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Journal of Chemical Neuroanatomy 23 (2002) 255–267

Journal of CHEMICAL NEUROANATOMY

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# Developmental expression of three different prepro-GnRH (Gonadotrophin-releasing hormone) messengers in the brain of the European sea bass (*Dicentrarchus labrax*)

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Received 8 October 2001; received in revised form 4 January 2002; accepted 4 January 2002

**Abstract**

In this study, we have analyzed the ontogenic expression of three gonadotrophin-releasing hormones (GnRH) systems expressed in the brain of a perciform fish, the European sea bass, using in situ hybridization. The riboprobes used correspond to the GnRH-associated peptide (GAP) coding regions of the three prepro-GnRH cDNAs cloned from the same species: prepro-salmon GnRH, prepro-seabream GnRH and prepro-chicken GnRH II. On day 4 after hatching, the first prepro-chicken GnRH-II mRNA-expressing cells appeared in the germinal zone of the third ventricle. They increased in number and size from 10 to 21 days, reaching at day 30 their adult final position, within the synencephalic area, at the transitional zone between the diencephalon and the mesencephalon. First prepro-salmon GnRH mRNA-expressing cells became evident on day 7 arising from the olfactory placode and migrating towards the olfactory nerve. On day 10, this cell group reached the olfactory bulb, being evident in the ventral telencephalon and preoptic area from days 15 and 45, respectively. Weakly labeled prepro-seabream GnRH mRNA-expressing cells were first detected at 30 days in the olfactory area and ventral telencephalon. On day 45, prepro-seabream GnRH mRNA-expressing cells were also present in the preoptic region reaching the ventrolateral hypothalamus on day 60. The results obtained in sea bass indicate that sGnRH and sbGnRH cells have a common origin in an olfactory primordium suggesting that both forms might arise from a duplication of a single ancestral gene, while cGnRH-II cells develop from a synencephalic primordium. 

*Keywords:* Ontogenesis; GAP; Central nervous system; Perciform; In situ hybridization

## 1. Introduction

Recently, it has been demonstrated that perciforms express three different gonadotrophin-releasing hormone (GnRH) forms in their brains: salmon GnRH (sGnRH), seabream GnRH (sbGnRH) and chicken II GnRH (cGnRH-II, Powell et al., 1994; White et al., 1995; Gothilf et al., 1996; Fernald and White, 1999; González-Martínez et al., 2001). However, it is more and more considered that the presence of three GnRH forms in the brain is not restricted to perciform fish but is likely to be prevalent in most, if not all, vertebrates (Powell et al., 1985; Sherwood et al., 1986; Lescheid et al. 1997; Montaner et al., 1998, 1999, 2001; Yahalom et al., 1999; Carolsfeld et al., 2000). In addition, the three GnRH forms seem to exhibit a distinct pattern of expression, sGnRH being restricted to cells of the olfactory bulbs, sbGnRH expressed in the preoptic area, and cGnRH-II synthesized in cells of the dorsal synencephalon (Gothilf et al. 1996; Okuzawa et al., 1997;

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0891-0618/02/$ - see front matter 
PII: S0891-0618(02)00004-2

White and Fernald, 1998; Senthilkumaran et al., 1999). Based on this expression pattern and ontogenic studies, several authors proposed that sGnRH cells develop from the olfactory placode and sbGnRH neurons from a preoptic primordium, whereas cGnRH-II cells have a midbrain origin (Northcutt and Muske, 1994; Parhar, 1997; Parhar et al., 1998; Ookura et al., 1999). This is in contrast with evidence obtained in amphibian, avian and mammal, in which all forebrain GnRH neurons seem to have a similar developmental origin in the olfactory placode and migrate centrally along the ventral surface of the brain, adopting their final positions in a continuum from the olfactory bulbs to the hypothalamus (Wray et al., 1989a,b; Akutsu et al., 1992; Muske, 1993; Muske and Moore, 1994; Norgren and Gao, 1994; Schwanzel-Fukuda, 1999).

In the last years, the cDNAs encoding the three GnRH isoforms expressed in sea bass have been cloned (Zmora et al., 2002). The sequences corresponding to the three GnRH-associated peptide (GAP)-coding regions of the prepro-GnRH cDNAs, salmon GAP (sGAP), seabream GAP (sbGAP) and chicken II GAP (cIIGAP), represent valuable tools for in situ hybridization techniques, because, they are longer when compared with GnRH sequences and there is a lower sequence identity among different GAPs. Using these GAP riboprobes we have confirmed that each of the three GnRH prepro-mRNAs is preferentially expressed in a specific brain region. However, we have described for the first time an overlapping distribution of sGAP- and sbGAP-expressing cells from the olfactory bulbs to the preoptic region (González-Martínez et al., 2001). These results suggest that, as in other vertebrates, the forebrain GnRH systems of the European sea bass arise from a common olfactory primordium, but are in contradiction to previous studies in perciforms showing distinct embryonic origins and a neuroanatomical segregation in the expression of both forebrain GnRH systems, i.e. sGnRH and sbGnRH (White et al., 1995; Gothilf et al., 1996; Okuzawa et al., 1997; White and Fernald, 1998; Parhar et al., 1998).

In order to clarify this controversy, in the present study we analyzed for the first time in a teleost species, the ontogenic development of prepro-sGnRH, prepro-sbGnRH and prepro-GnRH-II cells in the European sea bass using specific riboprobes to the corresponding GAPs and in situ hybridization techniques.

## 2. Material and methods

### 2.1. Animals

Sea bass pre-larvae, larvae, post-larvae and juvenile specimens ($n > 500$) of 1, 2, 3, 4, 5, 6, 7, 10, 15, 21, 26, 30, 45 and 60 days after hatching (DAH) were purchased from a local fishery (Cupimar, San Fernando, Spain) and immediately processed as indicated below. At least 20–30 animals were processed and analyzed at each developmental stage. According to Barnabé and Billard (1984), developing specimens were considered pre-larvae from 1 to 5 DAH, larvae from 6 to 45 DAH, post-larvae from 45 to 60 DAH and juvenile from 60 DAH to adulthood (Fig. 1). All animals were treated in agreement with the European Union regulation concerning the protection of experimental animals.

### 2.2. Isolation of the sequences coding for the sea bass GAPs and riboprobes synthesis

A detailed description of the isolation of sea bass GAP-coding sequences and riboprobes synthesis was reported previously (González-Martínez et al., 2001). Briefly, 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). The resulting products were then subjected to nested PCR using degenerate GAP primers and the amplified PCR products were cloned into the pGEMT vector (Promega). The full-length sequences of the three sea bass GnRH forms are available at the GenBank, accession numbers AF224279- sbGnRH, AF224280- sGnRH, AF224281- cGnRH-II.

