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Three Forms of Gonadotropin-Releasing Hormone in a Perciform Fish (*Sparus aurata*): Complementary Deoxyribonucleic Acid Characterization and Brain Localization[1]

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ABSTRACT

Three forms of GnRH—salmon (sGnRH), seabream (sbGnRH), and chicken (cGnRH-II)—have been described in the gilthead seabream (*Sparus aurata*) brain, and the cDNA encoding the sbGnRH precursor was recently isolated. In the present study, the cDNAs encoding the sGnRH and cGnRH-II were isolated and characterized, and the neurons producing the three GnRHs were localized in the seabream brain. Fragments of sGnRH and cGnRH-II cDNAs were amplified by polymerase chain reaction and used as probes to isolate the full-length cDNAs from a brain cDNA library. The cDNA encoding the cGnRH-II precursor is 573 nucleotides (nt) long, and the cDNA encoding the sGnRH precursor is 1971 nt in length with an unusually long 5′ untranslated region. Specific single-strand DNA probes for in situ detection of mRNA were designed according to nonconserved regions among the three GnRH cDNAs. Localization of GnRH mRNA-producing cells in the brain revealed five distinct populations of cells: sGnRH-producing cells in the ventromedial olfactory bulbs and the terminal nerve, sbGnRH-producing cells in the preoptic area and the ventral thalamus, and cGnRH-II-producing cells in the midbrain tegmentum. The discrete sites of expression of the three forms of GnRH indicate that only sbGnRH is directly involved in the control of gonadotropin secretion.

INTRODUCTION

Nine forms of GnRH (Fig. 1), with highly conserved structures, have been isolated and characterized from vertebrate brains [1–3]. Mammalian GnRH (mGnRH), the first form to be described, is present in all examined mammals and also has been reported to be present in amphibia and a number of primitive bony fish. Two GnRH forms have been isolated from chicken brains: chicken GnRH-I (cGnRH-I) and cGnRH-II. Chicken GnRH-II is present in all examined vertebrates except for Agnathans and placental mammals (see reviews [1, 2]). A teleost GnRH was first isolated from salmon (sGnRH) [4] and subsequently has been found to be broadly distributed among teleost species. Other species-specific GnRH variants were found in catfish (cfGnRH), dogfish (dfGnRH), and lamprey (lGnRH-I and lGnRH-III) [1, 2]. Recently, a novel GnRH was isolated and characterized from a perciform fish, the gilthead seabream (*Sparus aurata*) [3], and was named seabream GnRH (sbGnRH) (Fig. 1). This form was subsequently found in other fish of the order Perciformes: including the striped bass (*Morone saxatilis*) [5], African cichlid (*Haplochromis burtoni*), and pumpkinseed (*Lipomis gibbosus*) [6].

The cDNA sequences encoding the precursors for mGnRH, cGnRH-I, cfGnRH, sGnRH, cGnRH-II [1, 2] and very recently sbGnRH [7, 8] have been isolated and characterized from mammals, amphibia, birds, and teleosts. These cDNAs encode a precursor polypeptide consisting of a signal peptide at the N-terminal, the GnRH decapeptide, a 3-amino acid cleavage and processing site (Gly-Lys-Arg), and an additional peptide at the C-terminal referred to as GnRH-associated peptide (GAP). The bioactive decapeptide is rescued from the precursor by removal of the signal peptide and cleavage of the precursor at the dibasic amino acids (Lys-Arg).

It is widely accepted that nonmammalian vertebrates possess at least two forms of GnRH. The distribution of GnRH cell bodies and axons has been studied via immunocytochemistry in representatives of all taxa, revealing the existence of two major GnRH-producing systems. In the primitive fish of the class Agnathans, these two systems, both expressing lGnRH-I and lGnRH-III, are located in the rostral hypothalamus and in the preoptic area [9]. In all other nonmammalian vertebrates, the two GnRH systems are consistently located at 1) the terminal nerve-septum-preoptic area, which expresses the species-specific GnRH form and 2) the midbrain area, which expresses the ubiquitous cGnRH-II (for reviews see [10]). However, comparison of immunocytochemical studies reveals some species differences, thus making a definitive conclusion on the relevance of each GnRH system in the control of reproduction difficult. In birds [11] and reptiles [12], cGnRH-I is dominant in the hypothalamus and median eminence whereas cGnRH-II is widely distributed throughout the brain. Since only cGnRH-I is found in the median eminence, cGnRH-II is thought to have no direct involvement in the control of

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gonadotropin (GtH) release. In amphibia, although mGnRH is prevalent in hypothalamic areas, both mGnRH and c-GnRH-II are found in the portal blood that flows from the median eminence to the pituitary gland, indicating that both forms of GnRH may regulate GtH release [13]. Differences in the distribution of immunoreactive GnRH variants are also found among teleosts. In masu salmon [14] and gourami [15], sGnRH is produced in the terminal nerve-septum-preoptic system, whereas cGnRH-II cells are located in the midbrain tegmentum. In the pituitary, which in teleosts is directly innervated by GnRH axons, only sGnRH is found, indicating that in these species only sGnRH, originating from the septum-preoptic system, controls GtH secretion. In the eel, the terminal nerve-septum-preoptic GnRH system contains mGnRH, which is also dominant in the pituitary; yet a few cGnRH-II fibers project to the pituitary, indicating that midbrain cGnRH-II, as well as terminal nerve-septum-preoptic mGnRH, may regulate GtH secretion [16]. In the goldfish, the terminal nerve-septum-preoptic GnRH system is immunoreactive for both sGnRH and cGnRH-II, whereas the midbrain contains only cGnRH-II [17]. In the goldfish pituitary, both sGnRH and cGnRH-II are found [18], suggesting that both forms may regulate GtH secretion. The distribution of GnRH variants is even more complicated in the platyfish, where immunocytochemical studies indicate the presence of sGnRH, mGnRH, cGnRH-II, and lGnRH-I in cells of the brain and in the pituitary of mature fish [19]. The above differences in the distribution of GnRH variants may reflect species differences. However, in view of the multiplicity of GnRH forms in one species and their similar structure, one should be cautious when using antibodies to detect different GnRH forms, which may cross-react with other known or unknown forms of GnRH or with other peptides in the brain. For example, in the African cichlid, preoptic cells that were immunoreactive to sGnRH were later shown to express sbGnRH-mRNA by in situ hybridization [8, 20]. In the catfish, cGnRH-II antibodies reacted with cfGnRH cells, and specificity was achieved by using antibodies directed to the GAP region of the precursors [21].

