Differential Expression of Three Different Prepro-GnRH (Gonadotrophin-Releasing Hormone) Messengers in the Brain of the European Sea Bass (*Dicentrarchus labrax*)

DAVID GONZÁLEZ-MARTÍNEZ,[1,2] THIERRY MADIGOU,[2] NILLI ZMORA,[3] ISABELLE ANGLADE,[2] SILVIA ZANUY,[4] YONATHAN ZOHAR,[3] ABIGAIL ELIZUR,[3] JOSÉ ANTONIO MUÑOZ-CUETO,[1] OLIVIER KAH,[2]*

[1]Departamento de Biología Animal, Vegetal y Ecología, Facultad de Ciencias del Mar, Universidad de Cádiz, 11510 Puerto Real, Spain
[2]Endocrinologie Moléculaire de la Reproduction, UPRES-A CNRS 6026, Campus de Beaulieu, 35042 Rennes cedex, France
[3]National Center for Mariculture, Eilat 88112, Israel
[4]Instituto de Acuicultura de Torre de la Sal, CSIC, 12595 Ribeira de Cabanes, Castellón, Spain

ABSTRACT

The expression sites of three prepro-gonadotrophin-releasing hormones (GnRHs), corresponding to seabream GnRH (sbGnRH: Ser^8-mGnRH, mammalian GnRH), salmon GnRH (sGnRH: Trp^7Leu^8-mGnRH), and chicken GnRH-II (cGnRH-II: $His^5Trp^7Tyr^8$-mGnRH) forms were studied in the brain of a perciform fish, the European sea bass (*Dicentrarchus labrax*) by means of in situ hybridization. The riboprobes used in this study correspond to the three GnRH-associated peptide (GAP)-coding regions of the prepro-GnRH cDNAs cloned from the same species (salmon GAP: sGAP; seabream GAP: sbGAP; chicken GAP-II: cIIGAP), which show little oligonucleotide sequence identity (sGAP versus sbGAP: 42%; cIIGAP versus sbGAP: 36%; sGAP versus cIIGAP: 41%). Adjacent paraffin sections (6 mm) throughout the entire brain were treated in parallel with each of the three anti-sense probes and the corresponding sense probes, demonstrating the high specificity of the hybridization signal. The results showed that both sGAP and sbGAP mRNAs had a broader expression in the olfactory bulbs, ventral telencephalon, and preoptic region, whereas cIIGAP mRNA expression was confined to large cells of the nucleus of the medial longitudinal fascicle. In the olfactory bulbs, both the signal intensity and the number of positive cells were higher with the sGAP probe, whereas sbGAP mRNA-expressing cells were more numerous and intensely stained in the preoptic region. Additional isolated sbGAP-positive cells were detected in the ventrolateral hypothalamus. These results demonstrate a clear overlapping of sGAP- and sbGAP-expressing cells in the forebrain of the European sea bass, in contrast to previous reports in other perciforms showing a clear segregation of these two cell populations. J. Comp. Neurol. 429:144–155, 2001.

Indexing terms: in situ hybridization; teleost; perciform fish; reproduction

In all vertebrates, gonadotrophin-releasing hormone (GnRH), as indicated by its name, plays a key role in the central mechanisms regulating the reproductive axis, mainly by modulating the synthesis and release of pituitary gonadotrophins. First characterized in mammals (mammalian GnRH: mGnRH; Matsuo et al., 1971; Burgus et al., 1972), GnRH was next found in all vertebrates, and more recently two forms have been sequenced in protochordates (tun1-GnRH and tun2-GnRH; Powell et al., 1996b), so that the GnRH family now includes now 13

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*Correspondence to: O. Kah, Endocrinologie Moléculaire de la Reproduction, UPRES-A CNRS 6026, Campus de Beaulieu, 35042 Rennes cedex, France. E-mail: olivier.kah@univ-rennes1.fr

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TABLE 1. Primary Structures of the 13 Forms of GnRH Known in Procordates and Vertebrates[1]

Organism	GnRH type	Sequence
Mammalian	mGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
Chicken-I	cGnRH-I	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH_2
Chicken-II	cGnRH-II	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2
Salmon	sGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2
Catfish	cfGnRH	pGlu-His-Trp-Ser-His-Gly-Leu-Asn-Pro-Gly-NH_2
Seabream	sbGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly-NH_2
Dogfish	dfGnRH	pGlu-His-Trp-Ser-His-Gly-Trp-Leu-Pro-Gly-NH_2
Lamprey-I	1-lGnRH	pGlu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly-NH_2
Lamprey-III	1-lIIGnRH	pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2
Tunicate-1	tun1-GnRH	pGlu-His-Trp-Ser-Asp-Tyr-Phe-Lys-Pro-Gly-NH_2
Tunicate-2	tun2-GnRH	pGlu-His-Trp-Ser-Leu-Cys-His-Ala-Pro-Gly-NH_2
Guinea pig	gpGnRH	pGlu-Tyr-Trp-Ser-Tyr-Gly-Val-Arg-Pro-Gly-NH_2
Herring	hrGnRH	pGlu-His-Trp-Ser-His-Gly-Leu-Ser-Pro-Gly-NH_2

[1]Underlined residues indicate substitutions with respect to the mammalian sequence. Data from Carolsfeld et al., 2000.

variants (Carolsfeld et al., 2000; Table 1). Traditionally, these peptides have received the name from the species in which they were first found. Six of these GnRH forms have been discovered in the brain of different teleosts: salmon GnRH (sGnRH: Sherwood et al., 1983), mGnRH (King et al., 1990), catfish GnRH (cfGnRH: Ngamvongchon et al., 1992), seabream GnRH (sbGnRH: Powell et al., 1994), and chicken GnRH-II (cGnRH-II: King and Millar, 1982b; Miyamoto et al., 1984). This latter decapeptide isolated from chicken was named cGnRH-II since a first form had been found before in the same species (chicken GnRH-I: King and Millar, 1982a; Miyamoto et al., 1982).

Very recently, a new variant (His^5Ser^8-mammalian GnRH), has been characterized from the brain of the herring, a primitive teleost (Carolsfeld et al., 2000). Other variants include two GnRH peptides characterized in lamprey (lGnRH-I: Sherwood et al., 1986; lGnRH-III: Sower et al., 1993), one in dogfish (dGnRH: Lovejoy et al., 1992), and a last one in guinea pig (gpGnRH: Jiménez-Liñan et al., 1997). A major outcome of the numerous comparative studies based on biochemical, morphological, and physiological approaches in all vertebrate classes is the fact that the brain of many vertebrate species expresses at least two forms of GnRH. One GnRH is mainly synthesized in the forebrain and varies according to the species, whereas the second GnRH form is highly conserved in terms of both structure and site of expression. Indeed, cGnRH-II-expressing neurons have been consistently detected in the synencephalic/mesencephalic area of many species, including primates (Urbanski et al, 1999).