Messenger RNAs used for the riboprobes synthesis correspond to the GAP coding region of the three sea bass prepro-GnRHs (GenBank access numbers; seabream prepro-GnRH: AF224279; salmon prepro-GnRH: AF224280; chicken GnRH-II prepro-GnRH: AF224281). 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. At the nucleotide level, sequence identities were: sGAP GnRH versus sbGAP GnRH: 42%; cII GAP GnRH versus sbGAP GnRH: 36%; sGAP GnRH versus cIIGAP GnRH: 41%. The preparation of [$\alpha$-$^{35}$S] dUTP-labeled single-stranded

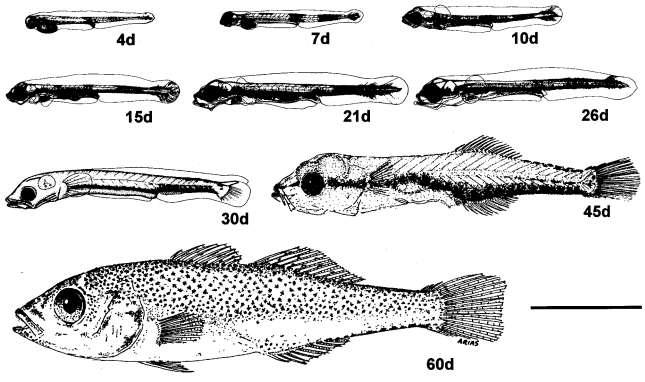


Fig. 1. Lateral drawings of developing sea bass at different ontogenic stages (from 4 to 60 DAH). Scale bar represents 5 mm. Drawings are modified from Barnabé (1991), Arias (1980).

sense and antisense RNA probes followed 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 using T7 and Sp6 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), dithiotreitol 10 mM (DTT), rATP, rGTP, rCTP (0.25 mM each), 100 μCi of [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 min at 37 °C. Probes 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% SDS). The fraction obtained never contained less than $4 \times 10^5$ cpm/μl. Then, 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 $2 \times 10^4$ cpm/μl.

### 2.3. In situ hybridization

In situ hybridization protocol was according to González-Martínez et al. (2001). Pre-larvae, larvae, post-larvae and juvenile specimens of sea bass were anaesthetized in phenoxyethanol (0.3 ml/l) and immersed in fixative solution (4% paraformaldehyde, 0.1 M phosphate buffer pH 7.4). After fixation, only juvenile brains were carefully extracted with the pituitary attached. Thus, whole fixed pre-larvae, larvae and post-larvae as well as fixed juvenile brains were dehydrated, embedded in paraffin, cut at 6 μm in horizontal, sagittal or coronal planes. Sections obtained were mounted on Tespa-treated slides (2% Tespa, Sigma) for further processing.

Tissue sections were equilibrated at room temperature, rehydrated and post-fixed 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 post-fixation in 4% PAF 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) and dehydrated through increasing concentrations of ethanol and air dried. The sections were covered with the hybridization mix ($20 \times 10^3$ cpm per slide), coverslipped and incubated overnight at 52 °C. Coverslips were then removed by immersion in a 5 × SSC, 10mM 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) 10 min 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 min 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 and exposed for 12 days at 4 °C, developed and counterstained with toluidine blue 0.02%. In order to control the specificity of riboprobes, adjacent sections were treated with the sense and anti-sense probes. Furthermore, the specificity of riboprobes used in the present study was previously demonstrated in adult sea bass brain (González-Martínez et al., 2001).

In situ hybridization sections were analyzed on a Olympus BH-2 photomicroscope and computer images were obtained with a Sony DKC-CM30 Digital Camera (Sony, Japan). The software used was ADOBE PHOTOSHOP 5.5 and no subsequent alterations have been made on images. For the precise localization of the different GAP-expressing cells, we have used toluidine blue-stained serial brain sections at the different stages studied and a detailed sea bass brain atlas developed in our laboratory (Cerdá-Reverter et al., 2001a,b) as a cytoarchitectonic reference.

## 3. Results

Sagittal, horizontal and coronal serial sections of the sea bass brain were examined with the anti-sense and sense riboprobes corresponding to the GAP-coding regions of the three prepro-GnRH cDNAs and results obtained are summarized in sagittal drawings of Fig. 2. After 12 days of exposure, positive-signaled sections showed a very clear hybridization labeling in specific brain nuclei while sense probes did not show any remarkable radiolabeling.

### 3.1. Ontogeny of chicken II GAP mRNA-expressing cells

The earliest prepro-GnRH expressed in the brain of sea bass corresponds to the cGnRH-II form, which was first detected at 4 DAH (Figs. 2 and 3A–B). These cIIGAP mRNA-expressing cells appeared bilaterally in the synencephalic area, in the germinal zone of the third ventricle, exhibiting a weak expression at this stage. From 7 to 21 DAH, there is a conspicuous increase in the number of positive cIIGAP cells as well as in the expression of the cIIGAP mRNA within these cells (Figs. 2 and 3C–E). Over this period, cIIGAP mRNA-expressing cells lie on the midline and migrate progressively to more dorsal positions (Compare Fig. 3D–E). On 30, 45 and 60 DAH cIIGAP mRNA-ex-