Gilthead seabream (*Sparus aurata*) is an interesting model for the study of comparative reproductive endocrinology [22]. First, it is a protandrous hermaphrodite—all individuals reach puberty as functional males and in later years undergo sex reversal to become functional females. Second, unlike many studied fish, the gilthead seabream females have an asynchronous ovarian development and undergo daily cycles of final oocyte maturation and ovulation for a period of 3 mo. Third, the seabream is an important farmed fish; thus it is beneficial to control its life cycle in captivity.

Recently, the presence of three forms of GnRH in the brain of the gilthead seabream has been demonstrated: sGnRH, sbGnRH, and cGnRH-II [3]. All three have been shown to induce GtH secretion after injection [23]. Molecular cloning and characterization of the sbGnRH precursor cDNA was described previously [7]. Here we report the isolation and characterization of the cDNAs encoding the sGnRH and cGnRH-II precursors, and the specific localization of the cells producing the three GnRH precursors in the gilthead seabream brain. Localization of GnRH-producing cells in the brain was carried out by means of in situ hybridization histochemical (ISHH) detection of mRNA, which circumvents the complication of antibody cross-reactivity encountered with the use of immunocytochemistry.

	1	2	3	4	5	6	7	8	9	10
mGnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
cGnRH-I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH_2
sbGnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly-NH_2
cfGnRH	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly-NH_2
sGnRH	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly-NH_2
cGnRH-II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH_2
dfGnRH	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly-NH_2
lGnRH-III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly-NH_2
lGnRH-I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly-NH_2

FIG. 1. Amino acid sequences of known GnRH forms. The structure of the nine known GnRH forms is shown along with the conventionally accepted nomenclature (GnRH peptides are usually named for the species from which they were first isolated). The different forms are listed in order of similarity to mGnRH. Amino acid differences are highlighted within a box.

MATERIALS AND METHODS

Isolation and Characterization of GnRH cDNAs

In order to isolate and characterize the cDNAs encoding the sGnRH and cGnRH-II precursors, we used an approach similar to that described previously for the isolation of the sbGnRH precursor [7]. Using seabream brain mRNA as a template, the respective cDNA fragments were polymerase chain reaction (PCR)-amplified, cloned, and identified. Brain cDNA libraries were then screened using the PCR products as probes, and positive clones were characterized.

Isolation of sGnRH Precursor cDNA

A fragment of sGnRH precursor cDNA was PCR-amplified from seabream brain mRNA using a set of degenerate primers, S(-16)-(-11) and S45–51 (Table 1), designed according to amino acids (-16)-(-11) and 45–51 of sGnRH precursor sequences of other fish species [24] (for convenience in numbering of amino acids, Gln^1 of the GnRH decapeptide is considered no. 1 of the precursor). PCR was carried out in a volume of 50 μl containing 3 μl template cDNA, 5 μl 10-strength PCR buffer (Promega, Madison, WI), 2.5 mM MgCl, 200 μM each dNTP, 2.5 μl formamide, 1.5 μM of each primer, and 1.0 U of *Taq* DNA polymerase (Promega). The amplification was performed using 12 cycles at 94°C, 45°C, and 72°C for 1 min each, then 21 cycles at 94°C, 55°C, and 72°C for 1 min each, followed by 7 min of extension at 72°C. Size fractionation of the PCR products through a 1.5% agarose gel revealed one product 200 bp in length. This product was purified, cloned into a plasmid vector, and propagated in *Escherichia coli* cells. Recombinant plasmid was then extracted, and the insert was identified by nucleotide sequence analysis. With the PCR product used as a probe, a positive clone of sGnRH precursor cDNA was purified from a seabream brain cDNA library that was constructed in phage λ-ZAP (Stratagene, La Jolla, CA). Recombinant plasmid pBluescript containing the sGnRH precursor cDNA was excised from the phage as recommended by the manufacturer, and the cDNA insert was sequenced and characterized.

Isolation of cGnRH-II Precursor cDNA

A short fragment, 97 bp long, of cGnRH-II cDNA was PCR-amplified from seabream brain mRNA using a set of

TABLE 1. Degenerate oligonucleotides used in the PCR amplification of fragments of GnRH precursors cDNAs.*

Peptide	Primer	Synthesis direction	Sequence (5′ to 3′)
sGnRH	S(-16)-(-11)	Forward	T(AT)(AG)T(AG)CTGGTG(GT)TG(GT)TG
	S-3	Reverse	ATTA(CT)AT(CT)(AG)ATAGGT(CT)T(AC)AG
cGnRH-II	C1-7	Forward	CA(AG)CA(CT)TGGTCNCA(CT)GGNTGG
	C30-35	Reverse	CA(CT)TCICCIGC(CT)TC(AG)CA
	C7-12	Probe	TGGTA(CT)CCNGGNGGNAA

* N, all nucleotides in one position; I, inosine.

degenerate primers, C1–7 and C30–35 (Table 1), designed on the basis of amino acids 1–7 and 30–35 of cGnRH-II precursors of catfish [25] and African cichlid [20]. PCR was carried out as described above except C1–7 and C30–35 were the primers used in the reaction. Size fractionation of the PCR products through an agarose gel (3.5%) revealed two products: 100 bp and 250 bp. In order to test these PCR products, Southern hybridization was performed using a degenerate oligomer, C7–12 (Table 1), designed according to amino acids 7–12, as a probe. The DNA was transferred to a MagnaGraph nylon membrane (MSI, Westborough, MA) and hybridized with a ^{32}P 5′ labeled C7–12 (spec. act. = 5×10^6 cpm/ng). Hybridization conditions were 37°C for 36 h in 7 ml hybridization buffer (6-strength SSC [single-strength SSC: 0.15 M sodium chloride and 0.015 M sodium citrate], 0.5% SSC, 5-strength Denhardt's solution, 100 μg/ml tRNA). The membrane was washed with 6-strength SSC, 0.05% sodium-pyrophosphate for 1 h at 37°C and subjected to autoradiography. Only one signal corresponding to the 100-bp band was obtained. This PCR product was purified, cloned, and identified as described above. With the PCR product used as a probe, a positive clone of cGnRH-II precursor cDNA was purified from a seabream brain cDNA library and characterized as described above.

