGnRH and cGnRH-II, remain still unknown. A critical step in the elucidation of the precise functions of different GnRH forms in the brain and pituitary is the study of their respective projections and targets. Recently, an immunohistochemical study performed in sea bass, using antibodies against the divergent GnRH-associated peptides of each GnRH precursor, unambiguously revealed the distinct pattern of projections of the three different GnRH systems expressed in this species [10].

The first step in GnRH action is the recognition of the hormone by specific membrane-associated receptors, which belong to the G-protein-coupled receptor family [1]. Since the first cloning of GnRH receptor (GnRH-R) in mouse [11, 12], cDNA encoding the GnRH-R has been characterized in a number of mammalian species [13–17]. The analysis of the primary sequence reveals that GnRH-R is a single polypeptide containing seven hydrophobic transmembrane domains connected by hydrophilic extracellular and intracellular loops [1]. Recently, cDNAs for GnRH-R have been cloned and functionally characterized in different teleosts [18–23]. Emerging data obtained in vertebrates and phylogenetic analyses have suggested the existence of different GnRH-R subtypes with distinct structural and/or pharmacological characteristics, different gene structures and/or tissue distributions [19, 23–28].

The correlation of the pattern of projections of the different GnRH systems with the expression of GnRH receptors could greatly contribute to improving the knowledge of real hormone-receptor interactions and to clarifying the precise functions of multiple GnRH forms in the control of reproduction and other physiological processes. To obtain such information, we have cloned a full-length GnRH-R cDNA and analyzed, using in situ hybridization techniques, the qualitative and quantitative expression of this receptor in the brain and pituitary of the European sea bass along the reproductive cycle.

MATERIAL AND METHODS

Animals

European sea bass, *Dicentrarchus labrax*, specimens from the experimental fish laboratories of the Instituto de Acuicultura de Torre La Sal

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(CSIC, Castellón, Spain) were kept in running sea water with natural photoperiod and temperature conditions. Twenty-four females were killed along the reproductive cycle, in November 1999 (late vitellogenesis; n = 6), December 1999 (maturation; n = 6), February 2000 (spawning; n = 6), and May 2000 (postspawning/resting; n = 6). All animals were treated in agreement with the European Union regulation concerning the protection of experimental animals.

Cloning of a Full-Length cDNA for a Sea Bass GnRH-R

Total RNA was prepared from pituitaries of vitellogenic females (GSI > 5%) using the TRIzol reagent (Gibco-BRL, Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Reverse transcription was performed with 5 μg of total RNA using 400 U of M-MLV Reverse Transcriptase (Gibco-BRL) in the presence of random hexanucleotide primers (200 ng) for 1 h at 37°C.

The full-length coding region was obtained by polymerase chain reaction (PCR) using degenerate primers designed from conserved sequences surrounding the start and the stop codon in other perciform fish, i.e., the striped bass (*Morone saxatilis*; [22]), the amberjack (*Seriola dumerili*; GenBank AJ130876), and the sea bream (*Sparus aurata*; N. Zmora, personal communication). Sequences of these primers were SB-for 5′ TCAGAAAATGRACACCACTCT 3′ and SB-rev 5′ CTCATAKGWTGCTSTCAGAG 3.′ PCR products of the expected size (about 1300 base pairs [bp]) were purified and cloned in the *Eco*RV site of the Bluescript plasmid for sequencing.

The cloning of the 3′ and 5′ extremities of the cDNA was then performed using the 5′/3′ RACE kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocols. Briefly, for the amplification of the 5′ flanking region, 1.5 μg of total RNA were reverse transcribed using a specific primer (SB-5R1: 5′ ATTGATAGCCAGAGGGTTGAG 3′). After the addition of a poly(A) tail at the 3′ flanking region of the first-strand cDNA using a terminal transferase, a first amplification was performed using an internal specific primer (SB-5R2: 5′ GGGTTGAGGATGGCTGACTGC 3′) and an oligo-d(T)-anchor primer (Roche Diagnostics). A nested PCR was carried out using 1 μl of the first reaction as template with the primer SB-5R3 (5′ CACGGTGACAAAGGCGCAAGA 3′) and a second anchor primer (Roche Diagnostics). A single PCR product of about 700 bp was obtained and cloned in the pCDNA3.1-TOPO cloning vector (Invitrogen) for sequencing. For the amplification of the 3′ flanking region, reverse transcription was performed on 2 μg of total RNA using an oligo-d(T) primer. A first PCR was performed using a specific primer (SB-3R1: 5′ TTTCCTGCCTGTTCCTGC TGC 3′) and the oligo-d(T)-anchor primer (Roche Diagnostics). A second PCR was then realized using a nested specific primer (SB-3R2: 5′ GAATAACATCCCCAGAGCCCG 3′) and the second anchor primer (Roche Diagnostics). A single PCR product of about 800 bp was obtained and cloned in the same plasmid as above for sequencing. The cDNA sequence corresponding to the sea bass GnRH receptor has been submitted to the GenBank under the accession number AJ419594.

Sequence Analysis

Multiple alignments and phylogenetic analysis were carried out using ClustalW. This analysis was performed using the amino acid sequences of GnRH receptors available from GenBank. The sequence sources and GenBank accession numbers are as follows: *Drosophila melanogaster* (AF077299), japanese eel (AB041327), catfish (catfish-1, X97497; catfish-2, AF329894), goldfish (goldfish A, AF121845; goldfish B, AF121846), rainbow trout (AJ272116), medaka (medaka1, AB057675; medaka2, AB057674), amberjack (AJ130876), *Haplochromis burtoni* (AY028476), striped bass (AF218841), rubber eel (AF174481), *Xenopus* (*Xenopus* I, AF172330; *Xenopus* II, AF257320), bullfrog (bullfrog-1, AF144063; bullfrog-2, AF15913; bullfrog-3, AF144062), chicken (AJ304414), mouse (L01119), rat (S59525), brushtail possum [29], sheep (L22215), bovine (U00934), horse (AF018072), dog (AF206513), pig (AH009128), marmoset monkey [30], bonnet monkey (AF156930), rhesus monkey (AF353987), and human (human I, L03380; human II, NM057163).

In Situ Hybridization

The preparation of [α-^{35}S] dUTP-labeled single-stranded mRNA probes was carried out according to standard procedures. Sense and antisense sea bass GnRH-R riboprobes were synthesized using p-Bluescript-sbGnRH-R transcription vector as a template and then linearized with *Bam*HI or *Eco*RI with T3 and T7 RNA polymerase, respectively. One microgram of the linearized plasmid was incubated for 1 h at 37°C in a solution containing a transcription buffer (Tris-HCl 40 mM, $MgCl_2$ 6 mM, Spermidine 2 mM), 10 mM dithiothreitol (DTT), rATP, rGTP, rCTP (0.25 mM each), 100 μCi of [α-^{35}S] dUTP (MP Biomedicals, Irvine, CA), RNase inhibitor, and 2.5 U of the appropriate RNA polymerase. The DNA template was then digested with RQ-1 DNase for 15 min at 37°C. Probes were purified on a Sephadex G50 column (Amersham Biosciences, Little Chalfont, UK) equilibrated with 50 μg of yeast tRNA using a loading buffer (Tris-HCl 10 mM, pH 7.5; EDTA 1 mM; DTT 10 mM; 0.1% SDS). The fractions containing the highest amount of radioactivity were pooled and the probes were precipitated overnight at −20°C and resuspended in the hybridization mix (50% formamide; 0.3 M NaCl; 20 mM Tris-HCl, pH 8.5; 5 mM EDTA; 10% dextran sulfate; 1× Denhardt solution; 10 mM DTT; 0.5 μg/μl yeast tRNA) at a concentration of 2×10^4 cpm/μl.