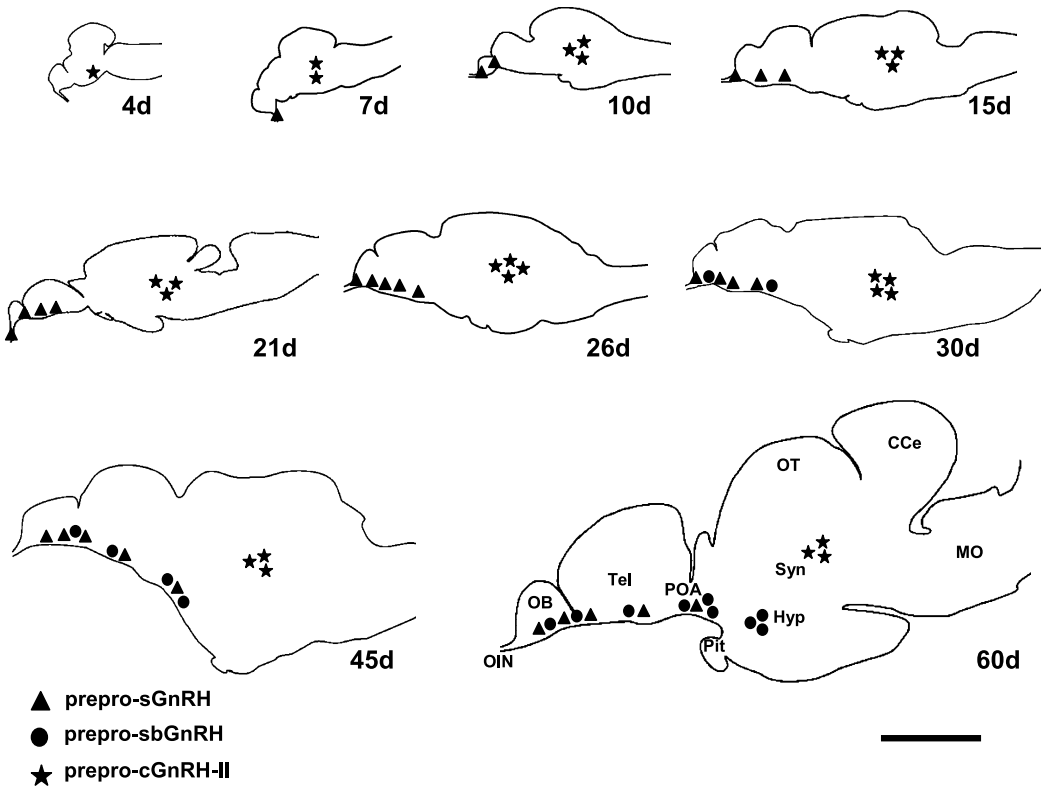


Fig. 2. Brain sagittal drawings summarizing the distribution of prepro-sGnRH- (triangles), prepro-sbGnRH- (circles) and prepro-cGnRH-II- (stars) expressing cells in pre-larvae (4 DAH), larvae (7, 10, 15, 21, 26 and 30 DAH), post-larvae (45 DAH) and juveniles (60 DAH) of sea bass. Scale bar represents 500 μm. Abbreviations: CCe, corpus of the cerebellum; Hyp, hypothalamus; MO, medulla oblongata; OB, olfactory bulbs; OlN, olfactory nerve; OT, optic tectum; Pit, pituitary; POA, preoptic area; Syn, synencephalon; Tel, telencephalon.

pressing cells increase in size, being recognized in their adult final position as a compact cell population in the proximity of the medial longitudinal fascicle and large blood vessels of this synencephalic area (Figs. 2 and 3F–H).

### 3.2. Ontogeny of salmon GAP mRNA-expressing cells

The first detection of sGAP mRNA expression in sea bass larvae occurred at 7 DAH. At the beginning, a weak and diffuse expression was evident at the transitional area between the olfactory placode and the olfactory nerve (Figs. 2 and 4A). Later, at 10 DAH, a cluster of sGAP mRNA-expressing cells was detected in the olfactory nerves, entering the rostral part of the olfactory bulbs (Figs. 2 and 4B). On 15 DAH, sGAP mRNA expression was visible in large terminal nerve ganglion cells, at the transition between the olfactory bulbs and the telencephalon (Fig. 4C), but also in some small cells lying laterally in the ventral part of the ventral telencephalon (Fig. 4D). At 21 DAH, the distribution of sGAP mRNA-expressing cell followed the same pattern but positive neurons were more numerous and exhibited a more intense sGAP mRNA expression (Figs. 2 and 4E–F). At 26 DAH, a higher sGAP-mRNA expression became evident in cells migrating from the olfactory nerve to the ventral telencephalon following the course of the terminal nerve (Figs. 2 and 5A–C). On 30 DAH, the distribution of sGAP mRNA-expressing cells was identical, exhibiting the most intense expression in terminal nerve ganglion cells (Figs. 2 and 5D). However, the mRNA expression seemed to decrease remarkably in cells of the ventral nucleus of the ventral telencephalon (compare Fig. 5E with Fig. 4F, Fig. 5A, C) and this reduced expression was maintained thereafter (Fig. 5F). At 45 and 60 DAH, the distribution pattern of sGAP mRNA-expressing cells did not markedly differ from the precedent stages but sGAP mRNA expression extended to the preoptic area, where few positive small cells were detected in the lateral aspects of the parvocellular preoptic nucleus (Figs. 2 and 5G). No other expressing cells could be detected further caudal in the brain of developing sea bass.

### 3.3. Ontogeny of seabream GAP mRNA-expressing cells

First detection of seabream prepro-GnRH expression occurred in larvae at 30 DAH. Positive cells exhibiting a weak hybridization signal were small in size and appeared in the olfactory bulbs, in a midline position, very close to large ganglion cells of the terminal nerve