Extensive studies in fish have clearly demonstrated the existence of two segregated GnRH systems, one anterior extending from the olfactory bulbs to the pituitary through the ventral telencephalon, preoptic area, and ventromedial hypothalamus, and the other restricted to the synencephalon (Kah et al., 1986). In different species, it

Abbreviations

AP	accessory pretectal nucleus
CCe	corpus of the cerebellum
CP	central posterior thalamic nucleus
Dc1	subdivision 1 of the central part of the dorsal telencephalon
Dd	dorsal part of the dorsal telencephalon
Dld	dorsolateral partof the dorsal telencephalon
Dlv2	subdivision 2 of the ventrolateral part of the dorsal telencephalon
Dm2	subdivision 2 of the medial part of the dorsal telencephalon
Dm3	subdivision 3 of the medial part of the dorsal telencephalon
Dm4	subdivision 4 of the medial part of the dorsal telencephalon
DP	dorsal posterior thalamic nucleus
ECL	external cellular layer
GL	glomerular layer
ICL	internal cellular layer
LSO	lateral septal organ
MaOT	marginal optic tract
NAPv	anterior periventricular nucleus
NAT	anterior tuberal nucleus
NC	nucleus corticalis
NDLIl	lateral part of the diffuse nucleus of the inferior lobe
NDLIm	medial part of the diffuse nucleus of the inferior lobe
NGa	anterior part of the nucleus glomerulosus
NGp	posterior part of the nucleus glomerulosus
NGT	tertiary gustatory nucleus
NLTd	dorsal part of the lateral tuberal nucleus
NLTi	inferior part of the lateral tuberal nucleus
NLTl	lateral part of the lateral tuberal nucleus
NLTm	medial part of the lateral tuberal nucleus
NLTv	ventral part of the lateral tuberal nucleus
nMLF	nucleus of the medial longitudinal fasciculus
NPC	central pretectal nucleus
NPGc	commissural preglomerular nucleus
NPGl	lateral preglomerular nucleus
NPGm	medial preglomerular nucleus
NPOav	anteroventral part of the parvocellular preoptic nucleus
NPOpc	parvocellular part of the parvocellular preoptic nucleus
NPPv	posterior periventricular nucleus
NPT	posterior tuberal nucleus
NRLd	dorsal part of the nucleus of the lateral recess
NRLl	lateral part of the nucleus of the lateral recess
NRLv	ventral part of the nucleus of the lateral recess
NRP	nucleus of the posterior recess
NSV	nucleus of the saccus vasculosus
NTe	nucleus of the thalamic eminentia
OB	olfactory bulbs
OLN	olfactory nerve fibers
OT	optic tectum
P	pituitary
PCo	posterior commissure
PMgc	gigantocellular part of the magnocellular preoptic nucleus
PMmc	magnocellular portion of the magnocellular preoptic nucleus
POA	preoptic area
PPd	dorsal periventricular pretectal nucleus
PPv	ventral periventricular pretectal nucleus
PSm	magnocellular superficial pretectal nucleus
PSp	parvocellular superficial pretectal nucleus
PT	posterior thalamic nucleus
PVO	paraventricular organ
rpo	preoptic recess
SOF	secondary olfactory fibers
SV	saccus vasculosus
TEG	tegmentum
TEL	telencephalon
TLa	nucleus of the torus lateralis
TLo	torus longitudinalis
TNgc	terminal nerve ganglion cells
TPp	periventricular nucleus of the posterior tuberculum
VAO	ventral accessory optic nucleus
Vc	central part of the ventral telencephalon
VCe	valvula of the cerebellum
Vd	dorsal part of the ventral telencephalon
Vl	lateral part of the ventral telencephalon
VM	ventromedial thalamic nucleus
VOT	ventral optic tract
Vs	supracommissural part of the ventral telencephalon
Vv	ventral part of the ventral telencephalon

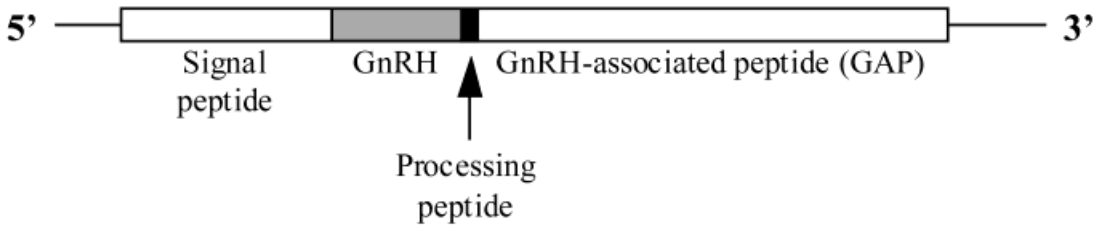

Fig. 1. Organization of the prepro-GnRH cDNA showing the peptide signal, the GnRH decapeptide, the processing peptide, and the GnRH-associated peptide (GAP).

was shown that the anterior system expresses either mGnRH in primitive fish (Leprêtre et al., 1993; Montero et al., 1994), sGnRH in cyprinids and salmonids (Yu et al., 1988; Amano et al., 1991), or cfGnRH in catfishes (Zandbergen et al., 1995), whereas the posterior system consistently expresses cGnRH-II.

More recently, it was shown that the brain of the gilthead seabream expresses three GnRH forms: sGnRH, cGnRH-II, and a third form referred to as seabream GnRH (sbGnRH, Ser^8-mGnRH), which was first isolated by means of high-pressure liquid chromatography and characterized by Edman degradation and mass spectrometry (Powell et al., 1994). The cDNAs encoding these three forms were sequenced (Gothilf et al., 1995, 1996) and shown to encode GnRH precursors having the same structural organization with a signal peptide, the GnRH decapeptide, a processing tripeptide, and a GnRH-associated peptide (GAP; Fig. 1). As the oligonucleotide sequences of the three GAPs were found to be highly divergent within the same species, probes corresponding to the GAPs have been used to localize the corresponding messengers in the brain of the gilthead seabream (Gothilf et al., 1996), allowing one to overcome the difficulty in obtaining specific antibodies against these highly related peptides. Interestingly, it was found that the cells expressing the new sbGnRH form appeared in the preoptic region, whereas those expressing sGnRH and cGnRH-II were localized in the olfactory bulbs and midbrain, respectively (Gothilf et al., 1996). In addition, sbGnRH was the predominant form in the pituitary gland (Powell et al., 1994; Holland et al., 1998).

The existence of three GnRH forms was confirmed, either by cDNA sequencing or biochemical characterization, in other perciform species including the African cichlid, *Haplochromis burtoni* (White et al., 1995), the red seabream, *Pagrus major*, the black seabream, *Acanthopagrus schlegeli*, the striped knifejaw, *Oplegnathus fasciatus*, and the Nile tilapia, *Oreochromis niloticus* (Senthilkumaran et al., 1999). However, recent studies also indicate that sbGnRH is not restricted to perciforms since it was also found in a scorpaeniform, *Sebastes rastrelliger* (Powell et al., 1996a) and a characiform, *Piaractus mesopotamicus* (Powell et al., 1997). In the African cichlid (White et al., 1995; White and Fernald, 1998) and the red seabream (Okuzawa et al., 1997), distribution of cells expressing the different GnRHs was found to be similar to that reported in the gilthead seabream by Gothilf et al. (1996), indicating a neuroanatomical segregation of the different GnRH systems.