The protocol for in situ hybridization was according to González-Martínez et al. [31]. Brains were collected from animals anesthetized in phenoxyethanol (0.3 ml/L), previously perfused with 0.65% NaCl and fixative solution (4% paraformaldehyde; 0.1 M phosphate buffer, pH 7.4; 5% picric acid). Transverse paraffin brain and pituitary sections (6 μm thick) were equilibrated at room temperature, rehydrated, and postfixed for 20 min. Sections were treated with protein kinase (20 μg/ml in 50 mM Tris-HCl, pH 8, and 5 mM EDTA) for 7.5 min and washed with PBS for 5 min, followed by a refixation in 4% paraformaldehyde for 5 min and a quick wash with distilled water. Thereafter, sections were acetylated for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8), dehydrated through increasing concentrations of ethanol, and air dried. The sections were covered with the hybridization mix (20×10^3 cpm/slide), coverslipped, and incubated overnight at 52°C. Coverslips were then removed by immersion in a 5× SSC, 10 mM DTT solution at 55°C for 30 min, and then washed in a 2× SSC, 50% formamide, 10 mM DTT solution at 65°C for 30 min followed by several washes in NTE buffer (10 mM Tris-HCl, 0.5 M NaCl, 5 mM EDTA) for 10 min at 37°C. To degrade single-stranded probe, the sections were incubated in a solution of NTE containing RNase A (20 μg/ml) for 30 min at 37°C. Sections were then rinsed in 2× SSC, 50% formamide, 10 mM DTT at 65°C, washed in 2× SSC and 0.1× SSC at room temperature, and dehydrated in ethanol. Slides were then dipped into an Ilford K5 photographic emulsion and exposed for 12 days at 4°C, developed, and counterstained with toluidine blue 0.02%. Adjacent sections were systematically treated with the sense and antisense probes.

In situ hybridization sections were analyzed on a Leica photomicroscope (Leica Microsystems AG, Wetzlar, Germany) equipped with bright field and dark field, and computer images were obtained with a Sony DKC-CM30 Digital Camera (Sony, Tokyo, Japan). The software used was Adobe PhotoShop 5.5 (Adobe Systems Incorporated, San Jose, CA) and no subsequent alterations were made. For the precise localization of GnRH-R-expressing cells, we have used a detailed sea bass brain atlas recently developed in our laboratory [32, 33].

For double in situ hybridization studies, nonisotopic riboprobes for sea bass luteinizing hormone (LH) and follicle-stimulating hormone (FSH) β subunits [34] were synthesized using a digoxigenin (DIG)-RNA labeling mix (Roche Diagnostics) according to the manufacturer's instructions. DIG-labeled riboprobes were dissolved in an appropriate volume of diethyl pyrocarbonate-treated H_2O to obtain 200 ng probe/μl. After 5 min incubation at 80°C, both ^{35}S-UTP and DIG riboprobes were simultaneously diluted 1:10 (final concentration of probes, 2×10^4 cpm/μl and 20 ng/μl, respectively) in hybridization buffer. Subsequently, 100 μl of hybridization solution containing either DIG-labeled LH-β or FSH-β riboprobes and ^{35}S-UTP labeled sbGnRH-R riboprobe were added to each pretreated slide. The posthybridization procedure was as above, but before dehydration, slides were washed for 10 min at room temperature in buffer A (100 mM Tris-HCl, pH 7.5; 150 mM NaCl) and then incubated in blocking solution (2% blocking reagent [Roche Diagnostics] in buffer A) for 30 min at room temperature. Subsequently, the slides were incubated with anti-DIG Fab fragments from sheep conjugated to alkaline phosphatase (Roche Diagnostics) diluted 1:1000 in blocking solution for 1 h at room temperature. The slides were then rinsed twice in buffer B (100 mM Tris-HCl, pH = 9.5; 50 mM $MgCl_2$; 100 mM NaCl). For signal detection, a NBT (4-nitroblue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolyl-phosphate) ready-to-use tablet (Roche Diagnostics) was diluted in buffer B containing 1 mM levamisole (Sigma, St. Louis, MO) and the slides were incubated in chromogenic solution for 1 h at room temperature. After color development, slides were dehydrated in ethanol solutions containing 0.3 M ammonium acetate, dipped in photographic emulsion (Amersham Biosciences), exposed under dry conditions at 4°C and developed after 12 days.

In addition, GnRH-R in situ hybridization was combined with GH immunohistochemistry using a streptavidin-biotin-peroxidase complex method. In this case, pituitary sections were first processed for in situ

hybridization using [α-^{35}S] dUTP-labeled GnRH-R riboprobes, as reported above, and subsequently they were incubated overnight in a moist chamber at room temperature with a rabbit antiserum against recombinant seabream GH (kindly donated by Dr. Valdivia) diluted 1:1000 in Coons buffer (CBT; 0.01 M Veronal, 0.15 M NaCl) containing 0.1–0.2% Triton X-100 and 0.5% casein. Sections were washed in CBT (2 × 15 min) and incubated for 1.5 h at room temperature with Biotin-sp-Conjugated-AffiniPure Goat Anti-rabbit-IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted 1:1000 in CBT. After washing in CBT (2 × 15 min), sections were incubated 1.5 h at room temperature with peroxidase-conjugated-streptavidin complex (Jackson ImmunoResearch Laboratories Inc.) diluted 1:1000 in CBT. Finally, sections were washed in CBT followed by Tris-HCl (0.05 M, pH 7.4) and peroxidase activity was visualized in 0.05 M Tris-HCl, pH 7.6, containing 0.025% 3,3 diaminobenzidine tetrahydrochloride (Sigma) and 0.01% hydrogen peroxide. Controls were performed by preabsorption of primary antisera with their respective antigens, replacement of primary antisera with the corresponding preimmune sera, and omission of primary or biotinylated antisera.

Quantitative Analysis of GnRH-R Expression in Brain and Pituitary

For quantitative analysis of GnRH-R expression along the reproductive cycle, at least 60 brain and pituitary sections (10 sections/animal), hybridized with the antisense or sense probe, were randomly selected at each reproductive stage (late vitellogenesis, maturation, spawning, and post-spawning/resting). Randomly selected bright field pictures (30 pictures/animal) covering 1100 μm^2 of surface in positive cell areas were obtained in the photomicroscope at high magnification with the help of a digital camera. To measure the percentage of the total pituitary area occupied by GnRH-R-expressing cells along the reproductive cycle, 60 antisense whole pituitary sections (10 sections/animal) were selected at random at each reproductive stage and randomly selected dark field pictures (30 pictures/animal) covering 0.146 mm^2 of surface were obtained at low magnification with the help of a digital camera. The area occupied by autoradiographic silver grains was measured with the help of the Scion Image Beta 4.0.2 software (NIH, Rockville, MD) and results were presented as mean ± SEM. The statistical analysis was performed with the help of the SPSS 10.0 program (SPSS Inc., Chicago, IL). As requirements of normality and homogeneity of variances were not satisfied, the Kruskall-Wallis nonparametric analysis of variance method was used followed by the Mann-Whitney and Wilcoxon tests. To compare the percentage of the total pituitary area occupied by GnRH-R-expressing cells along the reproductive cycle, a proportion test was used. Differences between mean values were considered significant at $P < 0.05$.

RESULTS

Cloning of a Full-Length cDNA Encoding a Sea Bass GnRH Receptor and Sequence Analysis

Using degenerate primers surrounding the start and the stop codons, we have cloned a cDNA containing the full-coding sequence of a sbGnRH-R. The sequence was then completed by obtaining the 5′ and 3′ untranslated region (UTR) using the rapid amplification of cDNA ends-PCR method. The complete cDNA was 1814 bp in length and encoded a predicted protein of 416 amino acids. The 5′ UTR and 3′ UTR were 239 bp and 324 bp in size, respectively. The complete nucleic acid sequence as well as the deduced amino acid sequence are shown in Figure 1. Hydrophobic analysis of the protein sequence showed an arrangement in seven transmembrane domains that is typical of a G protein-coupled receptor (Fig. 2). The structure of the molecule showed the presence of a C-terminal tail and restoration of Asp^{96}, which is shared by the rest of GnRH-R present in perciforms. Moreover, the conserved Cys^{25} and Cys^{123} were present and four potential N-linked glycosylation sites were evident in the NH_2-terminal region (Fig. 2). Sequence comparison was performed using ClustalW and a rooted phylogenetic tree was constructed by the addition of the *Drosophila* GnRH receptor-like sequence (Fig. 3). This analysis revealed a very high similarity with the GnRH-R cloned in other perciforms. Indeed, the sea bass GnRH-R shared about 96% and 90% of identity with striped bass GnRH-R and amberjack GnRH-R, respectively. The sbGnRH-R also showed 67% of identity with one of the receptors cloned in medaka and more than 55% of identity with an amphibian receptor cloned in the bullfrog, the bfGnRH-R-1. The identity with the GnRH-R of other vertebrates was about 40%.