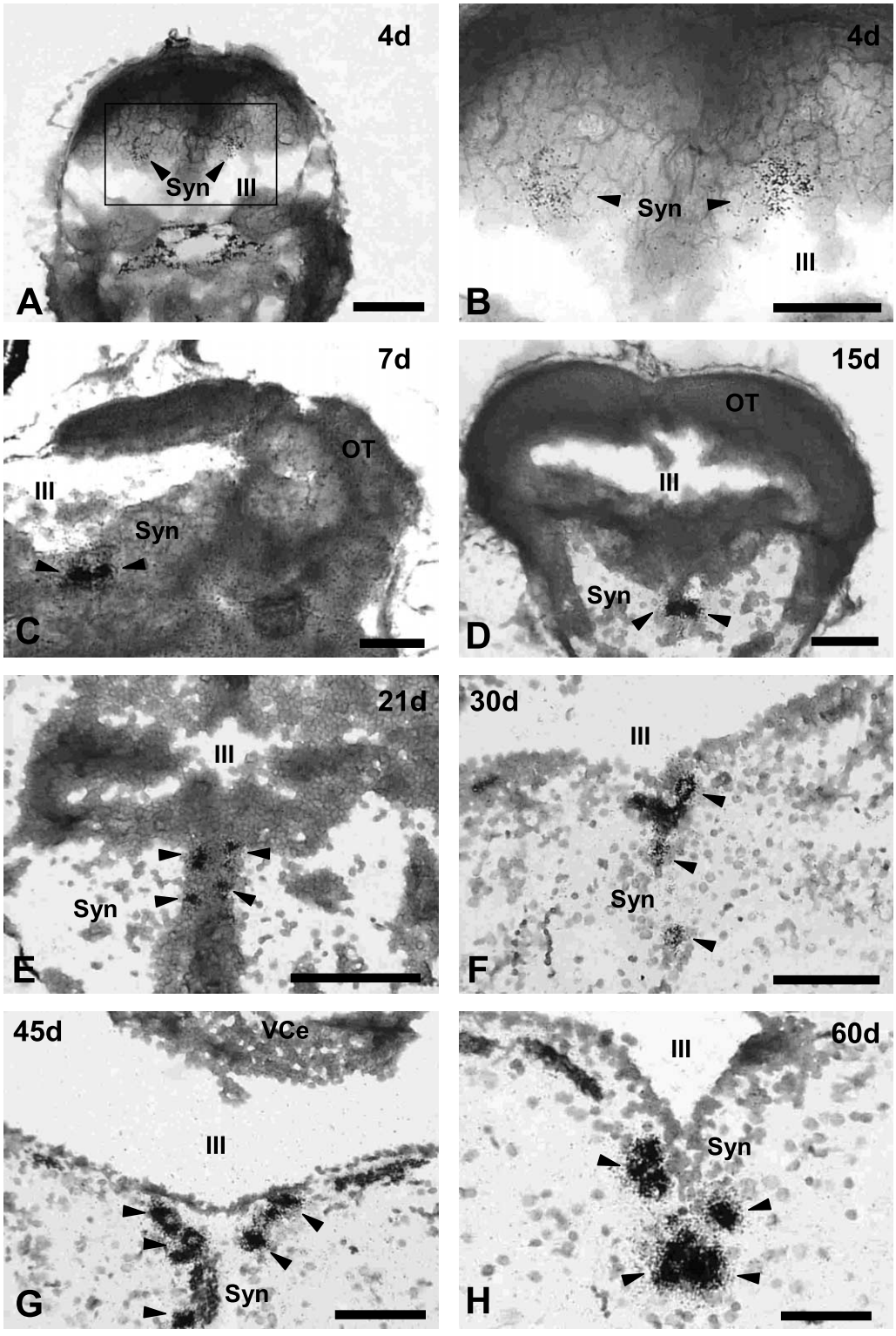


Fig. 3. Micrographs showing cIIGAP mRNA-expressing cells in the brain of developing sea bass using in situ hybridization. This expression starts at 4 days (A, B) and was restricted at all stages studied to the synencephalic area. (A) Four DAH. Squared area is magnified in B. (B) Four DAH. Detail of weakly cIIGAP mRNA-expressing cells in the germinal zone of the third ventricle. (C) Seven DAH. Positive cIIGAP cells in the synencephalon. (D) Fifteen DAH. Note that the midline cluster of migrating cIIGAP mRNA-expressing cells appear far away from their final dorsal position within the synencephalon. (E) Twenty one DAH. The cIIGAP mRNA-expressing cells are more numerous and approach their final position. (F) Thirty DAH. Positive cells have already reached their final position within the dorsal synencephalon. (G) Forty-five DAH. (H) Sixty DAH. It should be noted the marked increase in size of cIIGAP mRNA-expressing cells from 30 to 60 DAH. All pictures correspond to coronal sections. Scale bar represents 25 μm in B, and 50 μm in the remaining micrographs. Abbreviations: III, third ventricle; OT, optic tectum; Syn, synencephalon; VCe: valvula of the cerebellum.