Localization of GnRH-Producing Cells

To determine the distribution of the cells producing the different forms of GnRH in the brain, the distinct mRNAs encoding the three GnRH forms were localized by ISHH. The mRNAs of interest were detected using two single-strand DNA probes (54–55 bases long and 50–60% GC): one corresponds to the GAP coding region, and the other is directed to the signal peptide and/or 5′ untranslated region (UTR). For sGnRH mRNA, the two probes correspond to nt 1512–1566 (5′ UTR and signal peptide) and 1653–1707 (GAP); for sbGnRH mRNA [7], the two probes correspond to nt 3–56 (5′ UTR and signal peptide) and 174–228 (GAP); and for cGnRH-II mRNA, the two probes correspond to nt 33–86 (5′ UTR) and 242–295 (GAP). These sequences are highly diverse among the three different GnRH forms (see *Results*) and therefore enabled us to specifically localize each of the forms without the complication of cross-reactivity. To test the degree of specificity of the probes, each was labeled with ^{32}P and hybridized separately with the three sense RNAs. The three sense RNAs were transcribed from the recombinant plasmids using T3 RNA polymerase, fractionated through denaturing agarose gel, and transferred to a MagnaCharge nylon membrane (MSI) in six replicates. After a 5-h prehybridization treatment of the membranes (50% formamide, 4-strength SSC, 10-strength Denhardt's solution, and 250 μg/ml tRNA) at 40°C, the single-strand DNA probes were added. Hybridization and washing conditions were similar to those used for in situ hybridization (see below), and no cross-reactivity was detected under these conditions (data not shown). For the in situ hybridization on brain sections, probes were labeled with ^{33}P. To label the probes, 5 pmol of each were 3′ labeled with a tail of about ten ^{33}P-dATP using an overnight incubation with 50 pmol [α-^{33}P]dATP (Amersham Corp., Arlington Heights, IL) and 25 U terminal deoxynucleotidyl transferase (Promega) in a volume of 50 μl.

Seven captivity-reared seabream females undergoing final oocyte maturation were selected on the basis of their sexual behavior. The females were anesthetized in 200 ppm ethylene glycol-monophenylether, an ovarian biopsy was collected by inserting a glass catheter through the ovipore, and oocytes were examined under a light microscope to verify final oocyte maturation. Females were then killed, and brains were removed and immediately frozen on dry ice with the dorsal side facing the dry ice. The frozen brains were wrapped with parafilm and stored at −70°C. ISHH was performed according to methods previously described [26] with some modifications. Brains were transversely sectioned using a cryostat to 12-μm sections. From each brain, 140–260 sections were collected. The average distance between sections was 36 μm. Sections were mounted on acid-washed, chrom-alum gelatin-coated microscope slides and stored at −70°C. In total, seven brains were sectioned. Sections from brains 1–4 were hybridized with the six probes listed above, using only one of the probes per consecutive section. Each of the remaining three brains was hybridized with the two probes that are directed to one of the GnRH mRNAs, again using only one of the probes per consecutive section. Before hybridization, brain sections were fixed with 5% formalin in PBS for 5 min, acetylated with 1.5% triethanolamine:acetic anhydride (10:1) for 5 min, delipidated in chloroform for 5 min, and dehydrated with ethanol. Hybridization was carried out overnight at 40°C. Each slide was incubated with 70 μl hybridization solution (50% formamide, 4-strength SSC, 10% dextran sulfate, single-strength Denhardt's solution, and 250 μg/ml tRNA) containing 2 nM of one of the radiolabeled probes directed against a nonconserved region of a specific GnRH mRNA sequence (see above). After hybridization, sections were washed in double-strength SSC-50% formamide four times for 15 min at 42°C and twice with 0.2-strength SSC at room temperature for 30 min and were ethanol-dried. The slides were then dipped in Kodak NTB3 nuclear emulsion and exposed for 18 days in complete darkness at 4°C. After development of the emulsion, cell nuclei were counterstained with 0.01% toluidine blue dye for 5 min, and GnRH-producing cells were identified under microscopic observation as having clusters of silver grains associated with counterstained cell nuclei. In all our observations, brain nuclei expressing a certain GnRH form hybridized with the two probes. The precise localization of GnRH cells was determined by comparing the tissue morphology of la-

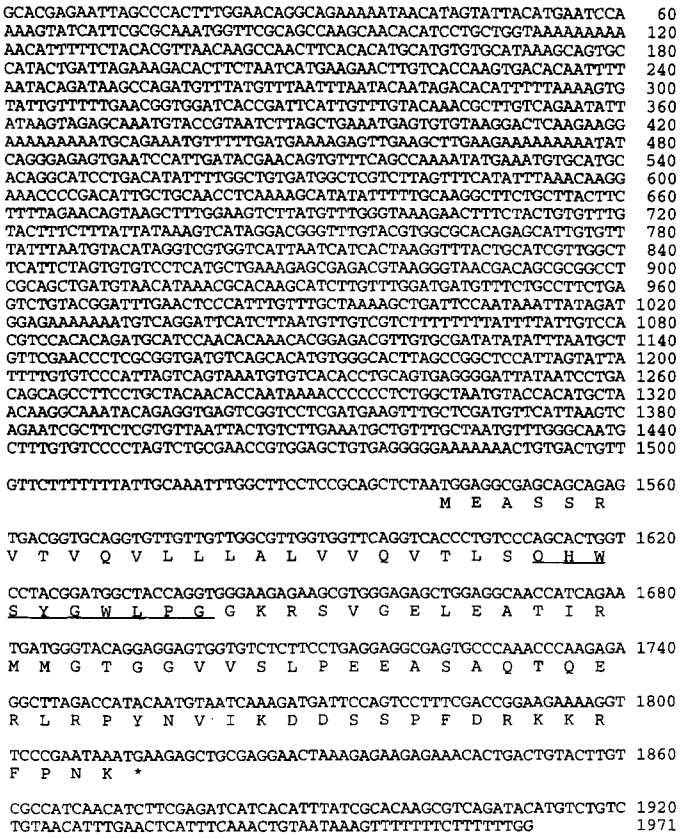

FIG. 2. The cDNA nucleotide sequence encoding the sGnRH precursor in seabream and the deduced amino acid sequence. The nucleotide sequence shown was obtained by sequencing both strands of one clone isolated from a brain cDNA library. The deduced amino acids are displayed using the one-letter code. The GnRH decapeptide is underlined. * = Stop codon.

beled sections to serial transverse sections of seabream (Muñoz-Cueto, Sarasquete, Zohar, and Kah, in preparation) and killifish [27] brain atlases. The counterstained brain nuclei in the vicinity of labeled cells, as well as some neuroanatomical references (terminal nerve, ventricle, lateral forebrain bundle, habenula, medial longitudinal fasciculus) also permit the determination, with accuracy, of the anatomical localization of GnRH labeled cells.