Localization of GnRH-R mRNA in the Brain and Pituitary of the European Sea Bass

The cloned sequence was revealed as a specific and useful probe for the precise localization of expression of GnRH-R gene in the brain and pituitary of sea bass by in situ hybridization. The specificity of the signal was systematically checked on adjacent control sections incubated with the sense probe, which only showed uniform background. In sea bass, GnRH-R was only detected in the forebrain, pineal, and adenohypophysis (Fig. 4). In the telencephalon, most GnRH-R-expressing cells were found in the ventral telencephalon (Figs. 4 and 5, A, B, and F) and, particularly, in the central (Fig. 5B), ventral (Fig. 5B) and intermediate (Fig. 5F) nuclei. Furthermore, a conspicuous GnRH-R expression was observed in cells of the central nucleus of the dorsal telencephalon (Fig. 5, C and D). In the diencephalon, most GnRH-R-expressing cells appeared in the preoptic area (Figs. 4 and 5, E–H), especially in the anteroventral (Fig. 5, E and F) and parvocellular (Fig. 5F) parts of the parvocellular preoptic nucleus. Further caudal, a weaker GnRH-R expression was evident in the anterior periventricular nucleus (Fig. 5G) and the gigantocellular part of the magnocellular preoptic nucleus (Fig. 5H).

In addition, a few GnRH-R-expressing cells were detected in the ventromedial thalamic nucleus (Figs. 4 and 5I). The most caudal GnRH-R-expressing cells appeared in the hypothalamus, particularly in the ventral part of the lateral tuberal nucleus (Figs. 4 and 5J) and in the nucleus of the lateral recess (Figs. 4 and 5K). A clear, specific radiolabeling was also evident in cells of the pineal gland (Figs. 4 and 5L). The highest expression of GnRH-R gene was observed in the pituitary (Fig. 6). Positive cells appeared as compact cell groups in the proximal pars distalis, but also in clusters and lining the external border of the pars intermedia (Fig. 6, A–D). Double in situ hybridization revealed that virtually all LH cells expressed GnRH-R (Fig. 6E) but only a few FSH cells exhibited a remarkable GnRH-R expression (Fig. 6F). However, sea bass GH-immunoreactive cells were not positive for GnRH-R (Fig. 6G).

Quantitative Analysis of Brain and Pituitary GnRH-R Expression along the Reproductive Cycle

In the brain, the quantitative analysis of cell masses exhibiting specific radiolabeling revealed the existence of changes in the expression of GnRH-R along the reproductive cycle. Values obtained were expressed as mean area occupied by silver grains in $\mu m^2/1000\ \mu m^2$ of brain section ± SEM, and appear represented in Figure 7. The highest GnRH-R expression was observed in the preoptic area, particularly in the anteroventral part of the parvocellular preoptic nucleus (NPOav). In this nucleus, the GnRH-R expression was low during late vitellogenesis and maturation periods and increased notably during the spawning season (78.25 ± 11.69; $P < 0.001$), decreasing significantly at the postspawning/resting period. In the remaining brain nuclei

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   1                                    agcgctggatagcagaggagctggacagctgcagagga
  39 tgtcggtgaacctgctaaacttacagcagactacccatggaagaccaccttacacgaagcttccgtc
 106 ctgcgaagatttttaaatacctggggtctcaaaagtgcgcaaaaggctaaacccagaactctctcca
 173 atgcacaagaaccgctgatcttcatggtgaatgagcagaatgtttcttcctctgcgaccatccgaaa
 240 ATG AAC ACC ACT CTG TGT GAC TCT GCC GTG GCC TTG TAT CAC CTG ACG ACA
     Met Asn Thr Thr Leu Cys Asp Ser Ala Val Ala Leu Tyr His Leu Thr Thr   17
 291 GAC CAC CAA CTG AAC GCC AGC TGC AAC TAC TCC TCG CCT ACG TCC AAC TGG
     Asp His Gln Leu Asn Ala Ser Cys Asn Tyr Ser Ser Pro Thr Ser Asn Trp   34
 342 ACA TCG GGG GGT GGC GCC CTG CAG CTG CCC ACA TTC ACC ACA GCG GCC AAA
     Thr Ser Gly Gly Gly Ala Leu Gln Leu Pro Thr Phe Thr Thr Ala Ala Lys   51
 393 GTC AGA GTG ATC ATC ACC TGC ATT CTC TGT GGT ATA TCG GCC TTT TGC AAC
     Val Arg Val Ile Ile Thr Cys Ile Leu Cys Gly Ile Ser Ala Phe Cys Asn   68
 444 CTT GCA GTG CTG TGG GCG GCA CAC AGC GAT GGG AAG CGT AAA TCC CAC GTC
     Leu Ala Val Leu Trp Ala Ala His Ser Asp Gly Lys Arg Lys Ser His Val   85
 495 AGG GTG TTG ATA ATC AAC CTG ACT GTG GCT GAT TTG CTC GTG ACC TTC ATC
     Arg Val Leu Ile Ile Asn Leu Thr Val Ala Asp Leu Leu Val Thr Phe Ile  102
 546 GTG ATG CCT GTG GAT GCC GTG TGG AAC ATC ACA GTC CAG TGG CTT GCT GGG
     Val Met Pro Val Asp Ala Val Trp Asn Ile Thr Val Gln Trp Leu Ala Gly  119
 597 GAC CTT GCC TGC AGG CTA CTG ATG TTC CTA AAG CTG CAG GCG ATG TAC TCT
     Asp Leu Ala Cys Arg Leu Leu Met Phe Leu Lys Leu Gln Ala Met Tyr Ser  136
 648 TGC GCC TTT GTC ACC GTG GTG ATT AGT CTG GAT AGG CAG TCA GCC ATC CTC
     Cys Ala Phe Val Thr Val Val Ile Ser Leu Asp Arg Gln Ser Ala Ile Leu  153
 699 AAC CCT CTG GCT ATC AAT AAG GCC AGA AAG AGG AAC AGA GTC ATG CTG ACT
     Asn Pro Leu Ala Ile Asn Lys Ala Arg Lys Arg Asn Arg Val Met Leu Thr  170
 750 GTG GCC TGG GGC ATG AGT GTC GTG CTG TCA GTC CCA CAG TTA TTC CTT TTT
     Val Ala Trp Gly Met Ser Val Val Leu Ser Val Pro Gln Leu Phe Leu Phe  187
 801 CAC AAT GTG ACC ATC ATC CAT CCC GAG GAC TTC ACT CAG TGT ACC ACA CGT
     His Asn Val Thr Ile Ile His Pro Glu Asp Phe Thr Gln Cys Thr Thr Arg  204
 852 GGA AGT TTT GTC ACT CAC TGG CAC GAA ACG GCC TAC AAC ATG TTC ACT TTT
     Gly Ser Phe Val Thr His Trp His Glu Thr Ala Tyr Asn Met Phe Thr Phe  221
 903 TCC TGC CTG TTC CTG CTG CCG CTG GTC ATC ATG ATC ACC TGC TAC ACC AGG
     Ser Cys Leu Phe Leu Leu Pro Leu Val Ile Met Ile Thr Cys Tyr Thr Arg  238
 954 ATC TTC TGT GAG ATC TCC AAA CGA ATG AAA AAG GAC AAC TTG CCC TCT AAT
     Ile Phe Cys Glu Ile Ser Lys Arg Met Lys Lys Asp Asn Leu Pro Ser Asn  255
1005 GAA GTG CAT TTG CGG CGT TCG AAG AAT AAC ATC CCC AGA GCC CGG ATG AGA
     Glu Val His Leu Arg Arg Ser Lys Asn Asn Ile Pro Arg Ala Arg Met Arg  272
1056 ACT CTA AAA ATG GGT ATT GTG ATT GTG TCG TCT TTC ATC GTC TGC TGG ACT
     Thr Leu Lys Met Gly Ile Val Ile Val Ser Ser Phe Ile Val Cys Trp Thr  289
1107 CCA TAC TAC CTG CTG GGA CTG TGG TAC TGG TTC TTC CCC GAC GAC CTG GAG
     Pro Tyr Tyr Leu Leu Gly Leu Trp Tyr Trp Phe Phe Pro Asp Asp Leu Glu  306
1158 GGG AAG GTC TCC CAT TCA CTG ACC CAC ATC CTG TTC ATC TTT GGG CTC GTC
     Gly Lys Val Ser His Ser Leu Thr His Ile Leu Phe Ile Phe Gly Leu Val  323
1209 AAC GCT TGC CTC GAC CCG GTC ATC TAC GGC CTG TTC ACC ATT CAC TTC CGA
     Asn Ala Cys Leu Asp Pro Val Ile Tyr Gly Leu Phe Thr Ile His Phe Arg  340
1260 AAG GGG CTC CGG AGG TAT TAC TGC AAC GCC ACC AAG GCA GCC GAC CTG GAT
     Lys Gly Leu Arg Arg Tyr Tyr Cys Asn Ala Thr Lys Ala Ala Asp Leu Asp  357
1311 AAC AAC ACG GTT ATA ACC GGA TCC TTC ATT TGT GCT GCC AAC TCG TTG CCA
     Asn Asn Thr Val Ile Thr Gly Ser Phe Ile Cys Ala Ala Asn Ser Leu Pro  374
1362 CTG AAA AGA GAG GCC AGC CAG GAG AGG TTC ATG TTG TAC AGC GAT AAC CAC
     Leu Lys Arg Glu Ala Ser Gln Glu Arg Phe Met Leu Tyr Ser Asp Asn His  391
1413 AGC AGA GCA GAG TCG ACG TCG CCA AGA AGC AGC TTT TTA AGA GAT CCA AAC
     Ser Arg Ala Glu Ser Thr Ser Pro Arg Ser Ser Phe Leu Arg Asp Pro Asn  408
1464 CAG TCC AGC TCC GAG AGC AAC CTA TGA ggagggaggagactatttttttactactgcta
     Gln Ser Ser Ser Glu Ser Asn Leu Stop                                 416
1522 tataactgttttatttattcagttttattcagtgtctgatgtgtgggggtcctgtattccttgaata
1589 ctacgtgtcgttacttcataaatcgcaatgtgcttctgcgtaacacaaagctgggtgggaaatacag
1659 aactttgtgtttccttgaaaaggggaagcttcataaaataaattcactgatcacactacactaacttt
1723 aatgagcttctgtcttctcgtttcagtcctctgtaaattgtaacctgtgttcatcttgaattaaatt
1790 tattcgctgtaaaaaaaaaaaaaaa
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FIG. 1. Nucleotide and deduced amino acid sequence of the sea bass GnRH receptor. Numbers on the left indicate the nucleotide positions. Amino acids are indicated below their respective codons and are numbered on the right.

