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Journal of Chemical Neuroanatomy 28 (2004) 1–15
Journal of CHEMICAL NEUROANATOMY
www.elsevier.com/locate/jchemneu

New insights in developmental origins of different GnRH (gonadotrophin-releasing hormone) systems in perciform fish: an immunohistochemical study in the European sea bass (*Dicentrarchus labrax*)

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Received 2 December 2003; received in revised form 1 April 2004; accepted 7 May 2004
Available online 24 June 2004

Abstract

The knowledge of the roles and origins of different gonadotrophin-releasing hormone (GnRH) systems could greatly contribute to improve the understanding of mechanisms involved in the physiological control of early development, puberty and spawning. Thus, in this study, we have analyzed the distribution of the cells expressing salmon GnRH, seabream GnRH and chicken GnRH-II forms in the brain and pituitary of developing sea bass using specific antibodies to their corresponding GnRH-associated peptides. The first prepro-chicken GnRH-II-immunoreactive cells arose in the germinal zone of the third ventricle at 4 days after hatching, increasing their number from days 10 to 30, in which they adopted their adult position. The prepro-chicken GnRH-II-immunoreactive fibers became conspicuous in the first week and from day 26 they reached almost all brain areas, especially the hindbrain, being never detected in the pituitary. First prepro-salmon GnRH-immunoreactive cells were detected in the olfactory placode at day 7 after hatching and reached the olfactory bulbs at day 10. Migrating prepro-salmon GnRH cells arrived at the ventral telencephalon at day 15, and became apparent in the preoptic area from day 45. The prepro-salmon GnRH innervation was more evident in the forebrain and increased notably between 10 and 30 days, at which fibers already extended from the olfactory bulbs to the medulla. A few prepro-salmon GnRH-immunoreactive fibers were observed in the pituitary from day 30. The prepro-seabream GnRH-immunoreactive cells were first detected at day 26 in the rostral olfactory bulbs. On day 30, prepro-seabream GnRH-immunoreactive cells were also present in the ventral telencephalon, reaching the preoptic area and the hypothalamus at 45 and 60 days, respectively. The prepro-seabream GnRH innervation appeared restricted to the ventral forebrain, increasing notably during the sixth week, when fibers also reached the pituitary. A significant prepro-seabream GnRH innervation was not detected in the pituitary until day 60.

Keywords: Ontogenesis; GnRH; Brain; Pituitary; Perciform; Immunohistochemistry

1. Introduction

With over 25,000 species, 58 orders and 468 families, teleosts form the largest and most diverse vertebrate radiation. In turn, perciforms, with 154 families and over 7000 species (37% of the teleosts, according to Nelson, 1984) represent the most successful group within ray-finned fishes and include many species with commercial interest. The European sea bass, *Dicentrarchus labrax*, is a predatory and pelagic perciform species with a high economical relevance for Mediterranean and Atlantic aquaculture. Most of the problems encountered in sea bass aquaculture are related to dysfunctions at early developmental stages, at the onset of puberty and during maturation and spawning (Carrillo et al., 1995; Felip et al., 1999; Rodríguez et al., 2000). Therefore,

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doi:10.1016/j.jchemneu.2004.05.001

the success of this activity is strongly dependent on the understanding of the mechanisms involved in the physiological control of early development, puberty and spawning under culture conditions. In this sense, increasing research efforts in sea bass have been developed to clarify different aspects of its physiology, especially the regulation of metabolic and reproductive processes (Roblin and Brusle, 1983; Gutiérrez et al., 1984; Cambré et al., 1990; Prat et al., 1990; Carrillo et al., 1995; Rodríguez et al., 2000; Sorbera et al., 2001).

It is clearly established that the brain, and especially the forebrain, plays an important role in the control of the reproductive axis (Kah et al., 1986). The gonadotrophin-releasing hormone (GnRH) represents the main cerebral factor controlling the reproductive process through its stimulatory actions on the release of gonadotrophins from the pituitary gland (Schally et al., 1971; Breton et al., 1972). The first GnRH form was isolated from extracts of pig hypothalamus and was named as mammalian GnRH (mGnRH, Schally et al., 1971; Matsuo et al., 1971). To date, fourteen different GnRH forms have been isolated and characterized in vertebrates (Matsuo et al., 1971; King and Millar, 1982a,b; Sherwood et al., 1983; Ngamvongchon et al., 1992; Lovejoy et al., 1992; Sower et al., 1993; Powell et al., 1994, 1996; Jiménez-Liñán et al., 1997; Carolsfeld et al., 2000; Okubo et al., 2000; Yoo et al., 2000; Montaner et al., 2001), but the family of GnRH-like peptides also include protochordate and invertebrate forms (Zhang et al., 2000; Iwakoshi et al., 2002; Adams et al., 2003).

At first it was thought that most vertebrates expressed two different GnRH forms in their brains (Yu et al., 1988; Muske and Moore, 1990; Lescheid et al., 1997; Urbanski et al., 1999), but recent evidence obtained in phylogenetically distant groups such as teleosts (Powell et al., 1994; White et al., 1995; Okubo et al., 2000; Montaner et al., 2001) and mammals (Montaner et al., 1998, 1999; Yahalom et al., 1999) suggest that the expression of three GnRHs could be prevalent in most vertebrates. As in other perciform species, the brain of the European sea bass expresses three GnRH decapeptides, salmon GnRH (sGnRH), seabream GnRH (sbGnRH) and chicken GnRH-II (cGnRH-II), each issued from a typical prepro-GnRH precursor also coding for a signal peptide, a cleavage tripeptide and a GnRH-associated peptide (GAP, González-Martínez et al., 2001; Zmora et al., 2002). These GAP sequences appear as useful tools to localize specifically the cells expressing the different GnRH forms, because it has been reported that the distribution of GnRH cells is consistently identical to that of the corresponding GAPs (Ronchi et al., 1992; Polkowska and Przekop, 1993; González-Martínez et al., 2002a). Furthermore, GAP sequences are longer and there is a lower sequence identity at the nucleotide and amino acid levels among different GAPs when compared to GnRH decapeptides, avoiding the problems of cross-reactivity often encountered when using riboprobes and antibodies to the highly-related GnRH decapeptides. Using specific GAP riboprobes and antibodies we have recently demonstrated a clear overlapping of prepro-sGnRH- and prepro-sbGnRH-expressing cells in the olfactory bulbs, telencephalon and preoptic area of the sea bass, whereas prepro-cGnRH-II expression appeared restricted to the dorsal synencephalon (González-Martínez et al., 2001, 2002a). These results are in contradiction with those obtained in other perciform species, in which sGnRH and sbGnRH cells appeared segregated in the olfactory bulbs and preoptic area, respectively (White et al., 1995; Gothilf et al., 1996; Okuzawa et al., 1997; White and Fernald, 1998a; Parhar et al., 1998). The multiplicity of GnRH forms within the brain of perciform species together with the disagreement in the expression pattern of forebrain GnRH systems raised basic questions with respect to the embryonic origins of cells expressing these different GnRHs. Thus, it appeared necessary to clarify if forebrain GnRH systems in sea bass are issued from different primordia, as suggested in other perciforms (Okuzawa et al., 1997; Parhar, 1997; Parhar et al., 1998, Ookura et al., 1999; Pandolfi et al., 2002), or from an olfactory primordium, as assumed in most vertebrate species (Wray et al., 1989a; Akutsu et al., 1992; Muske, 1993; Muske and Moore, 1994; Norgren and Gao, 1994; Schwanzel-Fukuda, 1999).