RESULTS

GnRH cDNA Sequences

Salmon GnRH. Using a PCR-amplified, 180-bp fragment of the sGnRH precursor cDNA as a probe, we screened a brain cDNA library and isolated and characterized a 1971-nucleotide (nt)-long cDNA. Nucleotide sequence analysis revealed that this cDNA encodes a 90-amino acid primary translation product (Fig. 2) composed of the three expected major regions: a 23-amino acid leader sequence; the biologically active sGnRH followed by a processing site (Gly-Lys-Arg); and a 54-amino acid associated peptide. This precursor has 72–98% identity with sGnRH precursors characterized from other fish, but only slight (21–33%) identity, mainly at the GnRH region, with precursors of other GnRH forms (Table 2). Similarly, at the nucleotide level, the cDNA has 60–75% identity with other sGnRH cDNAs but only 41–44% identity with cDNAs encoding other forms of GnRH. Even within the same species, the sGnRH cDNA has 44% identity with sbGnRH cDNA [7] and 40% with the cGnRH-II cDNA (this study).

TABLE 2. Percentages of amino acid identity between seabream GnRH precursors and other GnRH precursors characterized in various species.*

		Gilthead seabream GnRH precursors		
GnRH form	Species	sGnRH	cGnRH-II	sbGnRH
mGnRH	Human (M12578)	24	39	31
	Rat (M12579)	33	31	27
	Mouse (M14872)	29	35	28
	Frog (L28040)	27	39	34
cGnRH-I	Chicken (X69491)	33	32	27
sbGnRH	African cichlid (U31865)	27	32	60
	Gilthead seabream (U30320)	30	36	100
cfGnRH	Catfish (X78047)	21	34	27
sGnRH	Atlantic salmon (X79709)	75	30	27
	Masu salmon (D10946)	72	29	25
	Sockeye salmon (D31868)	74	29	27
	Plainfin midshipman [24]	77	26	24
	African cichlid (S63657)	88	29	26
	Red seabream (D26108)	98	30	32
	Gilthead seabream (U30311)	100	31	30
cGnRH-II	Catfish (X78049)	32	66	24
	African cichlid (L27435)	28	94	34
	Gilthead seabream (U30325)	31	100	36

* The sequences were extracted from the GenEMBL data base. The GenBank accession numbers or reference for each GnRH cDNA are given following the species name.

The coding sequence is flanked by a 1542-nt 5′ UTR and a 160-nt 3′ UTR (Fig. 2). A number of ATG start codons are found throughout the 5′ UTR but are followed by a stop codon after 3–46 codons. Therefore, initiation of translation from these sites would produce very short peptides. The ATG located at nt 1542–1544 together with its flanking nucleotides conforms more to the consensus sequence for initiation sites in eukaryotic mRNA [28] than the other ATGs and is the first ATG that initiates the translation of a polypeptide of a conceivable size (90 amino acids). Therefore, this ATG at nt 1542–1544 most likely encodes the first amino acid of the sGnRH precursor polypeptide. However, we cannot rule out the possibility that the other upstream ATGs are used to initiate translation and production of short peptides. The 5′ UTR of the seabream sGnRH-precursor cDNA is unusually long (1542 nt), and its possible significance is discussed below. A stop codon is located at nt 1812, and a typical polyadenylation signal (AATAAA) is present at nt 1949–1954.

Chicken GnRH-II. Using a short (97-bp) PCR-amplified fragment as a probe, we screened a seabream brain cDNA library and isolated and characterized a 571-nt cDNA (Fig. 3). Its coding region is located between the first ATG codon (nt 119–121) and a stop codon (nt 374–376). The 85-amino acid primary translation product is composed of a 23-amino acid leader sequence, the biologically active cGnRH-II followed by a processing site, and a 49-amino acid associated peptide. The associated peptide may be further cleaved at the dibasic amino acids Arg^{42}-Arg^{43} (Fig. 3, underlined) to produce two GAP peptides, as was suggested for the c-GnRH-II precursor from the African cichlid [20]. Overall, the cGnRH-II precursor has 66% and 94% identity with cGnRH-II precursors from catfish [25] and cichlid [20], respectively (Table 2). When compared with precursors of

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TTCGCCACGAGGTGAGATACTTTGAGCGACTGAAGGACAAGCAGTGGGAGCTTCTGGCAG  60

CCTGGACAGGTGCAGAGACAAAGTTGTGAGAATTGCGAACAAGGTGGGAATATCATGTGT 120
                                                        M  C

GTATCTCGGCTGGTTTTGCTGCTCGGGCTGCTTCTATGTGTGGGGGCTCAGCTGTCCAAC 180
 V  S  R  L  V  L  L  L  G  L  L  L  C  V  G  A  Q  L  S  N

GGCCAGCACTGGTCCCACGGTTGGTACCCCGGAGGCAAGAGGGAACTGGACTCTTTTGGC 240
 G  Q  H  W  S  H  G  W  Y  P  G  G  K  R  E  L  D  S  F  G

ACATCAGAGATTTCAGAGGAGATTAAGCTGTGTGAGGCAGGGGAATGCAGCTACTTGACA 300
 T  S  E  I  S  E  E  I  K  L  C  E  A  G  E  C  S  Y  L  T

CCCCAGAGGAGGAGTGTGCTGAGGAATATCTTGTTGGATGCTTTAGCAAGAGAGCTCCAG 360
 P  Q  R  R  S  V  L  R  N  I  L  L  D  A  L  A  R  E  L  Q

AAGAGGAAGTGACAGCTTTTCACTTCCAGCTGCTTTTCTATCTATAGTGACTCTCCTCTT 420
 K  R  K  *

CTTCACTATCCTGGTCTTGGTTGTGGAACAATTTCATGTTGATCCTGTGGTTTTGACTAT 480
TTGTTACATTTGTCAGTAAATGTGTTTTCCAGTGTTACATGGACTTCCCCCAGTGTAACA 540
ATGTCCCACAATAAAATATCTATTTTGA(n)                               568
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FIG. 3. The cDNA nucleotide sequence encoding the cGnRH-II precursor in seabream and the deduced amino acid sequence. The nucleotide sequence shown was obtained by sequencing both strands of two clones isolated from a brain cDNA library. The deduced amino acids are displayed using the one-letter code. The GnRH decapeptide and the second potential dibasic amino acid cleavage site are underlined. * = Stop codon.

other forms of GnRH, sequence identity is only 29–39% (Table 2) and is mainly confined to the GnRH and cleavage site regions. At the nucleotide level, the cGnRH-II cDNA has 57% and 87% identity with cGnRH-II cDNAs of other species, but very low identity (38–44%) with cDNAs encoding other forms of GnRH. Compared with the cDNAs encoding the two other GnRHs identified in seabream, homology is 40% and 42% for sGnRH and sbGnRH, respectively.