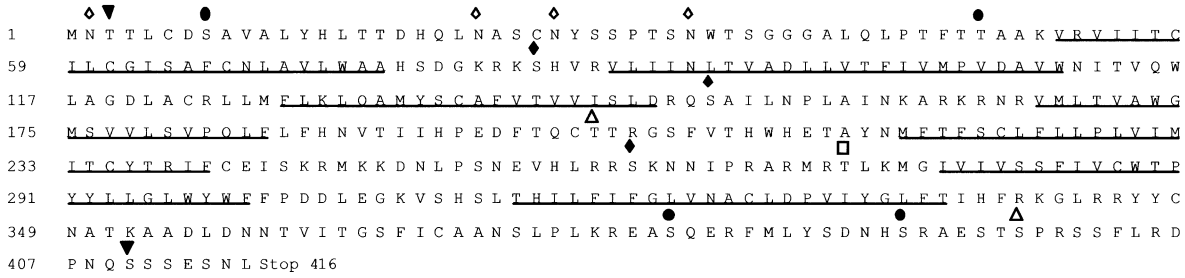

FIG. 2. Potential glycosylation sites (◊), casein kinase I (●) and II (▼) sites, calmodulin II (□), PKA (♦), and PKC (△) phosphorylation sites are shown in the sbGnRH-R sequence as indicated. Note that all the phosphorylation sites are present in intracellular or extracellular loop regions. The putative transmembrane domains are indicated in the underlined regions.

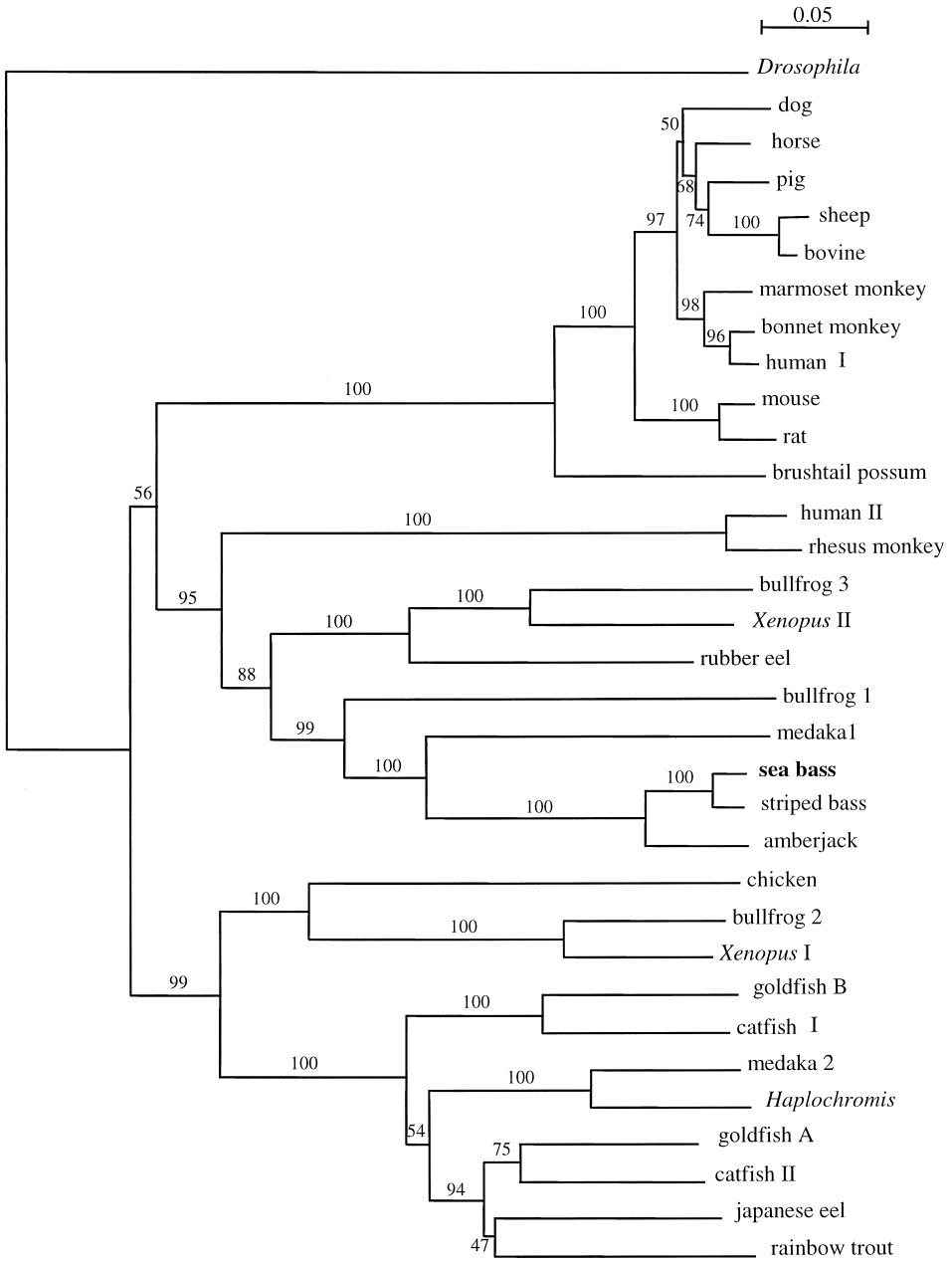

FIG. 3. Phylogenetic analysis of the GnRH-R sequences. Sequence alignment was performed according to the Lipman-Pearson algorithm using the Dayhoff matrix for amino acid homology. Distances for the different receptors were calculated with the neighbor joining method and one thousand bootstrap replicates were performed (expressed as percentages). The rooted tree was constructed by the addition of the drosophila GnRH-R like sequence.