Our previous in situ hybridization analysis of GnRH systems in developing sea bass using mRNA GAP probes showed that prepro-GnRH forms expressed in the forebrain of this species (sGnRH and sbGnRH) were first detected in the olfactory region but exhibited a different timing of appearance, while the most conservative cGnRH-II had an earlier time of expression and a synencephalic/midbrain origin (González-Martínez et al., 2002b). In this study, we use specific antibodies generated against sGAP, sbGAP and cIIGAP in order to examine these developmental expression patterns and confirm them at the protein level. We also provide new relevant information concerning the distribution of sGnRH, sbGnRH and cGnRH-II fibers in the brain and pituitary of developing sea bass.

2. Material and methods

2.1. Animals

Sea bass pre-larvae, larvae, post-larvae and juvenile specimens ($n > 500$) of 1–7, 10, 15, 21, 26, 30, 45 and 60 days after hatching (DAH) were purchased from a local fish farm (Cupimar, San Fernando, Spain) and immediately processed as indicated below. According to Barnabé (1991), 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 juveniles from 60 DAH to adult. All animals were treated in agreement with the European Union regulation concerning the protection of experimental animals.

2.2. Generation of specific antibodies against GAPs

The procedures for the isolation of the sequences coding for the three sea bass GAPs and the expression of recombinant GAPs were reported previously (González-Martínez et al., 2001; Zmora et al., 2002). Briefly, for the production of recombinant GAPs, two expression vectors were used: the His-tagged expression system for sGAP and sbGAP, and the gluthathione-*S*-transferase (GST) expression system for cIIGAP. Analysis on 17% SDS PAGE to detect the existence of recombinant GAP peptides resulted in clear bands of the expected size (6.5 KD) for sGAP and sbGAP. For cIIGAP, SDS PAGE analysis revealed two bands: the cIIGAP/GST fused protein, as a dominant band of 34 KD in size, and a smaller faint band of 27 KD which corresponds to the GST protein. The purified recombinants GAPs were used for the immunization of nine female guinea pigs (5–6 week-old; 345–365 g) obtained from CEGAV (France) and housed in laboratory cages designed for guinea pigs. Animals, three for each GAP, were acclimated for 1 week before the beginning of the immunization protocol. Each injection consisted of 25 μl of recombinant GAP (1 μg/μl), 75 μl distilled water, and 100 μg of incomplete Freund's adjuvant. Preimmune sera were collected on the day of the first injection and stored at −20 °C. The first injection was intradermal in the back (6–7 points) and the others, at 1 month interval, were given intramuscularly in the posterior legs. Eight days after the fourth injection, blood was collected under anesthesia by cardiac punction, centrifuged and the serum stored at −25 °C. The specificity of anti-sGAP, anti-sbGAP and anti-cIIGAP sera was previously assessed by dot–blot analysis using the recombinant sGAP, sbGAP and cIIGAP proteins as antigens (González-Martínez et al., 2002a).

2.3. Immunohistochemistry

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 cryoprotected overnight in 0.1 M phosphate buffer containing 15% sucrose, embedded in Tissue-Tek, frozen in cold isopentane and horizontal, coronal and sagittal serial sections of 16 μm thick were obtained with a cryostat.

Immunohistochemical staining was performed using a streptavidin-biotin-peroxidase complex method. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in Coons buffer (CBT, 0.01 M Veronal, 0.15 M NaCl) containing 0.1–0.2% Triton X-100 for 30 min. Before immunostaining, sections were transferred for 5 min to CBT and then saturated in CBT containing 0.5% casein for 30 min. Sections were incubated overnight in a moist chamber at room temperature with anti-sGAP, anti-sbGAP and anti-cIIGAP sera (1:500 to 1:1000 dilution in CBT 0.5% casein). Sections were washed in CBT (2 × 15 min) and incubated for 1.5 h at room temperature with Biotin-sp-Conjugated-AffiniPure Goat Anti-GuineaPig–IgG (Jackson Immuno Research Laboratories Inc., West Grove, PA) diluted 1:1000 in CBT. After washing in CBT (2 × 15 min), sections were incubated 1.5 h at room temperature with peroxidase-conjugated-streptavidin complex (Jackson Immuno Research Laboratories Inc., West Grove, PA) diluted 1:1000 in CBT. Finally, sections were washed in CBT followed by Tris–ClH (0.05 M, pH 7.4) and peroxidase activity was visualized either in 0.05 M Tris–HCl, pH 7.6 containing 0.025% 3,3 diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) and 0.01% hydrogen peroxide or 0.04% 4-chloro-1-naphthol (Sigma, St. Louis, MO) and 0.01% hydrogen peroxide. To confirm the specificity of the immunostaining, controls were performed by preabsortion of primary antisera with their respective antigens, replacement of primary antisera with the corresponding preimmune sera and omission of primary or biotinylated antisera. We have previously demonstrated that the distribution of immunoreactive GAP was consistently similar to that of the corresponding immunoreactive GnRH, using double immunofluorescence techniques in adult sea bass (González-Martínez et al., 2002a).

Immunohistochemical 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. For the precise localization of the different GAP-ir cells, we have used toluidine blue stained brain sections at different developmental stages and a detailed adult sea bass brain atlas developed in our laboratory (Cerdá-Reverter et al., 2001a,b).