It is therefore more and more considered that expression of sGnRH is restricted to the caudal olfactory bulbs, that of sbGnRH to the preoptic area, and that of cGnRH-II to the dorsal synencephalon. However, comparison of these data with those obtained previously in different teleosts by means of immunohistochemistry tends to indicate that the nature of the GnRH form expressed by many GnRH-immunoreactive neurons remains unknown. Indeed, none of the studies performed so far by in situ hybridization in perciforms mention the numerous GnRH-immunoreactive cells of the anterior olfactory bulbs, the ventral telencephalon, or the mediobasal hypothalamus reported in a number of species (Kah et al., 1986; Amano et al., 1991; Montero et al., 1994; Kim et al., 1995), including perciforms such as the European sea bass (Kah et al., 1991), and thus the precise identity of the variant synthesized in those neurons is still unknown. The recent cloning of three cDNAs encoding prepro-sGnRH, -sbGnRH, and -cGnRH-II in this latter species (N. Zmora, Y. Zohar, and A. Elizur, unpublished results) provided the opportunity of looking in detail at the distribution of cells expressing the corresponding GAPs by means of in situ hybridization. In this study, we present the precise localization of sGAP, sbGAP-, and cIIGAP-expressing cells in the brain of the European sea bass, by using probes obtained in that species. One of the main objectives of this study was to identify the prepro-GnRH forms expressed in GnRH-immunoreactive neurons of the anterior olfactory bulbs, the ventral telencephalon, or the mediobasal hypothalamus.

MATERIALS AND METHODS

Animals

Specimens of sea bass (n = 6) were purchased from a local fishery (Cupimar, San Fernando, Spain) and kept in the laboratory in running sea water. Two immature males (weight 205 and 280 g, gonadosomatic index 0.15 and 0.30, respectively), two vitellogenic females (weight 250 and 385 g, gonadosomatic index 0.33 and 0.76, respectively), and two spermiating males (weight 300 and 385 g, gonadosomatic index 1 and 2, respectively) were used. Animals were treated in agreement with the European Union regulation concerning the protection of experimental animals.

Isolation of the sequences coding for the seabass GAPs

Total RNA was extracted from hypothalamus of sexually mature female by using a scaled-down guanidinium thiocyanate method (Elizur et al., 1996). cDNA was synthesized by using 1 μg of total hypothalamus RNA, rtAMV (Promega, Madison, WI), and the universal oligo dT adaptor primer (Table 2).Primers specific for each of the three GAP regions were designed according to the striped bass and seabream GAP and flanking sequences (Gothilf et al.,

1996; Table 2). The first PCR was performed by using primers located 5′ to the GAP (sbGAP 1:, sGAP 1, and cIIGAP 1, respectively) and the 3′ adaptor primer (Table 2). The resulting products were then subjected to nested polymerase chain reaction (PCR) by using degenerate GAP primers (sbGAP 2: forward and reverse; sGAP 2 forward and reverse; cIIGAP 2 forward and reverse).

The amplified PCR products were cloned into the pGEMT vector (Promega). The full-length sequences of the three seabass GnRH forms are available at the Genbank, accession numbers AF224279 for sbGnRH, AF224280 for sGnRH, and AF224281 for cGnRH-II. A full report of the cloning of these sequences is currently in preparation.

Riboprobe synthesis

Messenger RNAs used for the probes correspond to the GAP coding region of the sea bass prepro-GnRH. The GAP probes consisted of 159 nt for the cGnRH-II GAP, 192 nt for the sbGnRH GAP, and 174 nt for the sGnRH GAP (Fig. 2). At the nucleotide level, sequence identities were as follows: sGAP GnRH versus sbGAP GnRH: 42%; cII GAP GnRH versus sbGAP GnRH: 36%; and sGAP GnRH versus cIIGAP GnRH: 41%. The preparation of [α-^{35}S]dUTP-labeled single-stranded sense and antisense RNA probes was according to standard procedures. The plasmids pGEM-T containing either sbGAP, sGAP, or cIIGAP cDNA were linearized with *Bam*HI or *Nco*I to synthesize sense and anti-sense riboprobes by using T7 and Sp6 RNA polymerase, respectively. One microgram of the linearized plasmid was incubated for 1 hour at 37°C in a solution containing a transcription buffer (Tris-HCl 40 mM, pH 8.25, $MgCl_2$ 6 mM, spermidine 2 mM), dithiotreitol 10 mM (DTT), rATP, rGTP, rCTP (0.25 mM each), 100 μCi [α-^{35}S] dUTP (ICN), RNase inhibitor, and 2.5 U of the appropriate RNA polymerase. The DNA template was then digested with RQ-1 DNase for 15 minutes at 37°C. Probe purification was achieved on a Sephadex G50 column 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% sodium dodecyl sulfate [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, Tris-HCl 20 mM, pH 8.5, EDTA 5 mM, 10% dextran sulfate, 1× Denhardt's solution, DTT 10 mM, 0.5 μg/μl yeast tRNA) at a concentration of 2×10^4 cpm/μl.

TABLE 2. Primers Used for Cloning of the GAPs[1]

Primer	Sequence
Oligo dT adaptor primer	5′ GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT 3′
sbGAP 1	5′ ACCTGAGCAAGAAGAATGGCT 3′
sGAP 1	5′ GTTGTTGGCGTTGGTGG 3′
cIIGAP 1	5′ CTCGGGCTGCTTCTATGTGT 3′
3′ adaptor primer	5′ GACTCGAGTCGACATCGA 3′
sbGAP 2 forward	5′ CATATGGGGAAG(A/G)GGGA(A/C)CTGGACG 3′,
sbGAP 2 reverse	5′ GGATCCTCATTT(T/C)TT(A/G)TA(G/A/T/C)GTTCTG(T/G)GTCC 3′
sGAP 2 forward	5′ CATATGGGGAAGAGAAG(C/T)GTGGGAGA 3′
sGAP 2 reverse	5′ GGATCCTCA(T/A)TT(A/G)TT(C/A)GGGAACCT(CT)TT(CT)TT 3′
cIIGAP 2 forward	5′ CATATGGGCAAGAGGGAACTGGACTCTTT 3′
cIIGAP 2 reverse	5′ GGATCCTCACTTCCTCTTCTGGAGCTC 3′

[1]Primers were designed according to the striped bass and seabream GAP and flanking sequences (Gothilf et al., 1996).

In situ hybridization

The in situ hybridization protocol was according to Mazurais et al. (1999). Brains were collected from animals anesthesized 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). Tissues were collected, fixed overnight at room temperature, dehydrated, embedded in paraffin, and cut transversally at 6 μm; then sections were mounted on slides treated with Tespa (2% Tespa, Sigma) for processing.

Tissue sections were equilibrated at room temperature, rehydrated, and postfixed for 20 minutes. Sections were treated with protein kinase (20 μg/ml in 50 mM Tris-HCl, pH 8, and 5 mM EDTA) for 7.5 minutes and washed with phosphate-buffered saline (PBS), pH 7.4, for 5 minutes, followed by a refixation in 4% PAF for 5 minutes and a quick wash with distilled water. Thereafter, sections were acetylated for 10 minutes 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.