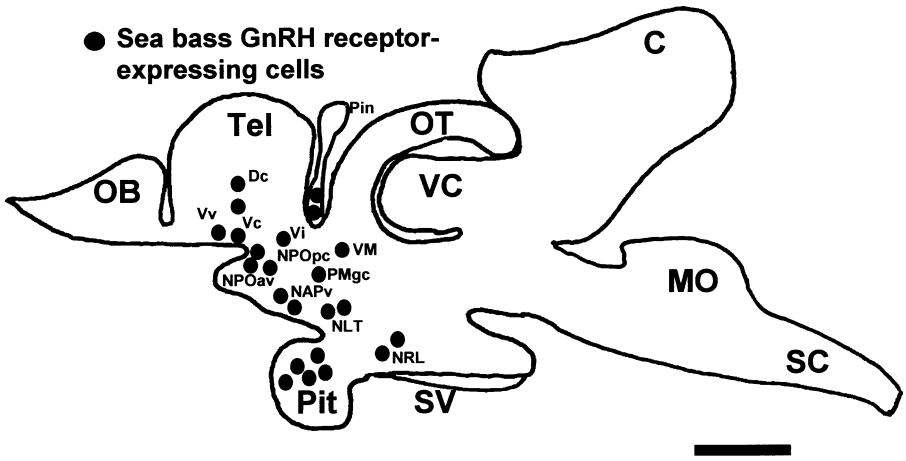

FIG. 4. Sagittal drawing of sea bass brain and pituitary summarizing the distribution of GnRH receptor-expressing cells. C, Corpus of the cerebellum; Dc, central nucleus of the dorsal telencephalon; MO, medulla oblongata; NAPv, anterior periventricular nucleus; NLT, lateral tuberal nucleus; NPOav, anteroventral part of the parvocellular preoptic nucleus; NPOpc, parvocellular part of the parvocellular preoptic nucleus; NRL, nucleus of the lateral recess; OB, olfactory bulbs; OT, optic tectum; Pin, pineal gland; Pit, pituitary; PMgc, gigantocellular part of the magnocellular preoptic nucleus; VC, valvula of the cerebellum; Vc, central nucleus of the ventral telencephalon; Vi, intermediate nucleus of the ventral telencephalon; VM, ventromedial thalamic nucleus; Vv, ventral nucleus of the ventral telencephalon; SC, spinal cord; SV, saccus vasculosus; Tel, telencephalon. Scale bar = 1 mm.

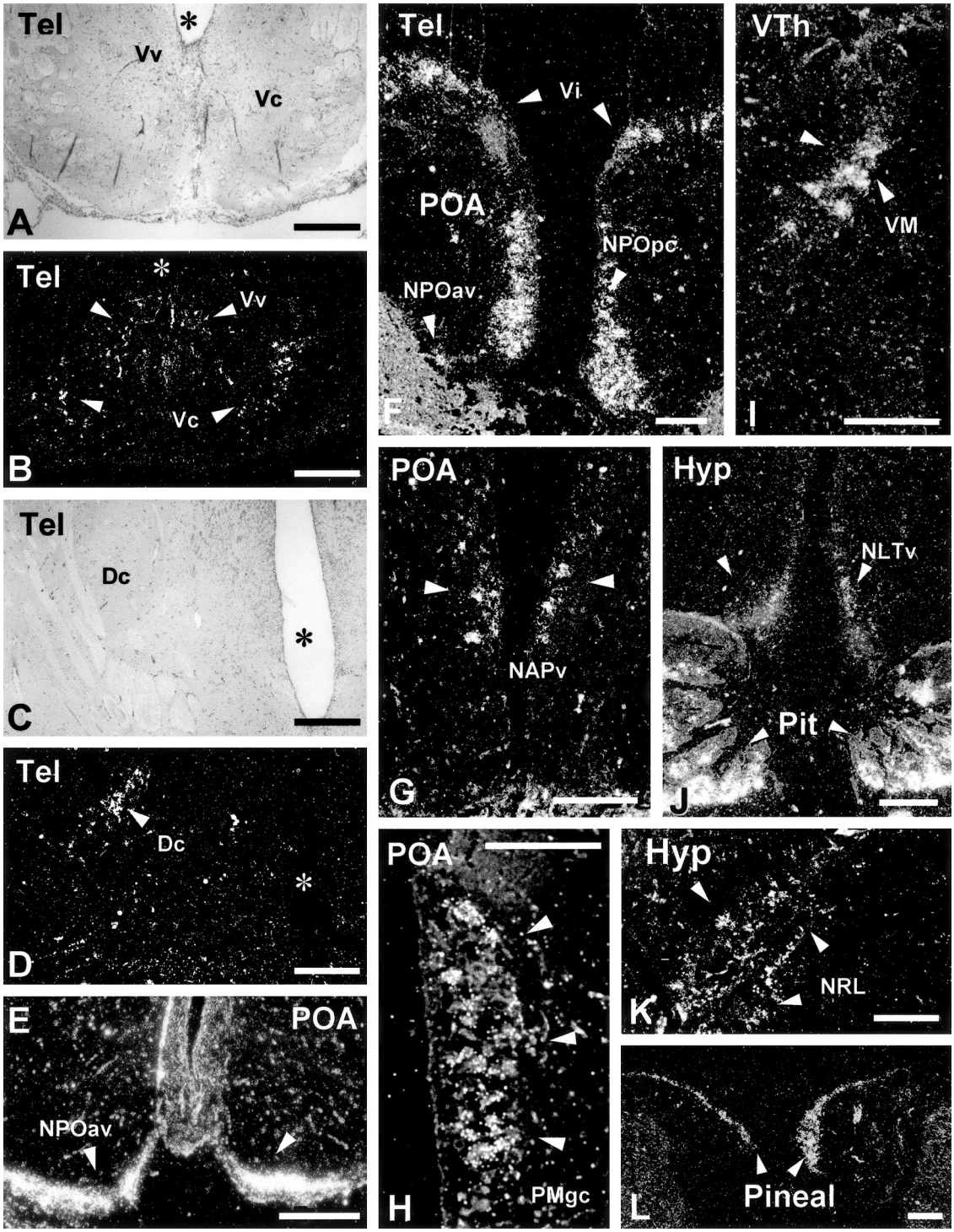

FIG. 5. Localization of sea bass GnRH receptor in the brain and pineal gland of sea bass using in situ hybridization. **A**) Bright field picture through the ventral telencephalon corresponding to the section presented in **B**. **B**) Positive cells (arrowheads) in the ventral (Vv) and central (Vc) nuclei of the ventral telencephalon. **C**) Bright field picture through the dorsal telencephalon corresponding to the section presented in **D**. **D**) GnRH-R expressing cells (arrowhead) in the central nucleus of the dorsal telencephalon (Dc). **E**) Intensely positive neurons (arrowheads) in the anteroventral part of the parvocellular preoptic nucleus (NPOav). **F**) GnRH-R positive cells (arrowheads) in the anteroventral (NPOav) and parvocellular (NPOpc) parts of the parvocellular preoptic nucleus and in the intermediate nucleus of the ventral telencephalon (Vi). **G**) GnRH-R expressing cells (arrowheads) in the anterior periventricular nucleus (NAPv). **H**) Note the presence of positive cells (arrowheads) in the gigantocellular part of the magnocellular preoptic nucleus (PMgc). **I**) Section through the thalamus of sea bass showing the expression of GnRH-R in cells (arrowheads) of the ventromedial thalamic nucleus (VM). **J**) Positive cells (arrowheads) in the ventral part of the lateral tuberal nucleus (NLTv) of the hypothalamus and in the pituitary (Pit). **K**) GnRH-R expressing cells (arrowheads) in the nucleus of the lateral recess (NRL). **L**) Pineal gland of the sea bass exhibiting intensely positive cells (arrowheads). Asterisks in **A** to **D** mark the ventricle. Tel, Telencephalon; POA, preoptic area; VTh, ventral thalamus; Hyp, hypothalamus. Scale bars = 500 μm in **A** to **D** and 250 μm in **E** to **L**.