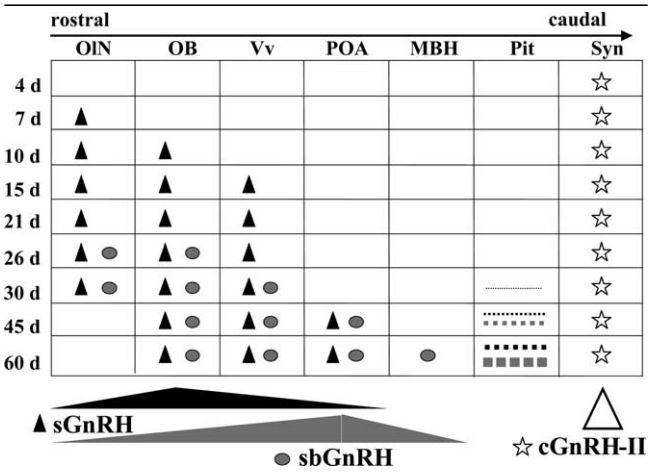
3. Results

All brain levels were analyzed with the three anti-GAP sera on sagittal, horizontal and coronal sections of 20–30 specimens per developmental stage. The results obtained are summarized in Table 1 and in sagittal drawings of Fig. 6. No positive immunoreactivity was evident before 4 DAH. Immunostaining was not observed when primary antisera were preabsorbed with their respective antigens or replaced with the corresponding preimmune sera, or when primary or biotinylated antisera were omitted.

3.1. Ontogeny of chicken II GAP-immunoreactive cells and fibers

The first cIIGAP-immunoreactive (ir) cells appeared in the synencephalic germinal zone of the third ventricle at 4 DAH (Figs. 1A and 6). At this stage, some small-sized and poorly immunostained cells, rounded to ovoid in shape, appeared scattered on the midline, in the germinal zone of the third

Table 1
Chronogram summarizing the temporal and regional expression of prepro-sGnRH- (black triangles), prepro-sbGnRH- (grey circles) and prepro-cGnRH-II- (white stars) immunoreactive cells in the brain of developing sea bass



The temporal pattern of arrival of prepro-sGnRH (dotted black line) and prepro-sbGnRH (squared grey line) fibers to the sea bass pituitary is also reflected. The thickness of the lines is proportional to the amount of entering GnRH fibers. In the bottom of the table, the apex of triangles marks the brain area exhibiting the highest number of each GnRH-immunoreactive cell type. Abbreviations: OlN, olfactory nerve; OB, olfactory bulbs; Vv, ventral nucleus of the ventral telencephalon; POA, preoptic area; MBH, mediobasal hypothalamus; Pit, pituitary; Syn, synencephalon.

ventricle. At 7 DAH, the number of cIIGAP-ir cells remains low. From 10 to 26 DAH, there is an evident increase in the number and size of cIIGAP-ir cells (Figs. 1B-E and 6). These cells lay at the transitional area between the diencephalon and the mesencephalic tegmentum (Fig. 1D and E). At 30 DAH and thereafter, cIIGAP-ir cells adopted their adult final position, dorsally and medially to the fibers of the medial longitudinal fascicle, in the proximity of the tectal ventricle and blood vessels (Figs. 1F, G and 6).

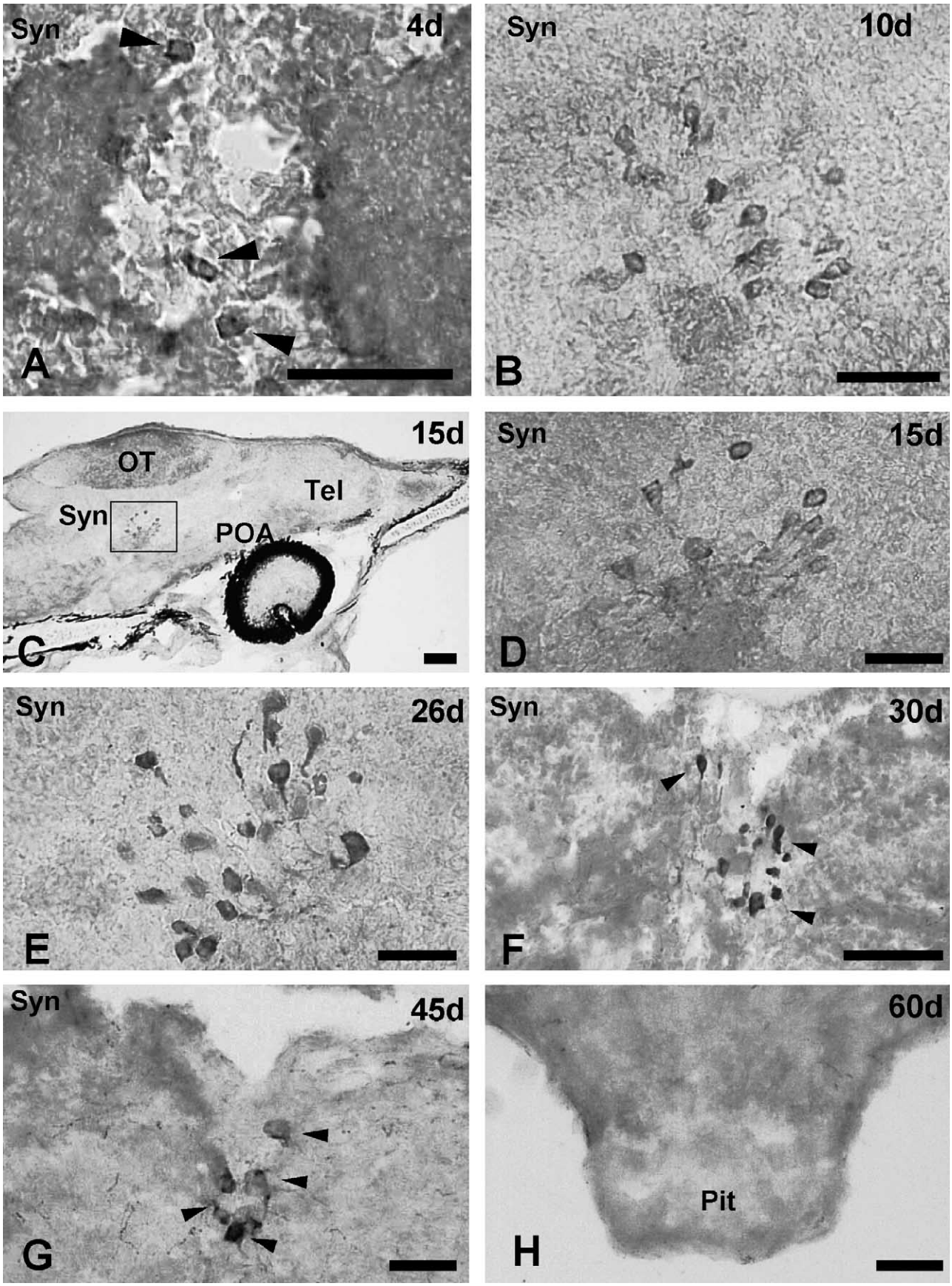
The cIIGAP-ir fibers became conspicuous during the second week after hatching, increasing on the third week in the diencephalon and synencephalon. From 26 DAH, cIIGAP-ir fibers reached almost all forebrain areas (olfactory bulbs, dorsal and ventral telencephalon, periventricular preoptic area and hypothalamus, thalamus, pretectum, posterior tuberculum), being more evident in the midbrain (mesencephalic tectum and tegmentum) and hindbrain (granular layer of the valvula, corpus and vestibulolateral lobe of the cerebellum and rhombencephalon, Fig. 6). No cIIGAP-ir fibers were detected in the pituitary at any developmental stage (Fig. 1H). Three different growing prepro-cGnRH-II-ir fiber tracts were evident during sea bass ontogeny (Fig. 6). The synencephalic-tectal tract courses in the caudo-rostral and ventro-dorsal directions and innervates the thalamus, hypothalamus and especially the optic tectum. The synencephalic-telencephalic tract runs rostro-medially up to the telencephalic hemispheres and olfactory bulbs coursing through diencephalic areas. Finally, the synencephalic-rombencephalic tract extends caudally and innervates the mesencephalic tegmentum, the cerebellum, medulla oblongata and spinal cord.