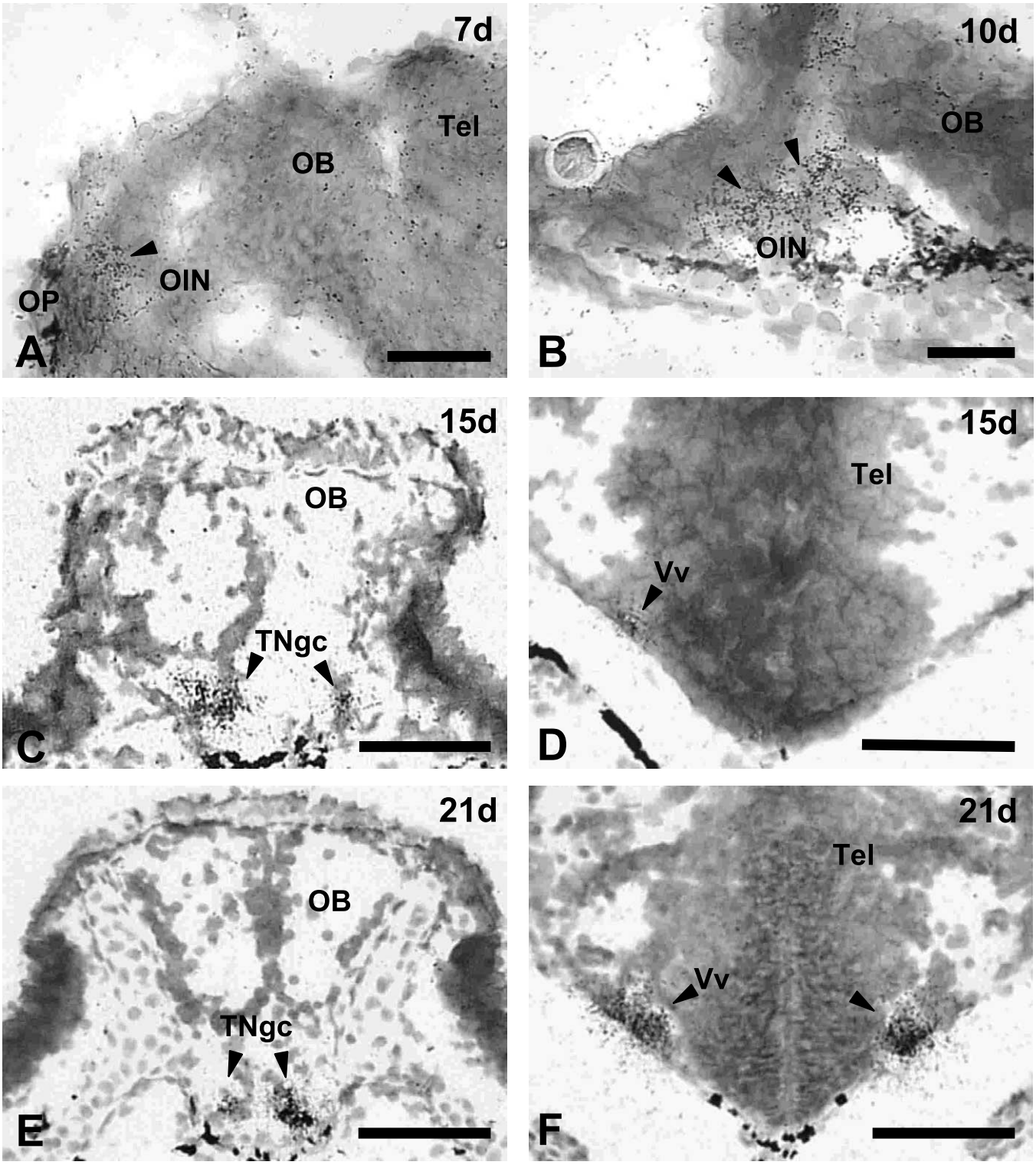


Fig. 4. Micrographs showing sGAP mRNA-expressing cells in the brain of developing sea bass using in situ hybridization (A) Seven DAH. sGAP mRNA-expressing cells migrating from the olfactory placode to the olfactory nerve. Sagittal section. (B) Ten DAH. Positive sGAP mRNA-expressing cells entering the olfactory bulbs from the olfactory nerve. Sagittal section. (C) Fifteen DAH. Terminal nerve ganglion cells, at the transitional area between the olfactory bulbs and the telencephalon, exhibiting a conspicuous sGAP mRNA expression. Coronal section. (D) Fifteen DAH. Weakly sGAP mRNA-expressing cells in the ventral nucleus of the ventral telencephalon. Coronal section. (E) Twenty-one DAH. sGAP mRNA expression in terminal nerve ganglion cells. Coronal section. (F) Twenty-one DAH. Positive sGAP cells in the ventral nucleus of the ventral telencephalon. Note the increase of mRNA expression in comparison to Fig. 4D. Coronal section. Scale bar represents 25 μm in A, B and 50 μm in the remaining micrographs. Abbreviations: OB, olfactory bulbs; OlN, olfactory nerve; OP, olfactory placode; Vv, ventral nucleus of the ventral telencephalon; Tel, telencephalon; TNgc, terminal nerve ganglion cells.

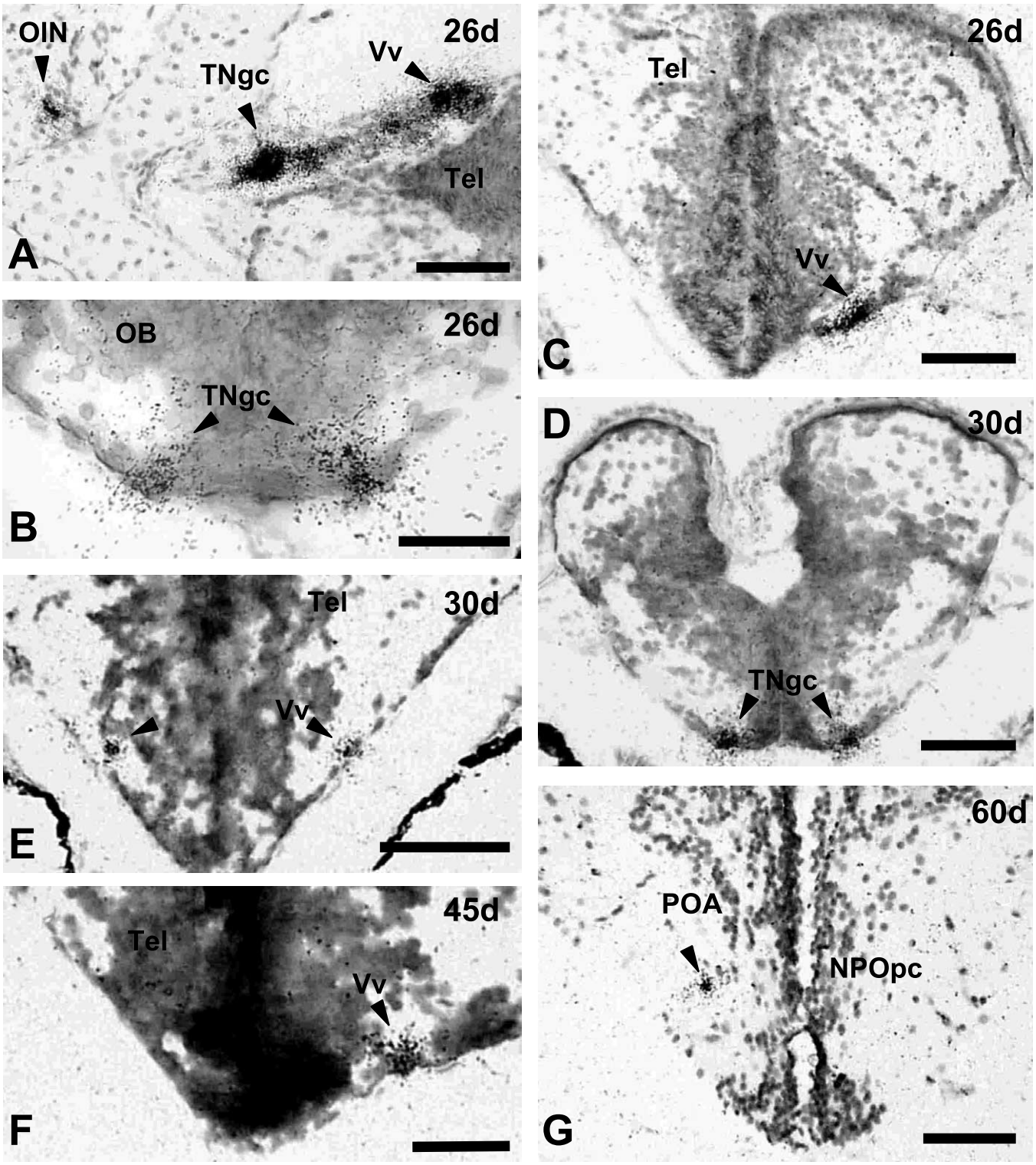


Fig. 5. Micrographs showing sGAP mRNA-expressing cells in the brain of developing sea bass using in situ hybridization (A) Twenty-six DAH. sGAP mRNA expression in olfactory nerve, terminal nerve and ventral telencephalic cells. Horizontal section. (B) Twenty-six DAH. sGAP mRNA expression in terminal nerve ganglion cells. Coronal section. (C) Twenty-six DAH. sGAP mRNA-expressing cells in the ventral nucleus of the ventral telencephalon. Coronal section. (D) Thirty DAH. sGAP mRNA expression in terminal nerve ganglion cells. Coronal section. (E) Thirty DAH. sGAP mRNA-expressing cells in the ventral nucleus of the ventral telencephalon. Note the reduced expression in comparison to 21 (Fig. 4F) and 26 (Fig. 5A,C) DAH. Coronal section. (F) Forty-five DAH. Positive sGAP cells in the ventral nucleus of the ventral telencephalon. Coronal section. (G) Sixty DAH. sGAP mRNA-expressing cells lateral to the parvocellular part of the parvocellular preoptic nucleus. Coronal section. Scale bar represents 25 μm in A, B, and F, and 50 μm in the remaining micrographs. Abbreviations: NPOpc, parvocellular part of the parvocellular preoptic nucleus; OB, olfactory bulbs; OlN, olfactory nerve; POA, preoptic area; Tel, telencephalon; TNgc, terminal nerve ganglion cells; Vv, ventral nucleus of the ventral telencephalon.