Brain Localization of GnRH-Producing Cells

Salmon GnRH-producing cells. Positive cells for sGnRH mRNA were detected in the ventromedial level of the olfactory bulbs (Figs. 4a, 5a, and 6). One or two labeled cells could be observed in each section. More caudally, in the transitional area between the olfactory bulbs and the ventral telencephalon, the ganglion cells of the terminal nerve were also positive for sGnRH mRNA (Figs. 4b, 5b, and 6). These positive cells were found in clusters of 3–10 cells in each section. Using the probes directed against mRNAs of the two other GnRH forms, no positive cells were detected in these areas.

Seabream GnRH-producing cells. Seabream GnRH mRNA was detected in the preoptic area at the level of the nucleus preopticus pars parvicellularis (Figs. 4c, 5c, and 6). These cells seemed to be smaller in size than the sGnRH cells in the terminal nerve and were more scattered. In consecutive sections, 4–6 cells were observed on each side of the ventricle. More caudally, approximately 800 μm, and dorsally, another group of sbGnRH mRNA-positive cells were located in the nucleus ventrolateralis of the thalamus (Figs. 4d, 5d, and 6). Positive cells were found ventrolateral to the nucleus habenularis and lateral to the nucleus ventromedialis rostral and extended caudally up to the posterior commissure. In this nucleus, 2–10 labeled cells were found in each section. These cells were somewhat similar in size to the preoptic sbGnRH cells but were more widely spread. Probes for the other forms of GnRH—sGnRH and cGnRH-II—did not hybridize to any cells in these areas.

Chicken GnRH-II-producing cells. Large cells positive for cGnRH-II mRNA were found only in the nucleus of the medial longitudinal fasciculus of the dorsal midbrain tegmentum (Figs. 4e, 5e, and 6). They were located along or close to the midline, caudal to the posterior commissure and rostral to the oculomotor nucleus. In each section, 2–7 cGnRH-II mRNA-positive cells were detected, and no reaction with probes to the other forms of GnRH was detected in this area.

DISCUSSION

Three forms of GnRH have recently been described in the brain of gilthead seabream [3]: sGnRH, sbGnRH, and cGnRH-II. In a previous paper, we reported the cDNA sequence of the sbGnRH precursor in seabream [7]. This paper reports on the molecular cloning and characterization of the cDNAs encoding the precursors for the two other forms of GnRH—sGnRH and cGnRH-II—and the localization of cells producing the mRNA encoding the three forms of GnRH in seabream brain.

The GnRH precursors characterized in the present study and previously [7] follow the same general organization of all other GnRH precursors: the primary translation product contains a signal peptide at the N-terminal followed by the GnRH decapeptide, the conserved cleavage site, and the GAP region at the C-terminal end of the molecule, suggesting that they have a common ancestral origin. However, the three GnRH precursors share amino acid similarity only in the bioactive decapeptide and the cleavage site while the remaining regions are highly diverse, suggesting that the genes encoding the three GnRHs were formed early in the evolution of the GnRH peptide family.

The cDNA encoding the sGnRH precursor has been isolated thus far from a number of teleost species. The sGnRH precursor reported here has 88–98% sequence identity with that of other perciforms and 72–75% with that of other fish classes (Table 2). A notable difference between the seabream sGnRH cDNA reported here and other previously reported sGnRH cDNAs is the unusually long 5′ UTR (Fig. 2). Comparison of the seabream sGnRH cDNA sequence with the Atlantic salmon sGnRH genomic DNA and cDNA sequences [29] reveals that 1) the region that corresponds to intron 1 in the Atlantic salmon sGnRH gene is not spliced out of the seabream sGnRH mRNA, 2) the transcription initiation site in the seabream sGnRH gene is about 1.3 kb upstream from that of the sGnRH gene in the Atlantic salmon, and 3), a sequence of 117 bp in the 5′ UTR of the seabream sGnRH cDNA (nt 1175–1291) is highly homologous (75% identity) to a region of the Atlantic salmon sGnRH promoter, which stretches upstream from and includes the TATA box. We have screened fractions of our cDNA library for additional clones of sGnRH cDNA using PCR and found only positive clones with long 5′ UTR that contained "intron 1." On the basis of sequence analysis of the Atlantic salmon sGnRH gene, Klungland et al. [29] suggested the presence of a second putative promoter, occurring 1244 bp upstream from the active promoter. However, using reverse transcriptase (RT)-PCR, they were able to show that only the downstream promoter is active [29]. In transgenic zebrafish carrying either the downstream active promoter or the putative upstream promoter attached to a reporter gene, both promoters were shown to be active, but at different locations in the brain (Alestrom, personal communication). Taken together, the above findings suggest that two promoters may be active in the sGnRH gene. In seabream females undergoing final oocyte maturation (the reproductive stage from which our