3.2. Ontogeny of salmon GAP-immunoreactive cells and fibers

The first sGAP-ir cells became evident at 7 DAH as a cluster of medium-sized, round and pyramidal neurons migrating from the olfactory placode to the olfactory nerve (Figs. 2A and 6). At 10 DAH, this cell group increased in size and contained more intensely immunostained cells which reached the rostral olfactory bulbs (Figs. 2B, C and 6). Migrating sGAP-ir cells reached the ventral telencephalon at 15 DAH (Figs. 2D and 6), and were apparent in the preoptic area from 45 DAH (Fig. 3C). No other sGAP-expressing cells could be detected further caudal at any developmental stage. From 15 DAH, the distribution of sGAP-ir cells exhibited an evident rostro-caudal gradient in the brain of developing sea bass (Fig. 6). Thus, the number of positive cells and the cell size were clearly greater in the olfactory terminal nerve population (TNgc, Figs. 2C-F, 2H, 3A, E and 6) than in telencephalic (Figs. 2D, F, 3B and 6) and preoptic (Figs. 3C and 6) areas.

The sGAP-ir innervation was more evident in the forebrain of developing sea bass and increased notably from 10 to 30 DAH, in which sGAP-ir fibers already extend from the olfactory bulbs to the medulla (Fig. 6). At early developmental stages, the sGAP-ir fibers were mainly observed in the olfactory bulbs and through the migratory cell route in the ventro-medial part of the telencephalon, but also in the preoptic region and the rostrolateral zones of the optic tectum (Fig. 6). Later stages showed how these sGAP-ir fibers adopted a more diffuse configuration reaching the olfactory bulbs, ventral and dorsal telencephalon, preoptic area, periventricular hypothalamus, thalamus, pretectum, mesencephalic tectum and tegmentum, and ventral rhombencephalon (Fig. 6). No sGAP-ir fibers were detected

Fig. 1. Distribution of immunoreactive (ir) cIIGAP cells and fibers in the brain of developing sea bass. (A) cIIGAP-ir cells in the synencephalon of a 4 DAH pre-larva (horizontal section). (B) Sagittal section showing cIIGAP-ir migrating cells in the synencephalon of a 10 DAH larva. Note the increase in the number of immunostained cells in relation to 4 DAH. (C) Sagittal section showing at low magnification cIIGAP-ir migrating cells in the synencephalon of a 15 DAH larva. Squared area is magnified in D. (D) Detail of cIIGAP-ir cells in the synencephalon of a 15 DAH larva (sagittal section). (E) Sagittal section showing cIIGAP-ir cells migrating dorsally in the synencephalon of a 26 DAH larva. (F) cIIGAP-ir cells adopting their midline final position close to the fibers of the medial longitudinal fascicle, in the dorsal synencephalon of a 30 DAH larva (coronal section). (G) cIIGAP-ir cells in the dorsal synencephalon of a 45 DAH post-larva (coronal section). (H) Coronal section of a 60 DAH juvenile specimen, showing the absence of immunoreactive fibers in the pituitary. Scale bar represents 50 μm in A and C, and 25 μm in the remaining pictures. Abbreviations: OT, optic tectum; Pit, pituitary; POA, preoptic area; Syn, synencephalon; Tel, telencephalon.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30d
F
Syn
45d
G
60d
Pit
H

in the cerebellum. Significantly, a few sGAP-ir axons were observed entering the pituitary from 30 DAH (Figs. 2G, 3D, F and 6).

3.3. Ontogeny of seabream GAP-immunoreactive cells and fibers

The sbGAP-ir cells were detected for the first time at 26 DAH as a small group of pyramidal cells in the rostral olfactory bulbs close to the olfactory nerve junction (Figs. 4A and 6). From 30 DAH, ovoid, round and pyramidal sbGAP-ir cells were also present in the ventral telencephalon (Figs. 4B, C, 5C and 6), reaching the preoptic area at 45 DAH (Figs. 4E and 6) and the hypothalamus at 60 DAH (Figs. 5F and 6). These bipolar hypothalamic sbGAP-ir cells exhibited a characteristic fusiform shape (Fig. 5F). The gradient of expression of sbGAP-ir cells follows an inverse pattern in comparison to sGAP-ir cells, with a number of cells lower in the rostral olfactory bulbs (Fig. 5A and 6) than in the ventral telencephalon (Fig. 5C and 6) or preoptic area (Fig. 4E and 6). Furthermore, sbGAP-ir cells located in the terminal nerve area were smaller in size compared to sGAP-ir terminal nerve ganglion cells (compare Fig. 5A and B).