(Figs. 2 and 6A). Furthermore, some weakly labeled small cells were also found in the ventral part of the ventral telencephalon (Fig. 6B). On day 45, the sbGAP expression was wider, with some faint sbGAP mRNA-expressing cells appearing in the terminal nerve region (Figs. 2 and 6C) and the ventral nucleus of the ventral telencephalon (Figs. 2 and 6D) and some intensely-labeled cells in the preoptic area (Figs. 2 and 6E). At 60 DAH, sbGAP mRNA-expressing cells extended from the olfactory bulbs to the ventrolateral hypothalamus (Figs. 2 and 6F–I), being this expression much more important in diencephalic nuclei (i.e. the parvocellular preoptic nucleus and the lateral region of the hypothalamic lateral tuberal nucleus, Fig. 6H–I) than in telencephalic cell masses (viz. terminal nerve area and ventral telencephalic cells, Fig. 6F–G). At this stage, preoptic sbGAP cells were more abundant, exhibited a higher mRNA expression as compared with sGAP positive cells in the same area, and also adopting a more lateral position in the preoptic area in comparison with the sGnRH-producing cells (compare Fig. 5G, Fig. 6H).

## 4. Discussion

In this study, we present a detailed description of the ontogenic development of the cells expressing three different prepro-GnRHs in the brain of the European sea bass using specific riboprobes corresponding to the sGAP, sbGAP and cIIGAP sequences. The results obtained indicate that, in sea bass, prepro-sGnRH and prepro-sbGnRH cells have a common origin in an olfactory primordium suggesting that both forms might arise from a duplication of a single ancestral gene, while prepro-cGnRH-II cells develop from a synencephalic primordium. The specificity of these GAP probes was previously demonstrated in adult sea bass brains (González-Martínez et al., 2001), and was corroborated in controls performed in the present study. Furthermore, the distinctive temporal pattern of expression of the different prepro-GnRHs during development argues for the absence of cross-reactivity and the accuracy of these GAP riboprobes in determining the expression of specific prepro-GnRHs. A similar conclusion can be reached out from other in situ hybridization studies performed in teleost species such as catfish (Zandbergen et al., 1995) or seabream (Gothilf et al., 1996) using GAP riboprobes.

This study in developing sea bass confirms the overlapping distribution of sGAP- and sbGAP-expressing cells described in the olfactory bulbs, ventral telencephalon and preoptic area of adult specimens (González-Martínez et al., 2001). Also, we provide for the first time unambiguous evidence showing that both GnRH systems expressed in the forebrain of a teleost species originate from an olfactory primordium. These results are in contradiction to most of the studies performed to date in perciform species (Gothilf et al. 1996; Parhar, 1997; Okuzawa et al., 1997; White and Fernald, 1998; Parhar et al., 1998; Ookura et al., 1999). These studies reported the existence of a neuroanatomical segregation in the expression of sGnRH and sbGnRH cells and suggested that sGnRH neurons differentiate from the olfactory placode, whereas, sbGnRH neurons originate from a preoptic primordium. However, our results agree with those obtained in amphibian, avian and mammal, in which all forebrain GnRH neurons from the olfactory bulbs to the hypothalamus seem to have a similar developmental origin in the olfactory placode (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989a,b; Murakami et al., 1992; Muske, 1993; Muske and Moore, 1994; Norgren and Gao, 1994; Schwanzel-Fukuda, 1999; Fiorentino et al., 2001).

The terminal nerve is a neuronal plexus found in the nasal cavity and rostral forebrain of most vertebrates, extending ventrally from the cranial olfactory nerves to the hypothalamus through the ventral telencephalon and the basal diencephalon (von Bartheld and Meyer, 1986; Wirsig and Leonard, 1986; von Bartheld et al., 1987; Wirsig-Wiechmann and Lee, 1999). This nerve system is thought to play an important role in establishing the migratory route that guides the GnRH neurons towards the forebrain (Schwanzel-Fukuda et al., 1989). Interestingly, prepro-sbGnRH cells in developing (this study) and adult (González-Martínez et al., 2001) sea bass extend from the olfactory bulb to the hypothalamus through the ventral forebrain, following the course of terminal nerve fibers.

Further evidence reinforcing that sGnRH and sbGnRH cells originate from a common olfactory primordium, is the fact that both sGAP- and sbGAP-expressing cells become apparent in the preoptic area of the sea bass at the same developmental stage, on day 45 after hatching. If sbGnRH cells of sea bass were issued from a preoptic primordia, as it has been proposed in other perciforms (Parhar, 1997; Okuzawa et al., 1997; Parhar et al., 1998), the presence of prepro-sbGnRH-expressing cells in the preoptic area would be expected when the first positive sbGnRH cells are detected, at 30 DAH, but it is not the case. Although, sGAP- and sbGAP-positive cells were co-expressed from the olfactory bulbs to the preoptic area, only the sbGAP cells reached the ventral hypothalamus. Hence, it seems improbable that sbGnRH cells in sea bass originate in a basal preoptic primordium, because, this would imply that they migrate in two different directions during development, rostrally towards the ventral telencephalon and olfactory bulbs, and caudally up to the ventral hypothalamus. Preoptic sGnRH and olfactory sbGnRH neurons constitute inconspicuous cell masses in sea bass, which in most cases appear evident only in