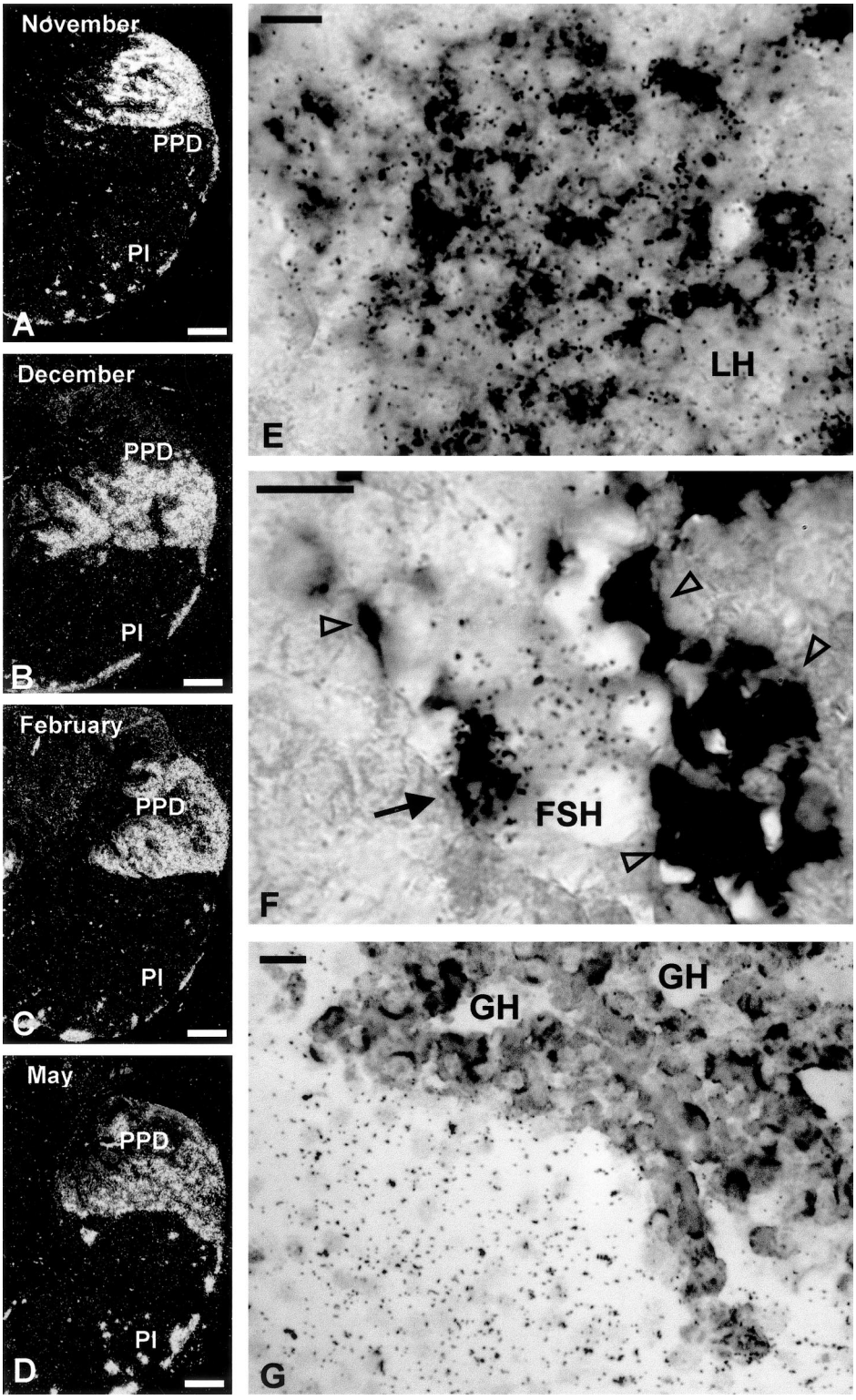

FIG. 6. Localization of sea bass GnRH receptor in the pituitary of sea bass using in situ hybridization. **A**) Pituitary of a sea bass specimen from November (late vitellogenesis). **B**) Pituitary of a sea bass specimen from December (maturation). **C**) Pituitary of a sea bass specimen from February (spawning). **D**) Pituitary of a sea bass specimen from May (postspawning/resting). **E**) Expression of GnRH-R (silver grains) in LHβ-expressing cells (dark staining). Note that all LH cells express the GnRH-R. **F**) Picture showing a FSHβ-expressing cell positive for GnRH-R (arrow) surrounded by several FSH cells that do not exhibit GnRH-R expression (open arrowhead). **G**) Picture showing immunostained GH cells that do not express GnRH-R but appear surrounded by GnRH-R-expressing cells (silver grains). Scale bars = 250 μm in **A** to **D** and 10 μm in **E** to **G**. PPD, Proximal pars distalis; PI, pars intermedia.

and in the pineal gland, significantly higher levels of GnRH-R were observed during the postspawning/resting season in relation to late vitellogenesis, maturation, and spawning stages (Fig. 7). This increase was especially evident in the pineal gland (56.03 ± 11.03; $P < 0.001$), ventromedial thalamic nucleus (VM; 52.43 ± 13.88; $P < 0.001$), and parvocellular part of the parvocellular preoptic nucleus (NPOpc; 46.38 ± 14.01; $P < 0.001$), but also in the anterior periventricular nucleus (NAPv; 38.66 ± 20.4; $P < 0.001$), the nucleus of the lateral recess (NRL; 38.38 ± 8.09; $P < 0.001$), the central nucleus of the dorsal telencephalon (Dc; 35.21 ± 20.2; $P < 0.001$), and the central (Vc; 33.54 ± 6.72; $P < 0.001$) and intermediate (Vi; 35.31 ± 10.58; $P < 0.001$) nuclei of the ventral telencephalon. The lowest increases in GnRH-R expression were observed in the ventral part of the lateral tuberal nucleus (NLTv; 29.28 ± 4.30; $P < 0.001$), the ventral nucleus of the ventral telencephalon (Vv; 22.98 ± 5.82; $P < 0.001$), and the magnocellular preoptic nucleus (PMgc; 18.86 ± 4.55; $P < 0.001$).