The sbGAP-ir innervation appeared restricted to the ventral forebrain (Figs. 4D, 5D, F and 6) and increased notably on the sixth week, in which sGAP-ir fibers also reached the pituitary (Figs. 4Fand 6). However, a significant and prominent sbGAP-ir pituitary innervation was evident at 60 DAH (Figs. 5E and 6). Furthermore, two conspicuous prepro-sbGnRH-ir fiber tracts were established during sea bass ontogeny: a fiber tract running rostro-caudally through the preoptic area (Figs. 4D and 5D) to the pituitary; and another fiber tract that arched in the ventrolateral hypothalamus and coursed caudo-rostrally to reach the sea bass hypophysis (Fig. 5F). Moreover, axons from the olfactory sbGAP-ir cells appeared to project locally, remaining in the vicinity of large sGAP-ir terminal nerve ganglion cells (Fig. 5A).

4. Discussion

Previous studies performed in our laboratory have permitted us to elucidate the ontogenic spatio-temporal pattern of mRNA expression for each prepro-GnRH form in the brain of sea bass (González-Martínez et al., 2002b). However, this study could not give any information about putative correspondence of GnRH mRNA-protein expression, innervation pattern and timing of presence of different GnRH projections in the pituitary and brain of developing sea bass.

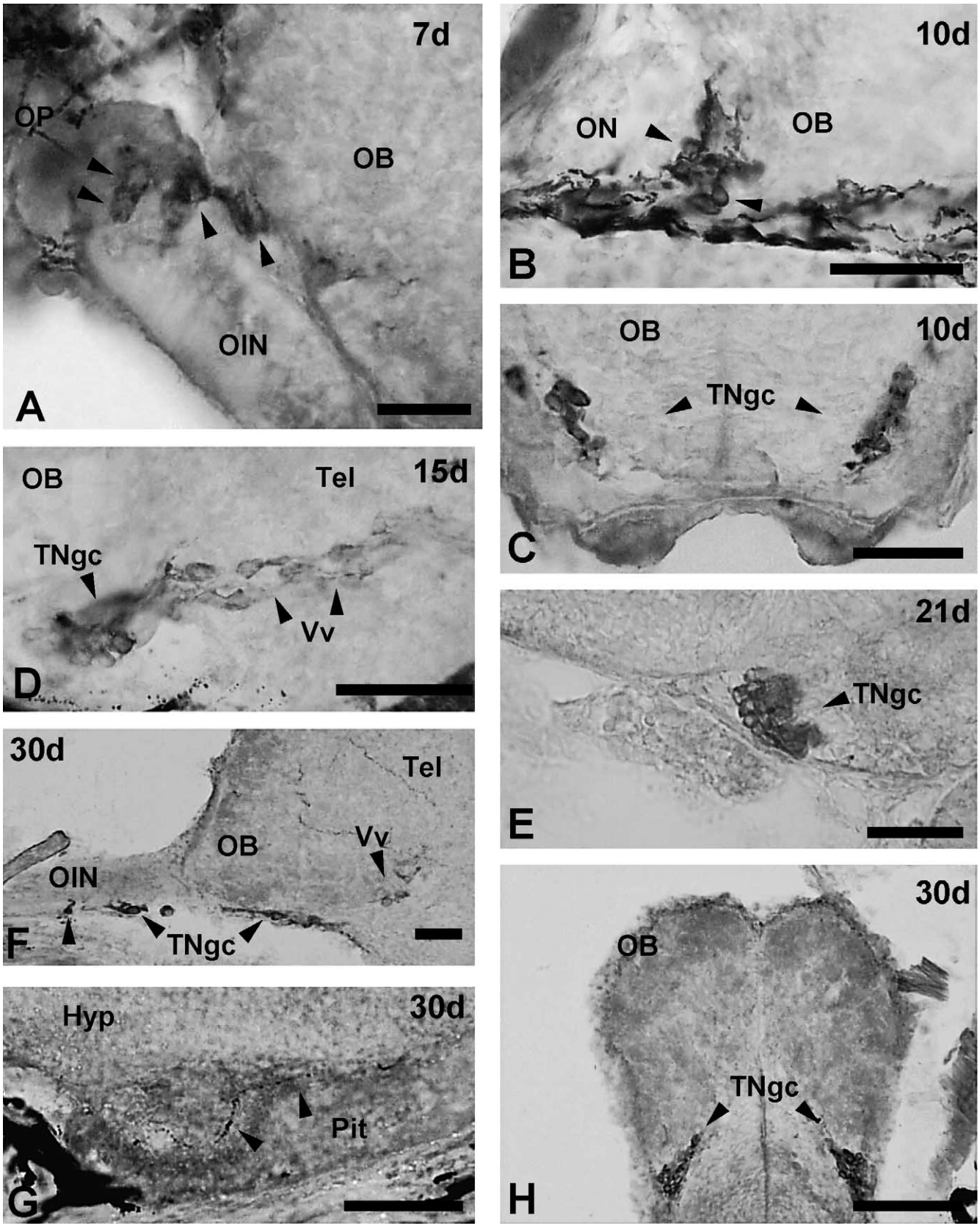
Therefore, herein we describe the ontogenic development and distribution of the three distinct prepro-GnRH-immunoreactive (ir) systems expressed in the brain of the European sea bass. We have used specific antisera against the GAP fragment of each prepro-GnRH, which are much more divergent in their amino-acid sequence than the corresponding GnRHs and represent accurate and specific markers of the different GnRH cell types (González-Martínez et al., 2002a; Zmora et al., 2002).

We show that the consistent expression of cGnRH-II in the synencephalon, and the overlapping of sGnRH and sbGnRH in the forebrain are maintained from developing to adult stages of the sea bass (González-Martínez et al., 2001, 2002a,b). As in other vertebrate species, early differentiation of sea bass mesencephalic cGnRH-II neurons occurs at the midbrain germinative primordium, whereas sGnRH neurons migrate from the olfactory region (Wray et al., 1989a,b; Muske and Moore, 1990; Muske, 1993; Dellovade et al., 1993; Northcutt and Muske, 1994; White and Fernald, 1998a,b; Parhar et al., 1998; González-Martínez et al., 2002b).