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sGAP cDNA       1   GGGAAGAGAAGUGUGGGAGAGCUA----GAGGCA----ACCAUCAGGAUGAUGGGUACAG   52
sbGAP cDNA      1   GGGAAGAGGGA-ACUGGACGGCCUCUCCGAGACACUGGGCAAUCAGAUAGUCGGGAGCUU   59
cIIGAP cDNA     1   GGCAAGAGGGA-GCUGGACU-CUU----UUGGCA------CUUCAGAGAUUUCGGAGGAG   48

sGAP cDNA      53   GAGAAGUGGUGUCUCUUCCUGAAGAGGC---GAGUGCCCA-A-ACCCAAGAGAGACUUAG  107
sbGAP cDNA     60   CCCACACGUGGCGACGCCCUGCAGAGUUUUAGGUUGUGCAGAGGAAUCACCUUUCCCCAA  119
cIIGAP cDNA    49   AUUAAGCUGUGUGAGG-CAGGAGAAUGC---AGCUAUUUG-AGACCCCAGAGGAG----G   99

sGAP cDNA     108   ACCAUACA--AUGUAAUUAAUGAUGAUUCCAGUCAUUUUGACCGAAAAAAGAGGUUCCCU  165
sbGAP cDNA    120   AAUAUACAGAAUGAAAGGAUUCCUUGACGCAGUCACUGACAGGGAGAACGGAAAUCGAAC  179
cIIGAP cDNA   100   AGUGUUC------UGAGAAAU-AUCAUUCUGGAUGCCUUAGCC--AGAGAGCUCCAGAAG  150

sGAP cDNA     166   ----AACAAAUGA   174
sbGAP cDNA    180   UUACAAGAAAUGA   192
cIIGAP cDNA   151   ----AGGAAGUGA   159
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Fig. 2. Oligonucleotide sequences of the full-length GAP-coding regions of the sGAP, sbGAP, and cIIGAP cDNAs used for the production of riboprobes.

Coverslips were then removed by immersion in a 5× standard saline citrate (SSC), 10 mM DTT solution at 55°C for 30 minutes and then washed in a 2× SSC, 50% formamide, 10 mM DTT solution at 65°C for 30 minutes followed by several washes in NTE buffer, pH 7.5 (10 mM Tris-HCl, 0.5 M NaCl, 5 mM EDTA) for 10 minutes at 37°C. In order to degrade single-stranded probe, the sections were incubated in a solution of NTE containing RNase A (20 μg/ml) for 30 minutes at 37°C. Sections were then rinsed in 2× SSC, 50% formamide, 10 mM DTT at 65°C, followed by washes of 2× SSC and 0.1× SSC at room temperature, and dehydrated in ethanol. Slides were then dipped into an Ilford K5 photographic emulsion, exposed for 12 days at 4°C, developed, and counterstained with toluidine blue 0.02%. Adjacent sections were systematically treated with the sense and anti-sense probes.

Micrographs were taken with an Olympus Provis photomicroscope using a 25 ASA Agfapan film. Negatives were scanned by using a high-resolution scanner under the TIFF format and processed with Adobe Photoshop 5.0 to generate numerized images. No alterations were made with the exception of light and contrast adjustment.

RESULTS

All levels of the brain were examined with the three anti-sense and three sense probes on adjacent serial sections. After 12 days of exposure, sections hybridized with the three anti-sense probes showed a strong hybridization signal in specific brain nuclei (Fig. 3), whereas adjacent sections treated with the corresponding sense probes only presented a very low uniform background (compare Figs. 4B and C, 5D and E, 6A and B). The results were highly consistent from one fish to the other, and no evident differences could be noted between males and females, although the number of cells positive for each probe was not quantified.

Salmon GAP mRNA-expressing cells

The highest concentration of sGAP mRNAs, in terms of both number of positive cells and signal intensity, was consistently observed in the olfactory bulbs, either in isolated cells of the glomerular layer (Figs. 3A, 4A) or in a bilateral group of large neurons located at the junction between the caudal olfactory bulbs and the telencephalon (Figs. 3B, 4B, 5C). Since these cells are associated with terminal nerve fibers, they are likely to correspond to ganglion cells of the terminal nerve, also referred to as the nucleus olfactoretinalis. At this level, up to 15 positive cells could be detected on a single transverse section. However, sGAP mRNA-expressing cells were not limited to the olfactory bulbs since isolated small neurons were also detected at different levels of the ventral telencephalon (Fig. 3C). Such cells were located laterally to the ventral part of the ventral telencephalon, being observed mainly in the rostral aspect of this structure (Fig. 4D). Furthermore, additional positive cells were found in the preoptic area slightly lateral to the parvocellular and anteroventral subdivisions of the parvocellular preoptic nucleus (Figs. 3D, 4E,F). More caudally, no other sGAP mRNA-expressing cells could be detected in the brain of the sea bass.

Seabream GAP mRNA-expressing cells

Of the three preproGnRH-expressing cells, those exhibiting a hybridization signal with the sbGAP probe had the wider distribution, extending from the olfactory bulbs to the ventrolateral hypothalamus (Fig. 3). The rostralmost sbGAP mRNAs were detected in cells of the glomerular layer of the olfactory bulbs (Figs. 3A, 5A). However, these cells were less numerous than those expressing sGAP in the same region, and only two to three positive cells could be observed in a single transverse section. Seabream GAP mRNA-positive cells were also observed in the vicinity of the terminal nerve ganglion cells (Fig. 3B). As shown in Figure 5B and C, these cells were different from those detected with the sGAP probe and smaller in size. More caudally, groups of positive cells were consistently located in the ventral telencephalon, although they were always larger in size, more numerous and lay in a more ventral position when compared with the location of the sGAP-expressing cells (Figs. 3C, 5D). The most important population of sbGAP mRNA-positive cells was observed in the preoptic region (Fig. 3D,E) at the level of, but slightly lateral to the parvocellular preoptic nucleus (Fig. 5F) and anterior periventricular nucleus (Fig. 5G). At these levels, up to 16 cells were frequently encountered on both hemispheres of a single transverse section. Finally, isolated cells were detected in the ventrolateral hypothalamus up to the level of the pituitary (Figs. 3F, 5H). The caudalmost cells always appear more ventral than the most rostral ones.

ChickenII GAP mRNA-expressing cells

Despite careful examination of all brain levels, the only cIIGAP mRNA-expressing neurons were found in the synencephalon, a transitional region between the diencephalon and the mesencephalon. These cells appeared just caudal to the posterior commissure, at the level of the nucleus of the medial longitudinal fascicle (Figs. 3G, 6A). They were large in size and always located in the vicinity of large blood vessels (Fig. 6A). No more than ten cells appeared at this level on a single transverse section.

DISCUSSION

The present study, whose results are summarized in Figure 7, provides more detailed information concerning the distribution of the cells expressing different preproGnRHs in the brain of a perciform fish, the European sea bass. In line with previous studies, we show that each of the three GAP mRNAs is preferentially expressed in a specific brain region. However, we describe, for the first time, a clear overlapping of the sGAP- and sbGAP-expressing cells in the telencephalon and diencephalon of a perciform species.

In terms of specificity, there is no doubt that the signal obtained with each of the three probes is highly specific. This assumption is based, first, on the fact that the oligonucleotide sequences of the three GAPs do not exhibit more than 42% of identity (sGAP versus sbGAP: 42%; cIIGAP versus sbGAP: 36%; cIIGAP versus sGAP: 41%), which, considering the highly stringent conditions of our washing steps, most likely prevents cross-hybridization. The sense controls systematically performed on parallel

Fig. 3. **A–F:** Schematic representation of the distribution of the cells expressing the sGAP (triangles), sbGAP (circles), and cIIGAP (stars) mRNAs on transverse sections of the brain of the sea bass. Scale bar = 1 mm.