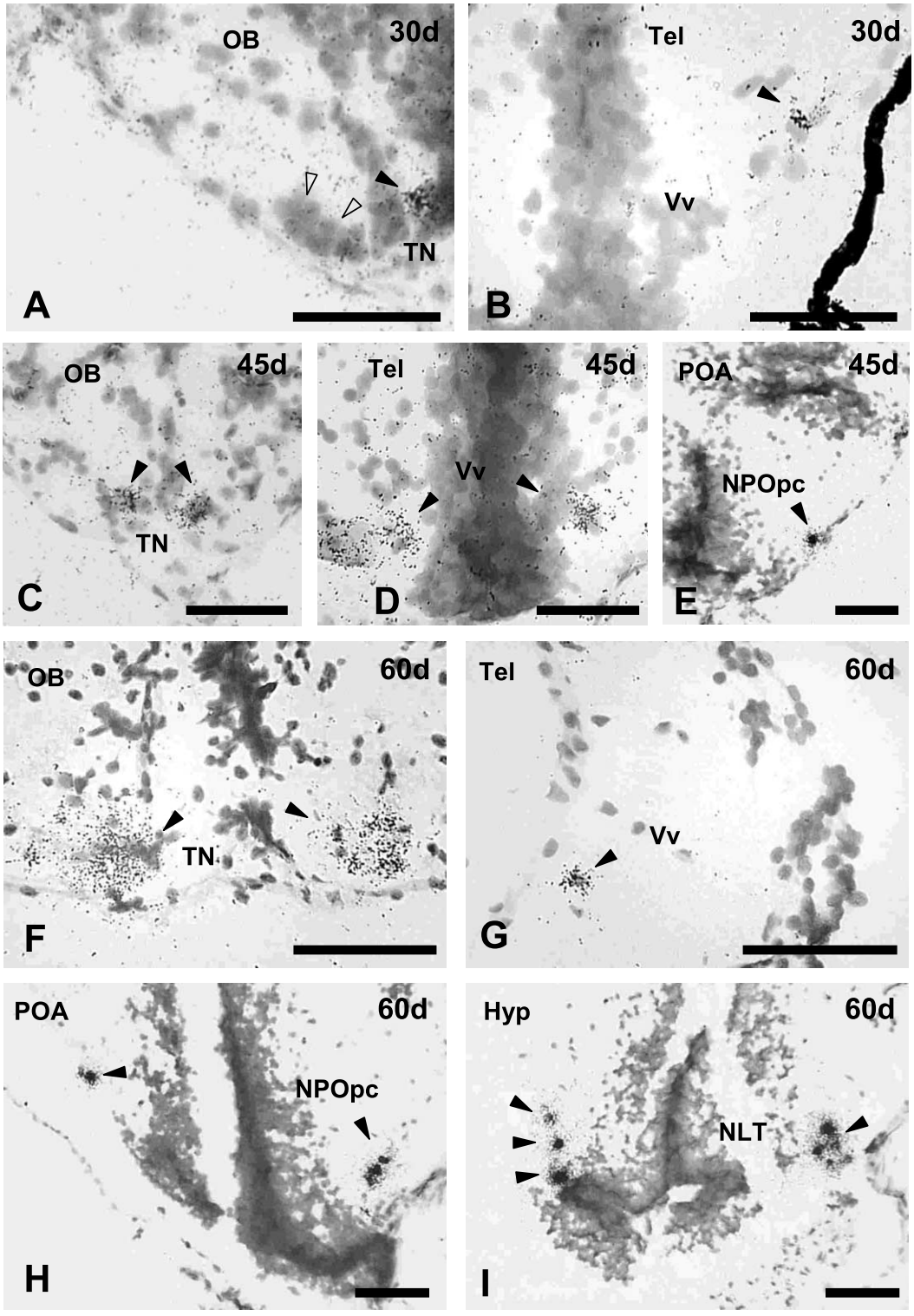


Fig. 6. Micrographs showing sbGAP mRNA-expressing cells in the brain of developing sea bass using in situ hybridization (A) Thirty DAH. sbGAP mRNA-expressing cells in the terminal nerve area. Radiolabeling was not evident in large terminal nerve ganglion cells (open arrowheads) expressing sGAP mRNA (see Fig. 5D). (B) Thirty DAH. Telencephalic sbGAP mRNA-expressing cells. (C) Forty-five DAH. Positive sbGAP cells in the terminal nerve area. (D) Forty-five DAH. sbGAP mRNA-expressing cells in the ventral nucleus of the ventral telencephalon. (E) Forty-five DAH. sbGAP mRNA-expressing cells in the rostral parvocellular part of the parvocellular preoptic nucleus. Note the intense radiolabeling in comparison to positive olfactory bulb (Fig. 6C) and ventral telencephalic (Fig. 6D) cells. (F) Sixty DAH. Positive sbGAP cells in the terminal nerve area. (G) Sixty DAH. sbGAP mRNA-expressing cells in the ventral nucleus of the ventral telencephalon. (H) Sixty DAH. sbGAP mRNA-expressing cells lateral to the parvocellular part of the parvocellular preoptic nucleus. (I) Sixty DAH. sbGAP mRNA-expressing cells in the ventrolateral hypothalamus. All pictures correspond to coronal sections. Scale bar represents 25 μm in B, and 50 μm in the remaining micrographs. Abbreviations: Hyp, hypothalamus; NLT, lateral tuberal nucleus; NPOpc, parvocellular part of the parvocellular preoptic nucleus; OB, olfactory bulbs; POA, preoptic area; Vv, ventral nucleus of the ventral telencephalon; Tel, telencephalon; TN, terminal nerve area.

one or two consecutive histological sections, making their identification difficult. Therefore, it is possible that preoptic sGnRH- and olfactory sbGnRH-expressing cells are also present in the brain of other perciform species but remain to be described.

In sea bass, the expression of prepro-sGnRH precedes that of prepro-sbGnRH, sGAP-expressing cells being evident on day 7 after hatching, whereas, sbGAP-expressing cells were not detected until day 30 after hatching. In this manner, sGAP mRNA expression became apparent at the beginning of the migratory course of sGnRH neurons from the olfactory placode (early expressing cells), while sbGnRH-expressing cells seem to remain silent during their early travel through the most rostral forebrain (late expressing cells). Interestingly, these observations agree with results obtained in a distant phylogenetic group as primates, using different LHRH antisera and immunocytochemistry (Quanbeck et al., 1997). These authors stated the existence of two populations of cells migrating from the olfactory placode, referred as 'early' and 'late' LHRH cells, which exhibited distinct temporal patterns of expression and differences in their morphology and neuroanatomical distribution. The 'early' LHRH neurons extend to the preoptic region whereas the 'late' LHRH cells reached the basal hypothalamus (Quanbeck et al., 1997).

In perciforms, sbGnRH represents the most abundant GnRH form in the pituitary, where it stimulates gonadotrophin release (Powell et al., 1994; Zohar et al., 1995; Gothilf et al., 1996; Yamamoto et al., 1997; Holland et al., 1998; Senthilkumaran et al., 1999; Rodriguez et al., 2000). In adult sea bass, the sbGAP-immunoreactive fibers can only be detected in the ventral forebrain running to the pituitary, which received a strong sbGnRH innervation (González-Martínez et al., 2002). This evidence reinforces the idea that physiological actions of sbGnRH are restricted to the hypophysis, having no relevant functions into the brain. During sea bass ontogeny, sbGnRH represents the most delayed GnRH form expressed into the brain, at 30 DAH. It should be noted that in sea bass the first primordial germinal cells are not detected until the fourth week and the genital crest appears at 43 DAH (Roblin and Brusle, 1983). In addition, gonadotrophin-immunoreactive cells are not evident in the sea bass pituitary during the first 26 DAH (Cambré et al., 1990).