Our results show that prepro-cGnRH-II-ir cells represents the earliest detectable GnRH population in sea bass development, at day 4 after hatching which corresponds to the same early age of mRNA detection by in situ hybridization (González-Martínez et al., 2002b). An early expression of the synencephalic/midbrain GnRH form was also reported in some vertebrate species (Muske and Moore, 1990; White and Fernald, 1998b). Moreover, the number of prepro-cGnRH-II-ir cells increased notably from 4 to 30 DAH, when cGnRH-II cells reached their final position, and seemed to decrease progressively from this stage to adults (González-Martínez et al., 2002a,b). Similar observations have been made in other teleosts (Parhar et al., 1998; White and Fernald, 1998b; Ookura et al., 1999) and amphibians (Muske and Moore, 1990).

In the sea bass pituitary, prepro-cGnRH-II-ir fibers were not detected during ontogenesis nor in adults (González-Martínez et al., 2002a), suggesting that a putative role of cGnRH-II in the control of reproduction does not involve any direct action on gonadotrophic cells, at least in

Fig. 2. Distribution of immunoreactive (ir) sGAP cells and fibers in the brain of developing sea bass. (A) sGAP-ir cells migrating from the olfactory placode to the olfactory nerve in a 7 DAH larva. Sagittal section. (B) 10 DAH larva. sGAP-ir cells entering the olfactory bulbs from the olfactory nerve (sagittal section). (C) 10 DAH larva. Terminal nerve ganglion cells, at the transitional area between the olfactory bulbs and the telencephalon, exhibiting a conspicuous sGAP immunoreactivity (horizontal section). (D) 15 DAH larva. sGAP-ir migrating cells through the terminal nerve area and the ventral telencephalon. Note the higher immunostaining in terminal nerve ganglion cells in comparison to ventral telencephalic cells (sagittal section). (E) Cluster of sGAP-ir terminal nerve ganglion cells in a 21 DAH larva (sagittal section). (F) 30 DAH larva. sGAP-ir cells in the olfactory nerve, olfactory bulbs and ventral telencephalon. Positive terminal nerve ganglion cells exhibit a more intense immunostaining than ir cells in the ventral telencephalon. Note the presence of sGAP-ir fibers in olfactory bulbs and telencephalon (sagittal section). (G) 30 DAH larva. sGAP-ir fibers entering the sea bass pituitary (sagital section). (H) Bilateral sGAP-ir terminal nerve ganglion cells (horizontal section). Scale bar represents 50 μm in A, C and E, and 25 μm in the remaining pictures. Abbreviations: Hyp, hypothalamus; OB, olfactory bulbs; OlN, olfactory nerve; OP, olfactory placode; Pit, pituitary; Vv, ventral nucleus of the ventral telencephalon; Tel, telencephalon; TNgc, terminal nerve ganglion cells.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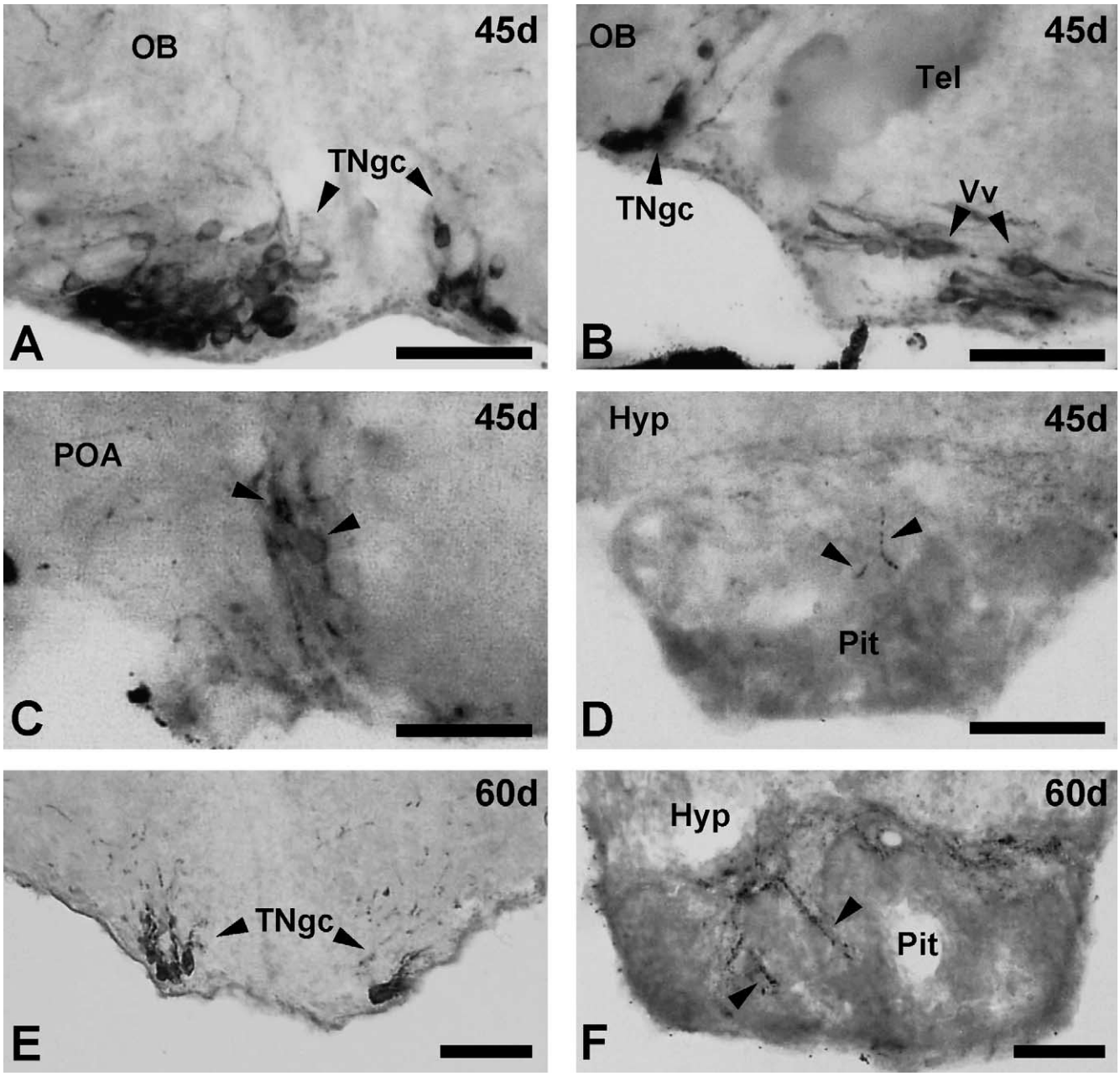