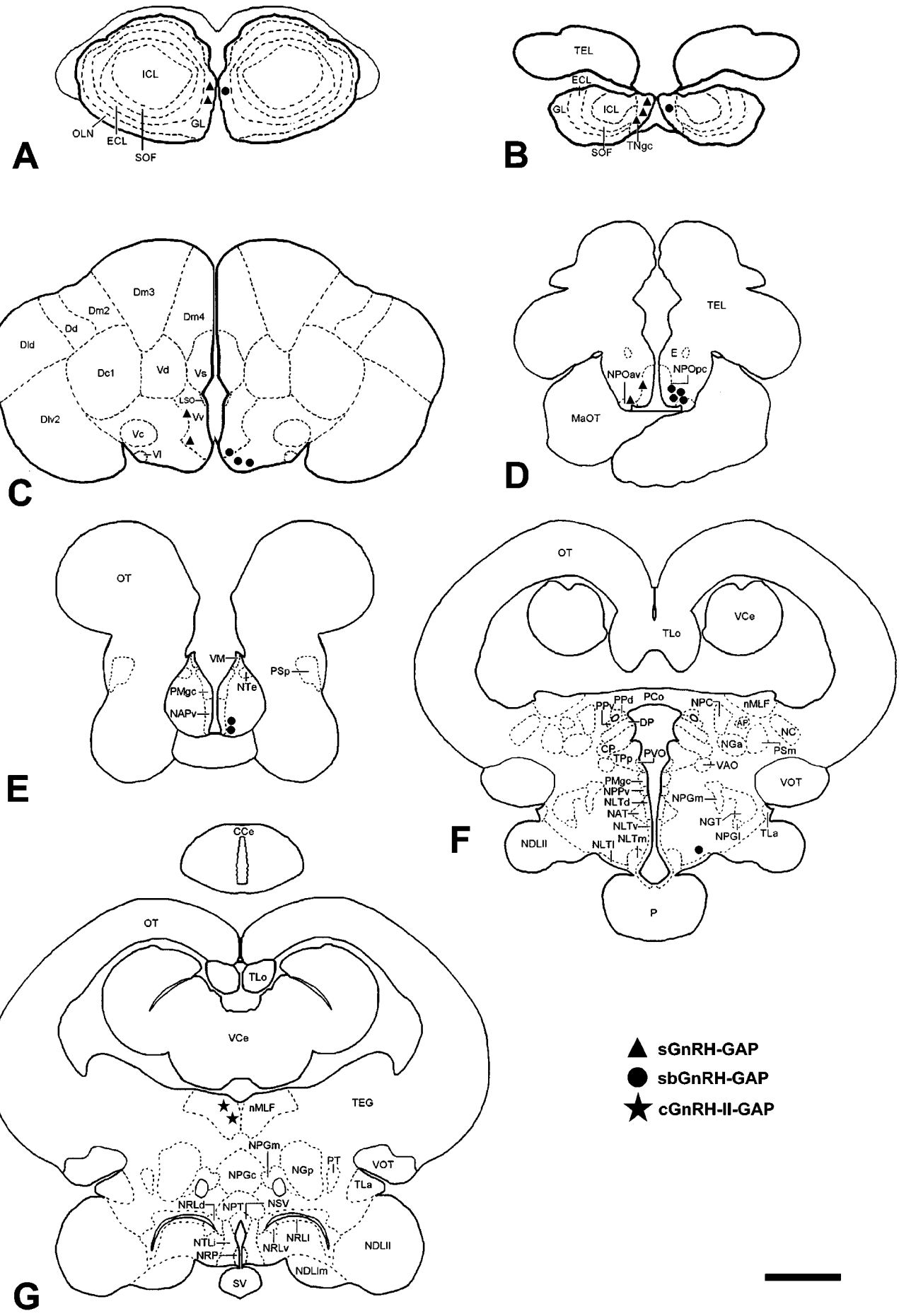
A
ICL
OLN
ECL
SOF
GL
B
TEL
ECL
GL
ICL
SOF
TNgc
C
Dm3
Dm2
Dm4
Dd
Dld
Dc1
Vd
Vs
Lso
Vv
Dlv2
Vc
Vl
D
TEL
E
NPOav
NPOpc
MaOT
E
OT
VM
PSp
NTe
PMgc
NAPv
F
OT
TLo
VCe
PPv
PPd
PCo
NPC
nMLF
O
DP
AP
NC
NGa
PSm
CP
TPp
PVO
VAO
VOT
PMgc
NPPv
NLTd
NAT
NLTv
NLTm
NPGm
NGT
NPGl
TLa
NDLII
NLTl
P
G
CCe
OT
TLo
VCe
nMLF
TEG
NPGm
NPGc
NGp
PT
VOT
TLa
NRLd
NPT
NSV
NTLi
NRP
NRLv
NRLl
NDLII
NDLlm
SV
sGnRH-GAP
sbGnRH-GAP
cGnRH-II-GAP