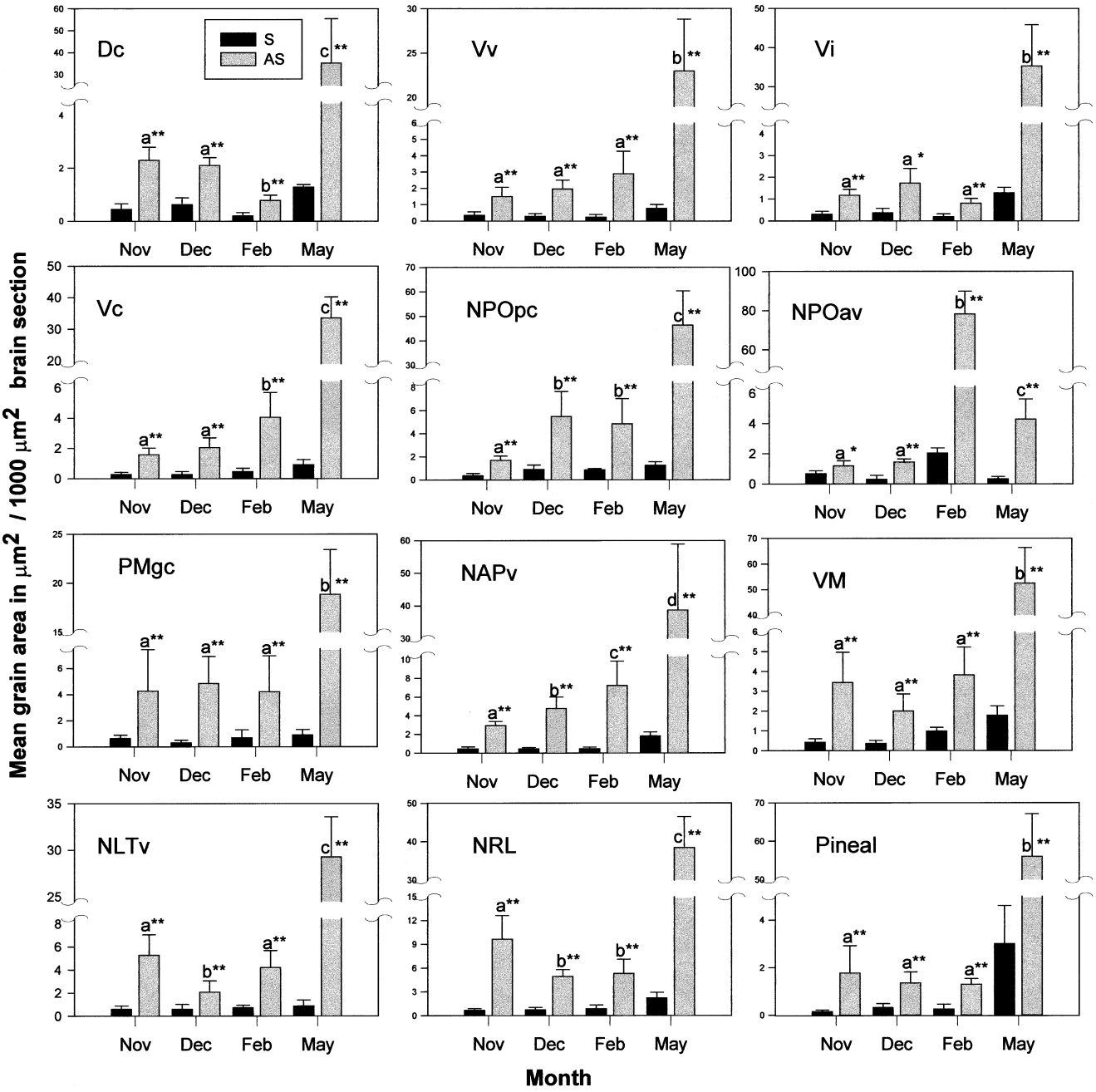

FIG. 7. Quantitative analysis of expression of sea bass GnRH-R gene in the pineal gland and different forebrain nuclei of sea bass along the reproductive cycle. Nov, November (late vitellogenesis); Dec, December (maturation); Feb, February (spawning), May (postspawning/resting). Black and grey columns represent mean values of sense (S) and antisense (AS) sections, respectively. Values are expressed as mean area occupied by silver grains in μm^2/1000 μm^2 of brain section ± SEM. Asterisk indicates significant differences between mean values of antisense and sense sections from the same period (*, $P < 0.01$; **, $P < 0.001$). Different letters indicate significant differences between antisense mean values from different reproductive periods ($P < 0.05$). For abbreviations, see Figure 4 except for NLTv, ventral part of the lateral tuberal nucleus; Pineal, pineal gland. Note the different *Y*-axes in histograms.

The pituitary of the sea bass exhibited an important expression of the GnRH-R gene at all stages of the reproductive cycle (Fig. 6, A–D). However, this expression was significantly higher in late vitellogenesis (318 ± 224.00; $P < 0.002$; Figs. 6A and 8A) in relation to maturation (Figs. 6B and 8A), spawning (Figs. 6C and 8A), and postspawning/resting periods (Figs. 6D and 8A), in which the GnRH-R expression was markedly reduced. This reduction is the consequence of a decrease in the expression of GnRH-R within positive cells (Fig. 8A) but also in the number or size of GnRH-R-expressing cells because the mean percentage of pituitary section occupied by GnRH-R-expressing cells also exhibited a dramatic reduction from vitellogenesis (22%, $F = 14.661$; $P < 0.001$) to maturation (7.75%), spawning (2.5%), and postspawning/resting (3%) periods (Fig. 8B).

DISCUSSION

The complete sequence of a sea bass GnRH-R is presented herein. The presence of a C-terminal tail and restoration of Asp^{96} (compared with the G-protein coupled receptors) are the characteristics that classify the sbGnRH-R as a member of the growing family of perciform GnRH-

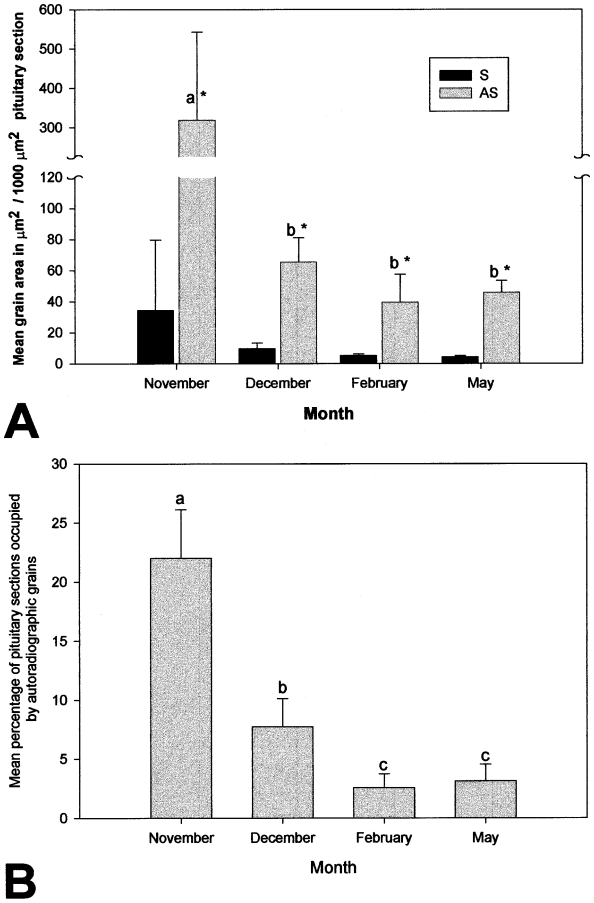

FIG. 8. **A**). Quantitative analysis of expression of sea bass GnRH-R gene in the pituitary of sea bass along the reproductive cycle in November (late vitellogenesis), December (maturation), February (spawning), and May (postspawning/resting). Black and grey columns represent mean values of sense (S) and antisense (AS) sections, respectively. Values are expressed as mean area occupied by silver grains in $\mu m^2/1000\ \mu m^2$ of pituitary section ± SEM. Asterisk indicates significant differences between mean values of antisense and sense sections from the same period (*, $P < 0.001$). Different letters indicate significant differences between antisense mean values from different reproductive periods ($P < 0.05$). **B**) Quantitative analysis of the mean percentage of pituitary area occupied by GnRH receptor-expressing cells along the reproductive cycle. Different letters indicate significant differences between mean values from different reproductive periods ($P < 0.05$).

Rs. A change in the signature sequence (Asp-Arg-Tyr) of the rhodopsin family of G-protein coupled receptors at the junction of the transmembrane domain III and second intracellular domain is unique to the vertebrate GnRH-Rs. A similar change has been observed in the case of the sbGnRH-R (^{147}DRQ^{149}), which is conserved in the GnRH-Rs identified in amberjack, rubber eel, and striped bass. The sbGnRH-R has several predicted phosphorylation sites, of which PKA-specific Ser^{83} and calmodulin II-specific Thr^{273} and most of the serine sites are also present in the GnRH-Rs of striped bass, amberjack, and rubber eel. The conserved single Cys^{25} in the first extracellular loop and Cys^{123} in the second extracellular loop that have been implicated for proper receptor folding in all the mammalian species [35] are also present in sbGnRH-R. Similar to the catfish GnRH-R1 [18], there are at least four potential N-linked glycosylation sites in the NH2-terminal of sbGnRH-R. Glycosylation is important for the GnRH-R expression in mammalian species [36].

To date, most information concerning the GnRH-R expression in the fish brain has been obtained by RT-PCR [21, 22, 24, 37, 38] and information on the precise sites of expression of the GnRH-Rs is limited to the rainbow trout [20]. This trout receptor is poorly expressed in the pituitary, but a conspicuous expression was detected in midbrain areas [20]. Surprisingly, this pattern of expression markedly differs from that observed in sea bass, in which a strong expression is detected in the pituitary and the forebrain, whereas no signal could be detected in the mesencephalon. Thus, the difference in the distribution of these receptors and their low level of identity probably reflects the fact that they represent different GnRH-R subtypes, most likely involved in different functions.