Fig. 3. Distribution of immunoreactive (ir) sGAP cells and fibers in the brain of developing sea bass. (A) 45 DAH post-larva. Bilateral clusters of intensely immunostained sGAP terminal nerve ganglion cells (coronal section). (B) 45 DAH larva. Sagittal section showing sGAP-ir perikarya and fibers in the olfactory bulbs and the ventral nucleus of the ventral telencephalon. Note the intense immunostaining of terminal nerve ganglion cells in comparison to the cells of the ventral telencephalon. (C) 45 DAH post-larva. sGAP-ir cells and fibers in the preoptic area. This immunostaining is markedly reduced in relation to terminal nerve ganglion cells (Fig. 4A and B) (sagittal section). (D) 45 DAH post-larva. sGAP-ir fibers reaching the pituitary of sea bass (coronal section). (E) 60 DAH juvenile. Large sGAP-ir cells in the terminal nerve area (coronal section). (F) 60 DAH juvenile. sGAP-ir fibers in the pituitary of sea bass (coronal section). Scale bar represents 50 μm in all micrographs. Abbreviations: Hyp, hypothalamus; OB, olfactory bulbs; Pit, pituitary; POA, preoptic area; Vv, ventral nucleus of the ventral telencephalon; Tel, telencephalon; TNgc, terminal nerve ganglion cells.

this species. According to our results, prepro-cGnRH-II-ir fibers were neither detected in the pituitary of adults (Zandbergen et al., 1995) nor in that of developing catfishes (Dubois et al., 2001). Furthermore, negative labeling of midbrain GnRH neurons after biocytin or DiI application into the pituitary of dwarf gourami, tilapia and catfish also provides evidences that cGnRH-II cells do not project to the pituitary in these species (Yamamoto et al., 1998; Dubois et al., 2001).

The structure and distribution of cGnRH-II is highly conserved across vertebrates suggesting that this neurohormone could serve different functions other than reproduction, for instance, as neurotransmitter, neuromodulator and/or autocrine/paracrine hormone in peripheral tissues (Penlington