Figure 3

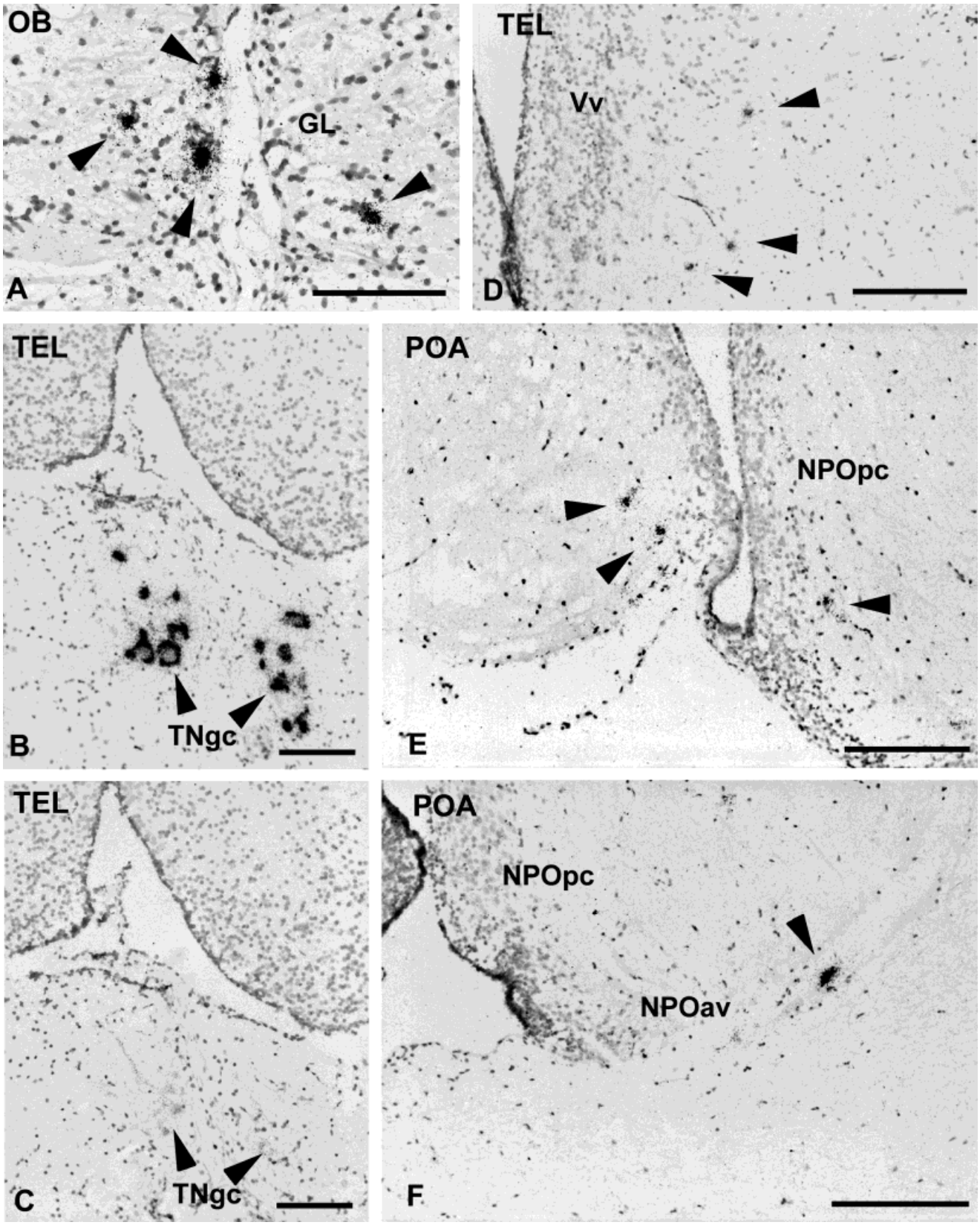

Fig. 4. Micrographs showing sGAP mRNA-expressing cells on transverse paraffin sections counterstained with toluidine blue. **A:** Positive cells (arrowheads) in the glomerular layer of the olfactory bulbs (Fig. 3A). **B,C:** Adjacent sections hybridized with the anti-sense (B) and sense (C) probes showing the strong specific expression of sGAP mRNA in the ganglion cells of the terminal nerve (arrowheads), just rostral to the junction of the olfactory bulbs with the telencephalic hemispheres (Fig. 3B). **D:** Scattered positive cells (arrowheads) lateral to the ventral part of the ventral telencephalon (Fig. 3C). **E:** Scattered cells (arrowheads) at the level of the parvocellular preoptic nucleus (Fig. 3D). **F:** Isolated positive cell (arrowhead) lateral to the anteroventral portion of the parvocellular preoptic nucleus (Fig. 3D). Scale bar = 100 μm.

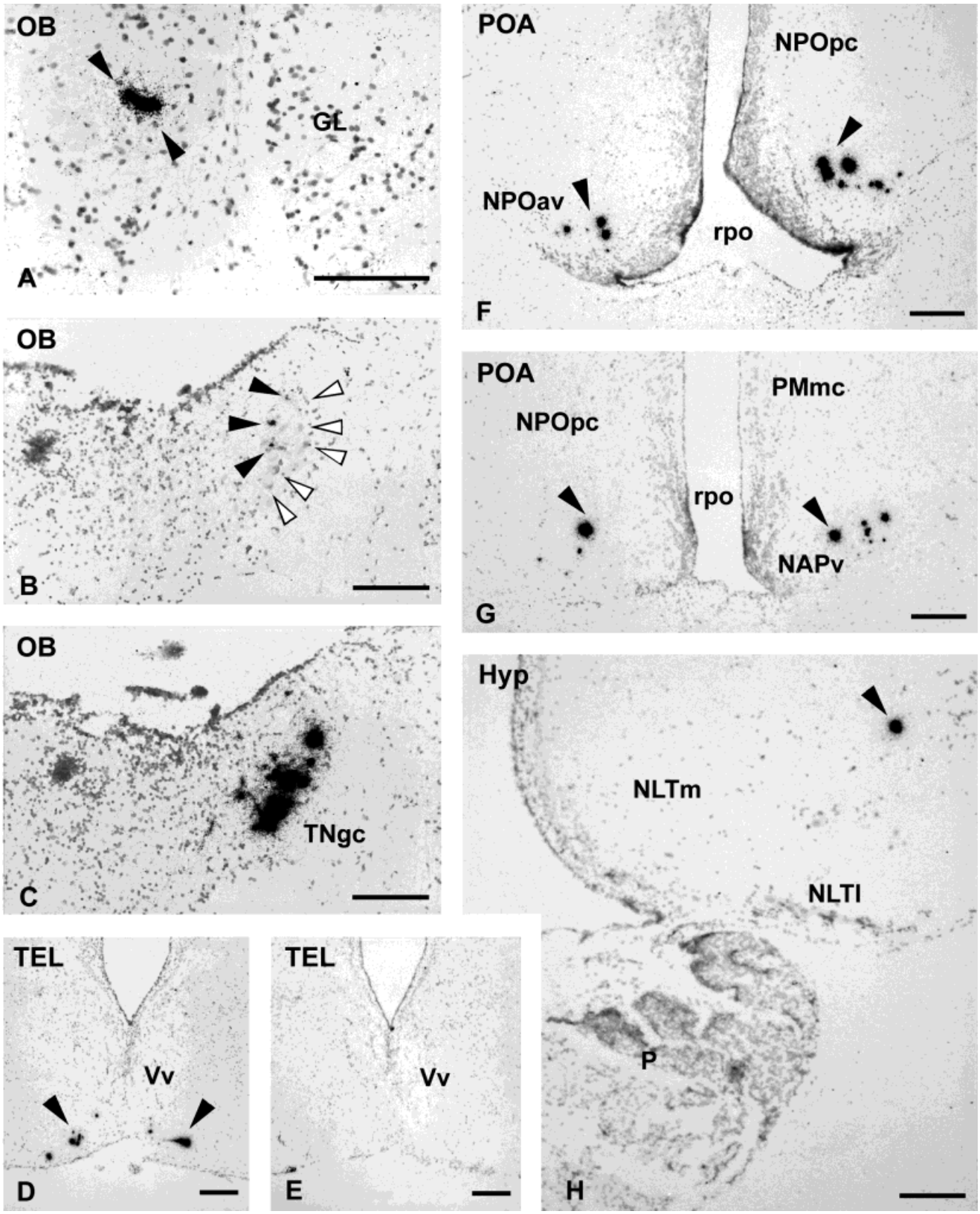

Fig. 5. Micrographs showing sbGAP mRNA-expressing cells on transverse paraffin sections counterstained with toluidine blue. **A:** Positive cells (arrowheads) in the glomerular layer of the olfactory bulbs (Fig. 3A). **B,C:** Adjacent sections hybridized with the sbGAP anti-sense (B) and sGAP anti-sense (C) probes (Fig. 3B). Note in B the presence of small sbGAP-positive cells (black arrowheads). Open arrowheads in B correspond to the large terminal nerve ganglion cells expressing sGAP mRNA in C. **D,E:** Adjacent sections hybridized with the anti-sense (D) and sense (E) probes showing the strong specific expression of sbGAP mRNA in cells (arrowheads) of the ventral part of the ventral telencephalon (Fig. 3C). **F:** Intensely labeled cells (arrowheads) at the level of the parvocellular preoptic nucleus (Fig. 3D). **G:** Positive cells (arrowhead) lateral to the anterior periventricular nucleus (Fig. 3E). **H:** Isolated positive cell (arrowhead) in the ventrolateral hypothalamus (Fig. 3F). Scale bar = 100 μm.