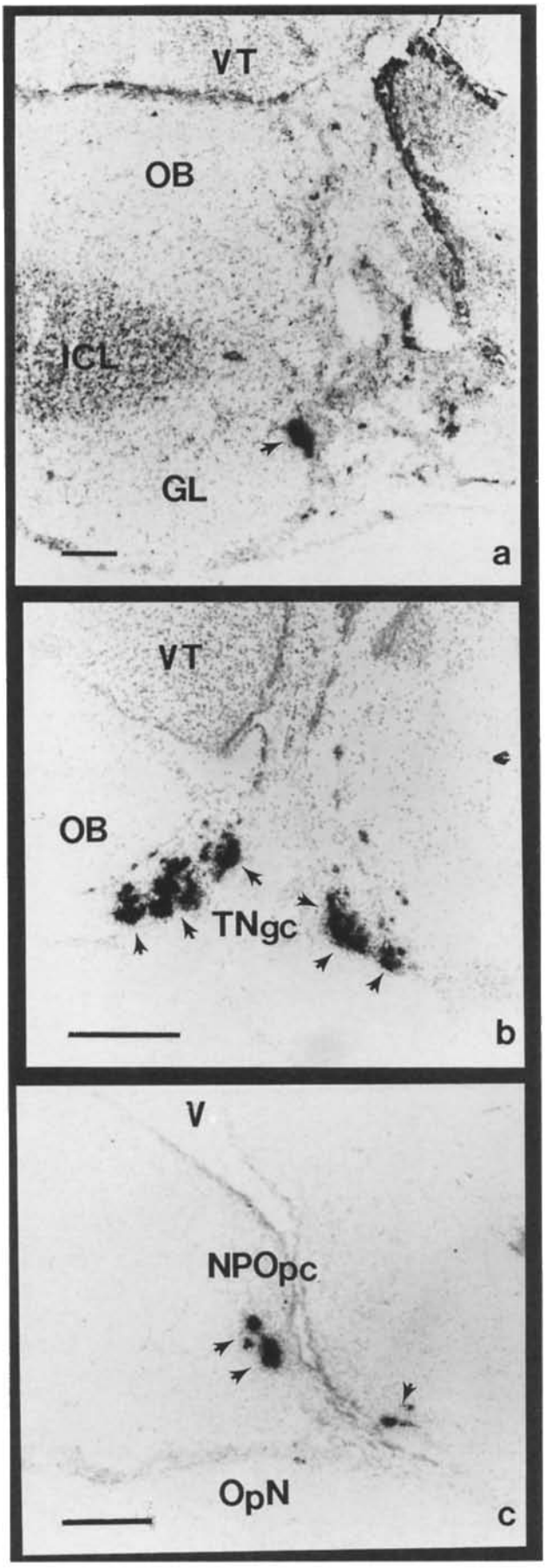

FIG. 4. In situ hybridization for the three different forms of GnRH in seabream brain. Photographs represent sections from the olfactory bulb (**a**), at the transitional zone between the olfactory bulb and telencephalon (**b**), preoptic area (**c**), thalamus (**d**), and midbrain (**e**) hybridized with ^{33}P-labeled synthetic oligonucleotide probes against the mRNAs encoding sGnRH precursor (**a,b**), sbGnRH precursor (**c,d**) and cGnRH-II precursor (**e**). Arrows mark positive cells. Scale bars represent 200 μm. For abbreviations refer to Figure 5 legend.