The high degree of phylogenic conservation of cGnRH-II and sGnRH could be related with a high selective pressure on GnRH forms exhibiting broader actions and target cells. Nevertheless, the precise functions of sGnRH remain to be clarified. It has been suggested that sGnRH is not essential for late reproductive events in fish but rather exert neuromodulatory functions, some of them possibly associated with reproduction (Kobayashi et al., 1994). Thus, it has been proposed that sGnRH might be implicated in the coordination of the sensory and motivational systems (White et al., 1995), playing a role in the processing of reproductive-related visual information reaching the retina (Stell et al., 1987) and the modulation of odorant sensitivity (Eisthen et al., 2000). In this context, the profuse distribution of sGnRH-immunoreactive fibers in the brain of teleosts, including sea bass, especially in visual and gustatory sensory areas (Kah et al., 1986, 1991; Oka and Matsushima, 1993; González-Martínez et al., 2002) could reinforce this assumption. According to the results of Rodriguez et al. (2000), sGnRH levels were 17-fold lower than sbGnRH levels in the hypophysis of male sea bass. The presence of sGnRH in the sea bass pituitary could be related with a direct regulation of gonadotrophic activity, but also with the effects of sGnRH on other adenohypophyseal cells. At least in other teleosts, stimulatory effects of sGnRH on growth hormone release have been reported (Marchant et al., 1989). It is also possible that sGnRH plays an important role during development since its expression in sea bass begins in early ontogenic stages. Prolactin-immunopositive cells became visible in the pituitary of sea bass between days 9 and 15 after hatching (Cambré et al., 1990), 2 days after that the first prepro-sGnRH-expressing cells were detected. Interestingly, sGnRH functions as a prolactin-releasing factor in *Oreochromis mossambicus*, a perciform teleost (Weber et al., 1997).

The sGnRH and sbGnRH decapeptides only differ in aminoacidic residues located in positions 7 and 8. The high aminoacidic and nucleotide identity, the common embryonic origin and the overlapping distribution of sGnRH and sbGnRH cells in the forebrain, as well as the hypophysiotrophic nature of both GnRHs (Rodriguez et al., 2000; González-Martínez et al., 2001, 2002), could argue in favor of the hypothesis considering that sGnRH and sbGnRH systems might arise by duplication–mutation processes from a single ancestral gene. Furthermore, the duplication–mutation processes that led to the second forebrain GnRH form appears to be more recent than those separating the midbrain (cGnRH-II) and the original forebrain GnRH form. Later, as a teleost derived condition, the second forebrain GnRH system could have progressively lost its brain projections and functions, emerging as the main hypophysiotrophic GnRH system.

In accordance with data obtained in fish, amphibian, bird and mammal (Muske and Moore, 1990; Muske, 1993; Dellovade et al., 1993; Northcutt and Muske, 1994; Kasten et al., 1996; Parhar, 1997; Parhar et al., 1998; White and Fernald, 1998; Mikami et al., 1988; Ookura et al., 1999), developing cGnRH-II cells in sea bass do not originate in the olfactory placode but in the germinal zone of the third ventricle, at a diencephalic/mesencephalic transitional area identified as the synencephalon. In sea bass, the earliest expression of a

prepro-GnRH corresponds to these cIIGAP mRNA-expressing cells, which were first detected on day 4 after hatching. Early expression of the synencephalic/midbrain GnRH form was also reported in some vertebrate species, including fish (Muske and Moore, 1990; White and Fernald, 1998), although, a simultaneous ontogenic expression of different GnRH forms has also been referred (Parhar, 1997; Parhar et al., 1998). As in other fish species, sea bass prepro-cGnRH-II cells migrate dorsally along the midline in the synencephalic area, the number, size and expression of these cells increasing notably during the first weeks after hatching (Parhar et al., 1998; White and Fernald, 1998; Ookura et al., 1999). This fact reflects that migrating cIIGAP cells are functional before reaching their final position, around 30 DAH, reinforcing the critical role of cGnRH-II in early development. Although, a quantitative study has not been carried out, the number of cIIGAP cells seems to be markedly reduced in adult sea bass (González-Martínez et al., 2001) in comparison to larva and post-larva specimens. In amphibians, tadpoles also show higher cGnRH-II expression than adult animals (Muske and Moore, 1990).

The cGnRH-II form is the most conserved and, probably, the most ancient GnRH form. Several studies stated that cGnRH-II is expressed in gonads (Yu et al., 1998; von Schalburg et al., 1999; Nabissi et al., 2000) and is involved in sexual behavior (Muske and Moore, 1994; Muske, 1993). Also, it has been suggested that cGnRH-II cells are responsive to feedback actions of sexual steroids (Montero et al., 1994; Rissman and Li, 1998). However, the selective pressure on cGnRH-II structure reinforces the assumption that this neurohormone could perform important functions other than reproduction, for instance, as neurotransmitter, neuromodulator and/or autocrine/paracrine hormone in peripheral tissues (White et al., 1998; Yu et al., 1998). In this context, this ontogenic study reveal that cGnRH-II is expressed very early during development, much before the differentiation of gonadotrophic cells and gonads (Roblin and Brusle, 1983; Cambré et al., 1990). Recently, it has been proposed that cGnRH-II might modify sensory-motor activity (White et al., 1995). Previous results obtained in our laboratory, showing that cGnRH-II-immunoreactive fibers strongly innervated the spinal cord and sensory areas and were detected in the cerebellum of sea bass (González-Martínez et al., 2002) might support this assumption. The neuromodulatory actions of cGnRH-II have been reported in sympathetic ganglion neurons of amphibian (Jan et al., 1980; Troskie et al., 1997). At least in mammals, cGnRH-II seems to be expressed in cells of the immune system, and especially, in mast cells (Silverman et al., 1994; Rissman et al., 1995; Marchetti et al., 1996). Based on these results, it has been proposed that perhaps cGnRH-II originated in the immune system and only later acquired a neuromodulatory function in the brain (White et al., 1998). Further studies analyzing the expression of cGnRH-II outside the brain in sea bass and other vertebrate species should contribute to clarify its functions and to elucidate its remarkable conservation in phylogeny.

## Acknowledgements

This work has been supported by UE (FAIR CT97-3785), CICYT (CYTMAR MAR95-1888-C03-02) and CNRS. We thank A. Santos for his excellent technical help and Dr F.J. Rodríguez for his useful advices. We are grateful to Dr Carlos Pendón and the Radioactive Isotopes Service of the University of Cádiz. We also thank A. Vidaurreta and CUPIMAR fish farm for supplying the biological material used in this study.

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