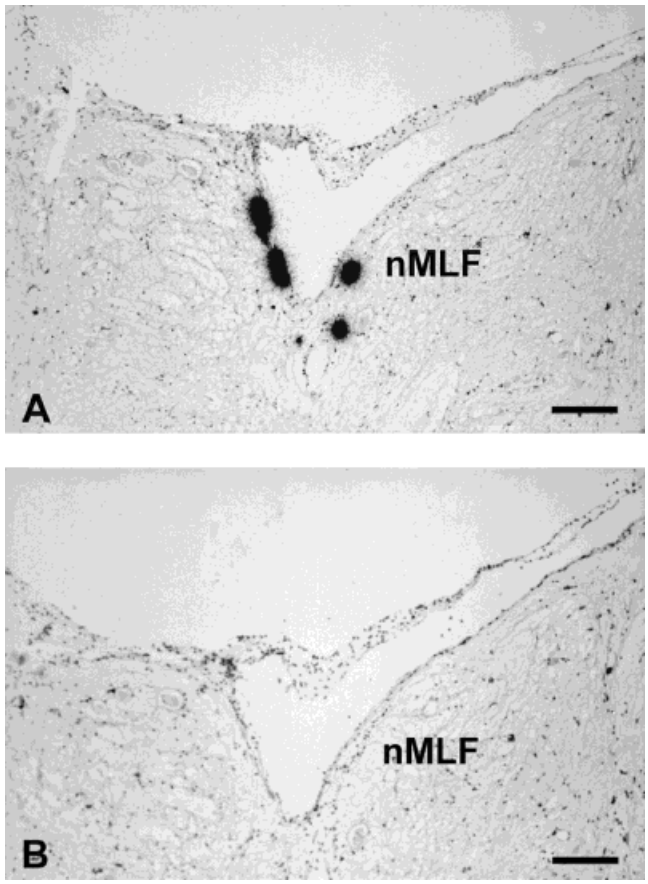

Fig. 6. Micrographs showing cIIGAP mRNA-expressing cells on transverse paraffin sections counterstained with toluidine blue. **A,B:** Adjacent sections hybridized with the anti-sense (A) and sense (B) probes showing the strong specific expression of cIIGAP mRNA in cells (arrowheads) of the nucleus of the medial longitudinal fascicle (nMLF; Fig. 3G). Scale bars = 100 μm.

sections only resulted in a very low and uniform background without preferential silver grain accumulation. Finally, careful examination of adjacent sections hybridized with the three probes clearly indicated the absence of cross-reactivity. This was particularly evident in the synencephalon, where only the cIIGAP probe produced a hybridization signal, which was absent from any other brain areas. Furthermore, the absence of cross-hybridization between the sGAP and sbGAP probes is evidenced by the fact that, at the same transverse level, clearly different cells were labeled by each of the probes. For example, the large ganglion cells of the terminal nerve, positive with the sGAP probe, were unstained on adjacent sections hybridized with the sbGAP probe, which on the contrary only stained small neurons in the vicinity of the large sGAP-labeled cells. Furthermore, the ventrolateral hypothalamus only contained sbGAP-expressing neurons and appeared devoid of sGAP hybridization signal. Taken together, these results indicate that, as shown previously (Zandbergen et al., 1995; Gothilf et al., 1996), probes corresponding to the GAP sequences are suitable for in situ hybridization studies.

With respect to the overall organization, the present results confirm previous studies based on immunohistochemistry in other species showing that GnRH neurons have a widespread distribution in the ventral forebrain (Kah et al., 1986; Oka and Ichikawa, 1990; Amano et al., 1991; Montero et al., 1994; Kim et al., 1995). In the European sea bass, a study performed before the characterization of sbGnRH and based on the use of antibodies to sGnRH showed the presence of numerous GnRH-immunoreactive neurons along a continuum of GnRH fibers extending from the olfactory bulbs to the pituitary (Kah et al., 1991). Positive neurons were notably detected in the anterior olfactory bulbs, at the junction of the olfactory bulbs and telencephalon, in the ventral telencephalon, and in the ventrolateral preoptic region. In addition, a few isolated neurons were reported in the ventrolateral hypothalamus along the immunoreactive fibers extending from the preoptic region to the pituitary (Kah et al., 1991). Therefore, the present study fully confirms this distribution and indicates that, most likely, the sGnRH antibodies used in this pioneer study recognize both sGnRH and sbGnRH.

An interesting finding of the present work is the fact that there is a strong overlapping of the sGAP and sbGAP-expressing cells from the olfactory bulbs to the preoptic region. However, it is clear that the gradient of expression of both cell types are different, with sGAP mRNA-expressing cells being predominant in the olfactory bulbs, whereas sbGAP-expressing cells were more abundant in the caudal telencephalon and preoptic area. This is in contrast to previous studies in perciforms showing a clear segregation of both cell populations (White et al., 1995; Gothilf et al., 1996; Okuzawa et al., 1997; Parhar et al., 1998; White and Fernald, 1998). Based on these results, it has been proposed that sGnRH neurons differentiate from the olfactory placode, whereas sbGnRH neurons originate from the preoptic area (Parhar et al., 1998; Ookura et al., 1999; Parhar, 1999). However, although it is likely that indeed sGnRH neurons originate from the olfactory placode, the present findings do not support the assumption that sbGnRH neurons develop in the preoptic area. First, the sbGnRH-expressing neurons are not confined to a particular brain nucleus, but on the contrary appear to be located along the continuum of GnRH-immunoreactive fibers reported in the sea bass (Kah et al., 1991). This is particularly obvious for the sbGnRH mRNA-positive cells isolated in the ventrolateral hypothalamus. Such immunoreactive cells have been described previously as located along the major bilateral preoptico-hypophyseal GnRH fiber tracts which tend to arch laterally before converging towards the pituitary stalk (Kah et al., 1991). This su ggests that these cells have migrated, probably from an embryonic primordium rostral to the preoptic region, since sbGnRH-expressing cells are found as anterior as the olfactory bulbs.

Although now it seems clear that sbGnRH is the physiologically relevant isoform with respect to gonadotrophin release in perciform fish (Powell et al., 1994; Zohar et al., 1995; Gothilf et al., 1996, 1997; Yamomoto et al., 1997; Holland et al., 1998; Senthilkumaran et al., 1999), the precise functions of sGnRH-expressing cells remain unsolved. The functions of GnRH-immunoreactive neurons of the terminal nerve have been best studied in the dwarf gourami (Yamamoto et al., 1995, 1997). In this species, the terminal nerve ganglion cells have been shown to have intrinsic rhythmic activities and to project widely in the brain, but not to the pituitary (Oka and Matsushima, 1993). Selective lesions of the terminal nerve ganglion

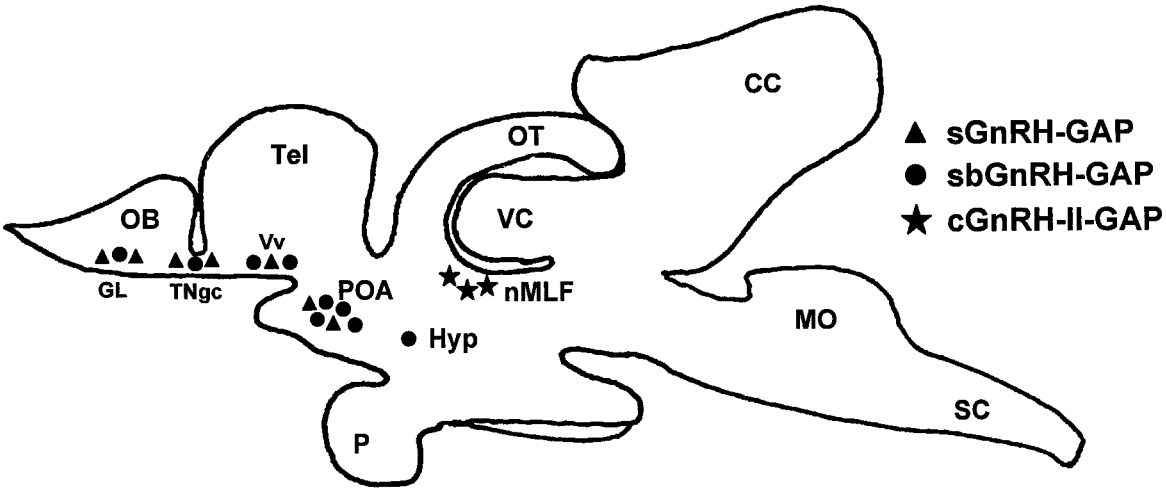

Fig. 7. Summary of the distribution of the cells expressing the sGAP (triangles), sbGAP (circles), and cIIGAP (stars) mRNAs on a representative sagittal section of the brain of the sea bass.