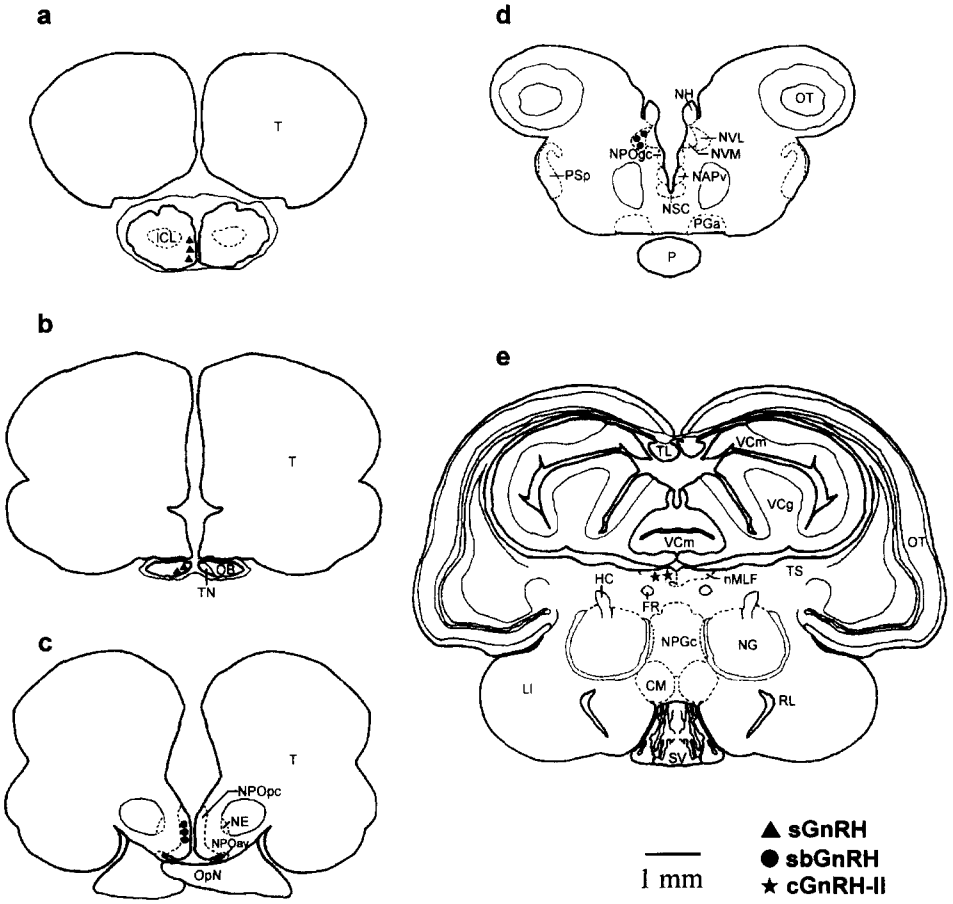

FIG. 5. Schematic illustration of the distribution of sGnRH-(**a,b**) sbGnRH-(**c,d**), and cGnRH-II-(**e**) producing cells in the seabream brain as seen on representative transverse sections. Abbreviations: CC, corpus cerebelli; CM, corpus mamillare; GL, glomerular layer of the olfactory bulb; FR, fasciculus retroflexus; ICL, internal cellular layer of the olfactory bulb; HC, horizontal commissure; LI, lobus inferior hypothalami; MO, medulla oblongata; NAPv, nucleus anterioris periventricularis; NE, nucleus entopeduncularis; NG, nucleus glomerulosus; NH, nucleus habenularis; nMLF, nucleus of the medial longitudinal fasciculus; NPGc, nucleus preglomerulosus commissuralis; NPOav, nucleus preopticus pars anteroventralis; NPOgc, nucleus preopticus magnocellularis pars gigantocellularis; NPOpc, nucleus preopticus pars paevocellularis; NSC, nucleus suprachiasmaticus; NVL, nucleus ventrolateralis thalami; NVM, nucleus ventromedialis thalami; OB, olfactory bulb; olfN, olfactory nerve; OpN, optic nerve; OT, optic tectum; P, pituitary; PC, posterior commissure; PGa, nucleus preglomerulosus anterior; PSp, nucleus pretectalis superficialis, pars paevocellularis; RL, recessus lateralis; RP, recessus posterioris; SV, saccus vasculosus; T, telencephalon; TL, torus longitudinalis; TN, terminal nerve; TNgc, ganglion cells of the terminal nerve; TS, torus semicircularis; V, ventricle; VC, valvula cerebelli; VCg, granular layer of the valvula cerebelli; VCm, molecular layer of the valvula cerebelli; VL, vagal lobe; VT, ventral telencephalon.

library of brain cDNA was constructed), the upstream promoter is active, producing a transcript with a long 5′ UTR and retaining "intron 1." However, it is also possible that the sGnRH cDNA sequence reported here represents a premature transcript. We are currently examining the possibility that the putative downstream promoter is active at other stages of sexual development in the seabream. Alternative promoter usage for the sGnRH gene is analogous to the expression of mGnRH in humans. In the human mGnRH gene, one promoter is active in the hypothalamus, and a second upstream promoter, active in reproductive organs, gives rise to an mRNA that has a longer 5′ UTR and in which the first intron is retained in the mature mRNA [30]. The occurrence and biological significance of alternative promoter usage in the sGnRH gene should be further investigated in fish.

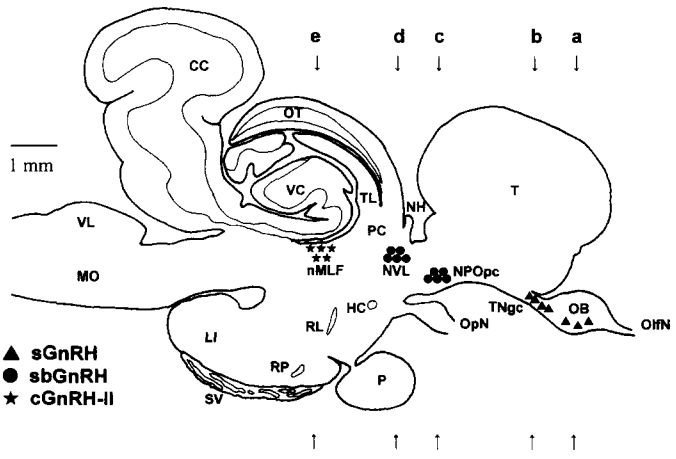

FIG. 6. Schematic presentation of the distribution of GnRH-producing cells in the seabream brain as seen in longitudinal section. The levels (**a** to **e**) of the transverse sections shown in Figure 5 are indicated by arrows. For abbreviations refer to Figure 5 legend.

The cDNA encoding the sbGnRH precursor was first isolated from a seabream brain cDNA library [7]. It encodes a 95-amino acid primary translation product composed of a 25-amino acid signal peptide, the biologically active sbGnRH followed by a processing site (Gly-Lys-Arg), and a 57-amino acid associated peptide. Recently, the cDNA encoding the sbGnRH precursor was PCR-amplified from a second perciform fish, the African cichlid [8]. These two cDNA sequences share 80% identity at the nucleotide level, whereas at the amino acid level there is only 60% identity. Moreover, in the African cichlid sbGnRH cDNA, there is a deletion of 5 nt in the GAP encoding region. This deletion leads to a shift in the nucleotide reading frame, and the ten C-terminal amino acids of the sbGnRH precursor are distinctly different between seabream and African cichlid. This suggests that the C-terminal portion of the GAP is not important for a possible biological role of the GAP.

The cDNA encoding the cGnRH-II precursor has been isolated thus far from three species: cichlid [20], catfish [25], and gilthead seabream (this paper). Analysis of the deduced amino acid of the seabream cGnRH-II precursor suggests that in addition to the cGnRH-II decapeptide, two additional peptides may be released from the precursor. One would be 28 amino acids, from the first cleavage site

(Gly^{11}-Lys^{12}-Arg^{13}) to the second putative cleavage site (Arg^{42}-Arg^{43}). The rest of the GAP, 19 amino acids, would form the second peptide. The cGnRH-II precursor has high identity to that of the African cichlid, which may also be cleaved in the same way [20], suggesting a possible important biological role for the associated peptide(s) of c-GnRH-II.

Because of the long stretches of dissimilarity among the three cDNAs, we were able to design single-strand DNA probes for specific in situ detection of each form of GnRH mRNA without cross-reactivity with the other GnRH mRNAs. Using this approach, we observed five distinct populations of GnRH-producing cells in the gilthead seabream brain (Figs. 5 and 6): sGnRH is synthesized in cells of the ventromedial olfactory bulbs and in the ganglion cells of the terminal nerve; sbGnRH is produced at the level of the preoptic area, a region known to control GtH secretion in fish, and in the ventral thalamus; and cGnRH-II is synthesized exclusively in the midbrain tegmentum. Therefore, the general organization of the GnRH systems in the seabream is similar to that found in other species. However, in contrast to the present findings, in which three variants of GnRH are present, in most vertebrates the GnRH systems were reported to consist of two forms of GnRH, c-GnRH-II in the midbrain and a species-specific form in the terminal nerve-septum-preoptic system [10].

Neuropeptide function is, in part, reflected by its anatomical arrangements within the brain. The anatomical distribution of the different forms of GnRH in the brain may be used as an indication of their relevance in the regulation of GtH secretion. The GnRH neurons associated with the olfactory bulb and the terminal nerve, sGnRH in the seabream, are a subject of interest and have been studied in a variety of vertebrates (for review see [31]). It has been proposed that the terminal nerve functions as a neuromodulatory center that regulates many systems, including the olfactory and visual systems and the pineal gland, and influences sexual behavior and gonadal function [30]. However, in goldfish, these cells seem not to be hypophysiotropic [32]. Moreover, olfactory tract transection in gourami [15] and goldfish [33] did not affect pituitary GnRH content, ovarian development, reproductive behavior, or ovulation. Therefore, the terminal nerve GnRH may be supportive, but not essential, for reproductive functions and is unlikely to act as a hypophysiotropic hormone in these species [15, 33]. Since sGnRH content in the pituitary of mature seabream is very low [3], we hypothesize that in seabream, sGnRH cells of the olfactory bulb and terminal nerve are not directly involved in the control of GtH release, a hypothesis that should be further confirmed.