In 1998, Troskie et al. suggested that different GnRH receptor subtypes might have accompanied the evolution of the GnRH subtypes [25]. Analysis of the sequences of the extracellular loop 3 of these receptors, which is important for the ligand selectivity in mammalian receptors [39], allowed these authors to classify the receptors into three groups (type I, with subtypes IA and IB, and II). In fish, the presence of two different GnRH receptor subtypes has been described in goldfish [19], *Haplochromis burtoni* [22], medaka [23], and African catfish [24]. Phylogenetic analyses indicated that the sea bass GnRH-R is closely related to those of perciforms and beloniforms, notably striped bass and medaka GnRH-R1, all intensely expressed in the pituitary. In contrast, the trout sequence falls in another branch, together with the medaka GnRH-R2 [23], the gene of which, similar to the trout GnRH-R gene, contains an additional exon [40]. Another fish species in which two GnRH-Rs have been reported is the goldfish [19], but the phylogenetic trees indicate that these are variant forms from the same subtype. In mammals, two different GnRH receptors have also been characterized [27, 28], and three distinct GnRH receptors seem to be present in several vertebrate classes [25, 26]. In the bullfrog, GnRH-R1 is intensely expressed in the pituitary whereas the GnRH-R2 has a low pituitary expression [26].

Comparing the distribution of GnRH-R and prepro-GnRH-ir fibers in sea bass brain [10], it seems evident that most GnRH-R-expressing cells appear in cell nuclei that mainly received sGnRH and sbGnRH projections. Thus, it appears that the GnRH-R of sea bass represents a receptor for the most abundant GnRHs in the forebrain of this species, sbGnRH and sGnRH. Supporting this assumption, this GnRH-R is highly expressed in sea bass pituitary gonadotrophic cells, which received sbGnRH and sGnRH fibers but not cGnRH-II projections [10], and seems to represent a GnRH receptor responsible for direct sbGnRH and/or sGnRH stimulatory actions on gonadotroph release. Moreover, significant differences in sea bass pituitary GnRH-R expression was observed during the reproductive cycle, showing the highest levels in November (late vitellogenesis). Interestingly, sbGnRH levels are also higher in this period, at least in male sea bass [9]. However, it is probable that more than one form of GnRH-R exists in the brain of sea bass, as it occurs in other vertebrates [25, 26]. In this sense, two types of GnRH-R sequences are expressed in *Haplochromis burtoni* (Perciformes) and medaka (Beloniformes) [22, 23].

In sea bass, GnRH-R-expressing cells were only evident in the pituitary, forebrain, and pineal. In the pituitary, labeling was restricted to the proximal pars distalis (PPD), and the external border of the pars intermedia, two regions that contain gonadotrophic cells [41]. This GnRH-R expression in sea bass pituitary was mainly detected in LH

cells but also in some FSH cells, whereas somatotrophs appeared devoid of GnRH-R expression. Interestingly, in tilapia, gonadotrophic cells (FSH and LH) but not GH cells expressed type IA and IB GnRH-Rs, whereas GH cells but not gonadotrophic cells expressed a different type III GnRH-R [42].

Qualitative and quantitative analyses reveal that the pituitary expression of GnRH-R was higher in November (late vitellogenesis) than in December (maturation), February (spawning), or May (postspawning/resting). However, in this study, we are detecting GnRH-R mRNAs and not the functional proteins, and their profiles could differ along the sea bass reproductive cycle. In other fish species, it has also been shown that the number of pituitary GnRH receptors [43] or pituitary GnRH-R mRNA levels [21] varies over the maturation process. Furthermore, there is evidence demonstrating that GnRH induces the expression of its own receptor [44, 45]. Thus, in rat, the administration of low concentrations of GnRH is followed by an upregulation of GnRH receptor in the anterior hypophysis [44]. The treatment with sGnRH analogs also increases the number of high-affinity GnRH receptors in the goldfish pituitary [45].

In the sea bass brain, the highest GnRH-R expression was observed in the anteroventral part of the parvocellular preoptic nucleus during the spawning season. The presence of sGnRH and sbGnRH cells in the immediacy of this nucleus has previously been reported in sea bass [10, 31]. This nucleus also contains catecholaminergic cells in sea bass [46] and other teleost species [47–49], which represent a source of dopaminergic projections reaching the pituitary and inhibiting the gonadotroph secretion [49]. Thus, the putative presence of GnRH-R in catecholaminergic cells of the anteroventral part of the parvocellular preoptic nucleus could represent a morphological substrate for GnRH-dopamine interactions, reinforcing the major role of this cell mass in the control of fish reproduction. However, such a role cannot be attributed to this nucleus in sea bass because the role of dopamine in regulating gonadotroph release in this species is rather dubious [50].

Other preoptic cell masses, as the parvocellular part of the parvocellular preoptic nucleus and the gigantocellular part of the magnocellular preoptic nucleus, also expressed GnRH-R in sea bass. These nuclei, which have been largely implicated in the control of pituitary functions and are considered as important neuroendocrine centers [51], represent a target for steroid hormones [47, 49, 52].

The presence of GnRH-Rs was also evident in ventral (Vv, Vc, Vi) and dorsal (Dc) telencephalic nuclei, in the anterior periventricular nucleus of the preoptic area, the lateral tuberal nucleus, and the nucleus of the lateral recess of the hypothalamus. In sea bass, these cell masses, as well as the parvocellular and magnocellular preoptic nuclei, have been implicated in the control of appetite and feeding and are known for the presence of neuropeptide Y (NPY), peptide Y (PY), peptide YY [53, 54], galanin [55], and/or catecholamines [46]. The stimulatory effects of NPY on gonadotroph secretion have also been largely referred in teleosts, including sea bass [56, 57]. These NPY actions on gonadotroph secretion seem to exhibit seasonal variations [58]. Furthermore, it has been suggested that GnRH and NPY could cooperate to modulate the interactions between the reproductive and metabolic processes [59]. Interestingly, the GnRH-R expression in these nuclei rise significantly at the postspawning/resting period, when sea basses recuperate their feeding habits after a natural fasting period coincident with the maturation/spawning season. In this way, the presence of GnRH-R- and NPY-related peptide expression in the same nuclei could be implicated in the mediation of these metabolic and reproductive interactions.

The ventromedial thalamic nucleus of sea bass also expressed GnRH-R. In gilthead seabream, the presence of sbGnRH-expressing cells was also evident in the ventral thalamus [60]. Moreover, the existence of hypophysiotrophic neurons in the thalamus has been described in goldfish [51] and *Apteronotus leptorynchus* [61]. The thalamus of goldfish [62] and rainbow trout [47] also expressed steroid receptors, and electrical stimulation of the thalamus elicits reproductive behavior, including sex color change and gamete release, in the hermaphrodite sea bass *Serranus subligarius* [63]. Although GnRH-expressing cells were not detected in the thalamus of sea bass [10, 31], a conspicuous sGnRH and cGnRH-II innervation was present in the ventral thalamus of this species [10]. Thus, GnRH acting on thalamic GnRH-R could have a role in the regulation of similar events in sea bass.

In sea bass, GnRH-Rs were also present in the pineal gland. In fish, the pineal gland has endocrine cells but retains a photoreceptive function. The presence of GnRH and/or GnRH-R in other visually related structures as the retina or the optic tectum has been extensively described [10, 20, 22, 64]. Based on these evidences, a role of GnRH in the coordination of visual sensory information has been proposed [22]. In this way, the presence of GnRH-R in a photoreceptive structure as the pineal gland of sea bass could reinforce this assumption.

In conclusion, the present study analyzes the expression of a GnRH-R cloned in the brain and pituitary of a perciform species along the reproductive cycle. The abundant expression of GnRH-R in pituitary gonadotrophs and forebrain neuroendocrine centers as well as the variation in GnRH-R expression along the reproductive cycle strongly suggest a relevant role of this receptor in the mediation of GnRH effects on the release of gonadotrophs and reproductive events. Furthermore, the quantitative analysis during the reproductive cycle reveals that GnRH-R expression is regulated in a different manner in the brain and the pituitary of sea bass.

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