cells affected the nesting behavior, but not the sexual behavior in the same species (Yamamoto et al., 1997). In the goldfish, electrophysiological studies have shown that terminal nerve cells are not sensitive to sexual pheromones (Fujita et al., 1991), and sectioning the olfactory tract in the female does not impair gonadal development and ovulation (Kobayashi et al., 1994). Therefore, it is believed that GnRH cells of the terminal nerve are not crucial for late reproductive events in fish, but rather exert general neuromodulatory functions, some of them possibly associated with reproduction. A lesioning study in the male hamster indicated that terminal nerve cells might facilitate odor-induced sexual excitation in the male hamster (Wirsig and Leonard, 1987). It is also possible that terminal nerve-associated GnRH neurons play a major role during development, and it will be crucial to identify the factors determining the final positioning of the two populations of GnRH neurons in the anterior forebrain upon migration.

On the other hand, the presence of neurons expressing GnRH in the synencephalon of sea bass was not detected by using antibodies to sGnRH (Kah et al., 1991), but they were observed by using an antiserum against cGnRH-II (J.A. Muñoz-Cueto, unpublished data). The present study shows that cIIGAP mRNA are strongly expressed in a discrete population of large neurons of the nucleus of the medial longitudinal fascicle. The presence of GnRH-immunoreactive neurons in this area was first reported in the platyfish by Münz et al. (1981), and then confirmed in the goldfish (Kah et al., 1986). Finally, it was shown in the Masu salmon that such cells express cGnRH-II (Amano et al., 1991). Since then, the presence of cGnRH-II neurons in the synencephalon/mesencephalon has been reported in all classes of vertebrates (Bennis et al., 1989; Leprêtre et al., 1993; Van Gils et al., 1993; King et al., 1994; Montero et al., 1994; Muske et al., 1994; Collin et al., 1995), including mammals (Dellovade et al., 1993; Lescheid et al., 1997; Urbanski et al., 1999), A second GnRH gene encoding cGnRH-II has been recently cloned in humans (White et al., 1998). In most species, expression of cGnRH-II was found to be restricted to the synencephalic/mesencephalic area, although there are a few exceptions, notably in goldfish (Kim et al., 1995), frog (Collin et al., 1995), and macaque (Urbanski et al., 1999). In these species, cGnRH-II expressing cells were found to have a wider distribution, particularly in the preoptic area. The precise functions and sites of projections of these synencephalic cGnRH-II neurons are still highly enigmatic. In some, but not all, species of teleosts, cGnRH-II has been detected in the pituitary (Yu et al., 1988; Schultz et al., 1993; Montero et al., 1995) although its origin is still uncertain. In the goldfish, large neurons of the midbrain tegmentum were found to be labeled following retrograde transport of DiI from the pituitary (Anglade et al., 1993). In several species, there is evidence that cGnRH-II neurons project towards the most caudal portions of the brain, including the spinal cord (Leprêtre et al., 1993; Chartrel et al., 1998). There are also indications that such cells could be involved in sexual behavior (Muske and Moore, 1994) and are sensitive to sexual steroids (Montero et al., 1994). In several species, it was shown that cGnRH-II cells differentiate from a mesencephalic primordium (Muske, 1993; Northcutt and Muske, 1994; Parhar, 1997; Parhar et al., 1998; White and Fernald., 1998; Ookura et al., 1999).

Until a third GnRH gene, possibly encoding an already identified GnRH variant, is identified in more species, it will not be known whether the existence of three GnRH genes is a characteristic of perciforms or if the situation is similar in other fish families or even in other vertebrate classes. The phylogenetic analyses indicate that sbGnRH-coding sequences form a branch different from that of sGnRH and cGnRH-II-coding sequences (J.A. Muñoz-Cueto and O. Kah, unpublished data; White et al., 1998), which suggests that the duplication that gave rise to sbGnRH is ancient. In agreement, recent data in herring have demonstrated that three GnRH forms, including a new variant (His^5Ser^8-mammalian GnRH), are expressed in the brain of a primitive teleost (Carolsfeld et al., 2000), indicating that three GnRH genes were present early in the teleost stem line. In addition, recent studies indicate that a third GnRH form, possibly sGnRH, is present in the brain of mammals, including humans (Yahalom et al., 1999). Supporting the assumption that distinct populations of GnRH neurons develop from the olfactory placode

of mammals, it has been shown in the rhesus macaque that two different populations of GnRH-immunoreactive neurons arise during development. These cell populations exhibit different morphological features and different final localization in the brain (Quanbeck et al., 1997), which could suggest the transient expression of another GnRH-like peptide. On the other hand, a recent study based on promoter transgenics in mouse suggests the existence of different GnRH neuronal populations of different embryological origins during development (Skynner et al., 1999).

Until recently, the nomenclature commonly used to name the different GnRH variants was based on the species in which a given form had been found for the first time. Although strongly established, this nomenclature is sometimes confusing. Recently, a valuable effort has been made to introduce a new classification of the GnRH genes on the bases of a phylogenetic analysis of known GnRH sequences and their respective sites of expression (White et al., 1998). This phylogenetic tree shows three branches: the GnRH1 hypothalamic branch contains genes known in vertebrates to express a hypophysiotrophic peptide; the GnRH2 mesencephalic branch clearly contains all chicken GnRH-II coding sequences of vertebrates; and the GnRH3 telencephalic branch only includes fish representatives. The present results fit well in this scheme with respect to cGnRH-II, which is not the case of all other studies in which cGnRH-II mRNA and/or protein were found outside the midbrain (Kim et al., 1995; Collin et al., 1997; Urbanski et al., 1999). However, the fact that sGnRH- and sbGnRH-expressing cells have a rather similar distribution pattern in sea bass highlights the need for more information, particularly on the existence of a third gene in tetrapods, before definitive conclusions can be drawn.

In conclusion, we have reported in this study the distribution of cells expressing prepro-sGnRH, -sbGnRH, and -cGnRH-II in the brain of the sea bass. These results indicate that cIIGAP mRNAs were only expressed in the nucleus of the medial longitudinal fascicle, as previously shown. However, sGnRH GAP- and sbGnRH GAP-expressing cells had a much broader distribution than that previously reported in other perciforms. In particular, there was clearly an overlapping in the expression sites of these two forms, raising interesting questions in terms of ontogenesis of the GnRH neurons in perciforms and possibly other species.

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