GnRH cells of the preoptic area are known to directly innervate the pituitary gland and to regulate reproduction in a number of teleosts [1, 34]. This hypophysiotrophic system is very conserved and is found even in the primitive Agnathans [9, 35]. Immunoreactive GnRH cells were previously demonstrated in this region of the seabream brain using antibodies against mGnRH [36]. In the present study, in accordance with the results in cichlid [8], we have shown that of the three forms of GnRH, only sbGnRH is produced in the preoptic area, specifically in the nucleus preopticus pars parvicellularis. This nucleus has been found to be hypophysiotropic in both the goldfish [32] and electric fish (*Apteronotus leptorynchus*) [37]. Moreover, sbGnRH is dominant in pituitaries of sexually mature seabream, having a 500-fold higher content than sGnRH [3], which is an indication of its transport towards the nerve terminals within the pituitary gland. Seabream GnRH is the dominant form also in the pituitary gland of striped bass (1000-fold higher; Powell, Gothilf, Sherwood, and Zohar, unpublished data) and the only form present in pituitaries of African cichlid and pumpkinseed (*Lepomis gibbosus*) [6]. Hence, we believe that sbGnRH, originating from the preoptic area, is the most relevant form of GnRH for the control of GtH secretion in the seabream.

We have also identified sbGnRH-producing cells in the ventral thalamus. GnRH cells in the thalamus were reported in chicken [38] and two fish species: three-spined stickleback [39] and molly [40]. Moreover, retrograde-labeling experiments revealed the existence of hypophysiotrophic neurons in the thalamus of goldfish and electric fish [32, 37]. In goldfish [41] and rainbow trout [42], the thalamus contains estrogen receptor-positive cells. And electrical stimulation of the thalamus elicits reproductive behavior, including sex color change and gamete release in hermaphrodite sea bass, *Serranus subligarius* [43]. Taken together, these results indicate the possibility that the thalamic sbGnRH cells described in this paper may have a role in the control of GtH release. However, the functional significance of the existence of two populations of sbGnRH cells—one in the preoptic area and one in the thalamus—is, at present, unknown.

The presence of cGnRH-II-producing cells in the midbrain tegmentum of seabream is in agreement with findings in other vertebrates, including teleosts [1, 2, 10]. Neurons from this area of the brain have been shown to project to the spinal cord in the hime salmon (*Onchorhynchus nerka*) [44] and goldfish [45], and it has been suggested that the cGnRH-II present there acts mainly as a neurotransmitter [2]. In the molly, midbrain cGnRH-II cells were shown to innervate the urophysis, a caudal neuroendocrine organ that secretes urotensins [46] and were accordingly implicated in controlling gonadal duct motility and gamete release. In sharks and rays, the midbrain GnRH cells were speculated to be involved in controlling movement of the claspers during copulation and to stimulate the release of GtH from the pituitary [47]. In the eel [16] and goldfish [18], cGnRH-II-containing fibers have been shown to directly innervate the pituitary gland. In addition, retrograde-labeling experiments in the goldfish showed hypophysiotrophic neurons in the midbrain tegmentum that resemble cGnRH-II neurons in size, shape, and position [32]. These data suggest a hypophysiotropic function for midbrain cGnRH-II cells in some fish species. However, in the seabream, cGnRH-II is not detectable in pituitaries of mature fish [3], and it is unlikely that cGnRH-II directly controls GtH release in this species.

In tetrapod vertebrates, the hypothalamic GnRH neurons originate from the olfactory placode and migrate along the olfactory tract to the preoptic area and hypothalamus [10]. Developmental studies in platyfish [48] and salmon [49, 50] have provided evidence that, as in higher vertebrates, a group of GnRH neurons follow the same migration pathway. This route of GnRH cell migration in fish results in a "continuum" of GnRH cells in the terminal nerve-septum-preoptic system of adult fish such as seen in salmonids [14], cyprinids [51], and eel [16]. The other group of GnRH cells in the midbrain express cGnRH-II and are thought to have a different embryonic origin [10]. The overall organization of the GnRH systems in the seabream is similar to that reported in most other vertebrates, that is, having groups of GnRH cells in the olfactory bulb, the terminal nerve, preoptic area, and midbrain. However, unlike other vertebrates, in which cells of the olfactory bulbs, terminal nerve, and

preoptic area have a common origin and contain one form of GnRH, these nuclei in the seabream express two different forms of GnRH: sGnRH in the olfactory bulb and terminal nerve, and sbGnRH in the preoptic area. Furthermore, another population of sbGnRH cells is present in the thalamus of seabream, in a more dorsal position than all other GnRH-producing cells. Thus, it is possible that in seabream the terminal nerve-septum-preoptic GnRH system has a dual embryonic origin.

In this paper, we described the characterization of c-DNAs encoding for precursors of three forms of GnRH: sGnRH, sbGnRH, and cGnRH-II. Using specific molecular probes, we have shown that the three forms of GnRH are expressed at different nuclei in the brain. On the basis of their brain localization, presence in the pituitary [3], and bioactivity [23], we suggest that sbGnRH is the most relevant form of GnRH for the direct stimulation of pituitary GtH secretion in seabream and that the other two forms, sGnRH and cGnRH-II, have a different function, yet to be discovered. A similar situation probably exists in other fish belonging to the order Perciformes [6, 8]. Moreover, chromatographic evidence from fish of other orders such as Pleuronectiformes [52], Gasterosteiformes [53], and even the distant Characiformes [54] suggest the possibility that the presence of sbGnRH as a third form of GnRH is phylogenetically widespread.

In the last thirteen years, the number of GnRH forms discovered in vertebrates has increased from one to nine. Furthermore, the multiplicity of GnRH variants in a single species has changed from the exception to the rule. The low levels of these peptides in the brain has made their isolation and identification difficult. It is thus anticipated that sensitive molecular methods, which thus far have been used only to isolate cDNAs encoding known GnRH forms, may be used to confirm the identity of GnRHs in fish brains and to discover additional forms that until now have been undetectable by conventional biochemical and immunological techniques.

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