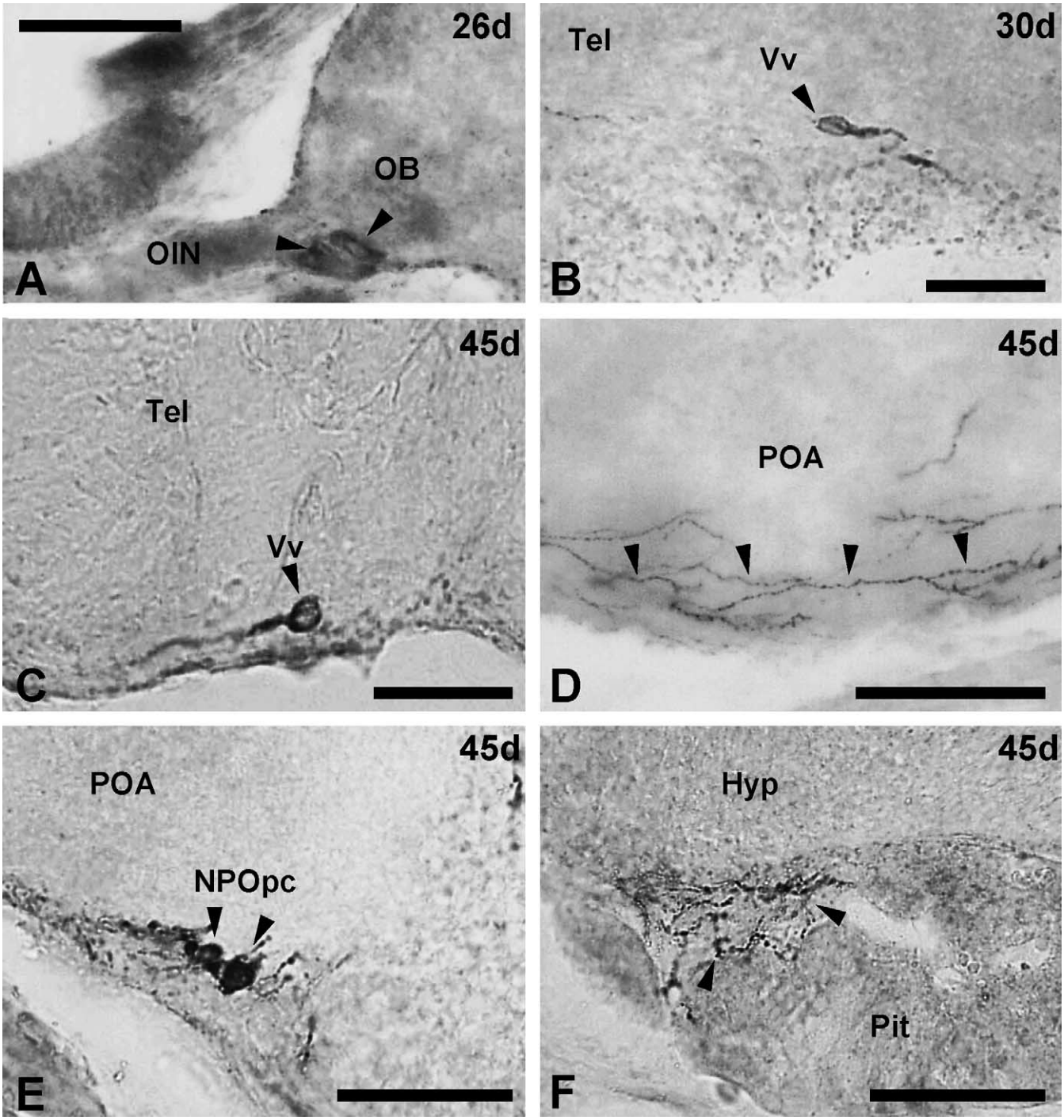

Fig. 4. Distribution of immunoreactive (ir) sbGAP cells and fibers in the brain of developing sea bass. (A) 26 DAH larva. sbGAP-ir cells at the transitional area between the olfactory nerve and olfactory bulb. (B) 30 DAH larva. sbGAP-ir cell in the ventral nucleus of the ventral telencephalon. (C) 45 DAH post-larva. sbGAP-ir cell in the ventral nucleus of the ventral telencephalon. (D) 45 DAH post-larva. Varicose sbGAP-ir fibers running through the ventral preoptic area of sea bass. (E) 45 DAH post-larva. Immunostained sbGAP cells and fibers in the parvocellular part of the parvocellular preoptic nucleus. (F) 45 DAH post-larva. sbGAP-ir fibers entering the pituitary of sea bass. All micrographs correspond to sagittal sections. Scale bar represents 25 μm in B and C and 50 μm in the remaining micrographs. Abbreviations: Hyp, hypothalamus; NPOpc, parvocellular part of the parvocellular preoptic nucleus; OB, olfactory bulbs; OlN, olfactory nerve; Pit, pituitary; POA, preoptic area; Vv, ventral nucleus of the ventral telencephalon; Tel, telencephalon.

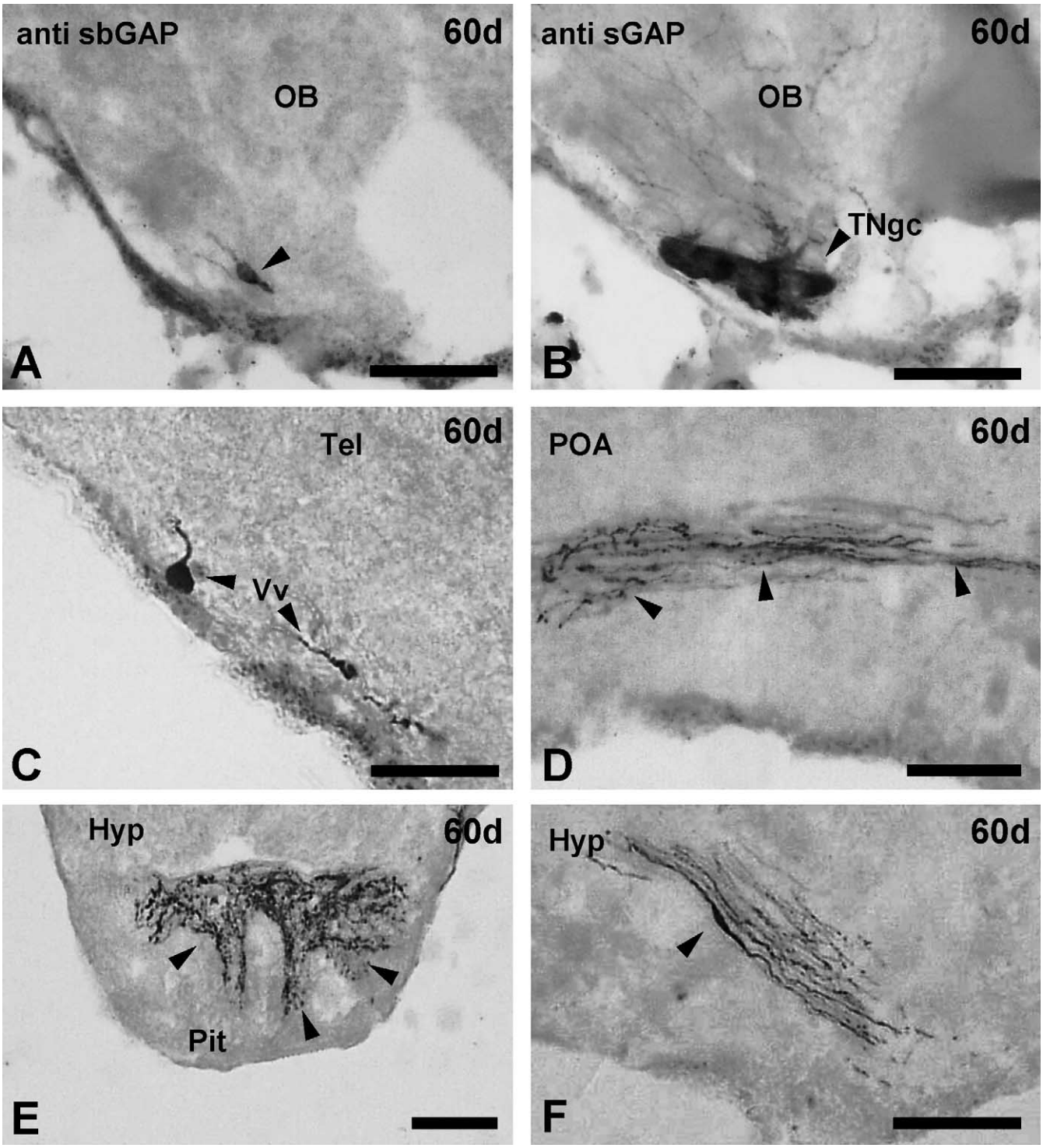

Fig. 5. Distribution of immunoreactive (ir) sbGAP cells and fibers in the brain of developing sea bass. (A and B) 60 days after hatching (DAH) juvenile. Adjacent coronal sections immunostained with anti-sbGAP (A) and anti-sGAP (B) sera. Note that anti-sbGAP serum immunostained only one small sbGAP cell in the terminal nerve area (A), whereas the anti-sGAP serum intensely labeled many large terminal nerve ganglion cells adopting a more superficial position in the same area (B). (C) 60 DAH juvenile. sbGAP-ir cells and fibers in the ventral nucleus of the ventral telencephalon (coronal section). (D) 60 DAH juvenile. sbGAP-ir fiber tract running along the ventral preoptic area of sea bass (sagittal section). (E) 60 DAH juvenile. Abundant sbGAP-ir fibers entering the pituitary of sea bass (coronal section). (F) 60 DAH juvenile. Fusiform sbGAP-ir cell in the ventral hypothalamus of sea bas. The presence of a conspicuous sbGAP-ir fiber tract can also be observed (sagittal section). Scale bar represents 25 μm in C and D and 50 μm in the remaining micrographs. Abbreviations: Hyp, hypothalamus; OB, olfactory bulbs; Pit, pituitary; POA, preoptic area; Vv, ventral nucleus of the ventral telencephalon; Tel, telencephalon; TNgc, terminal nerve ganglion cells.

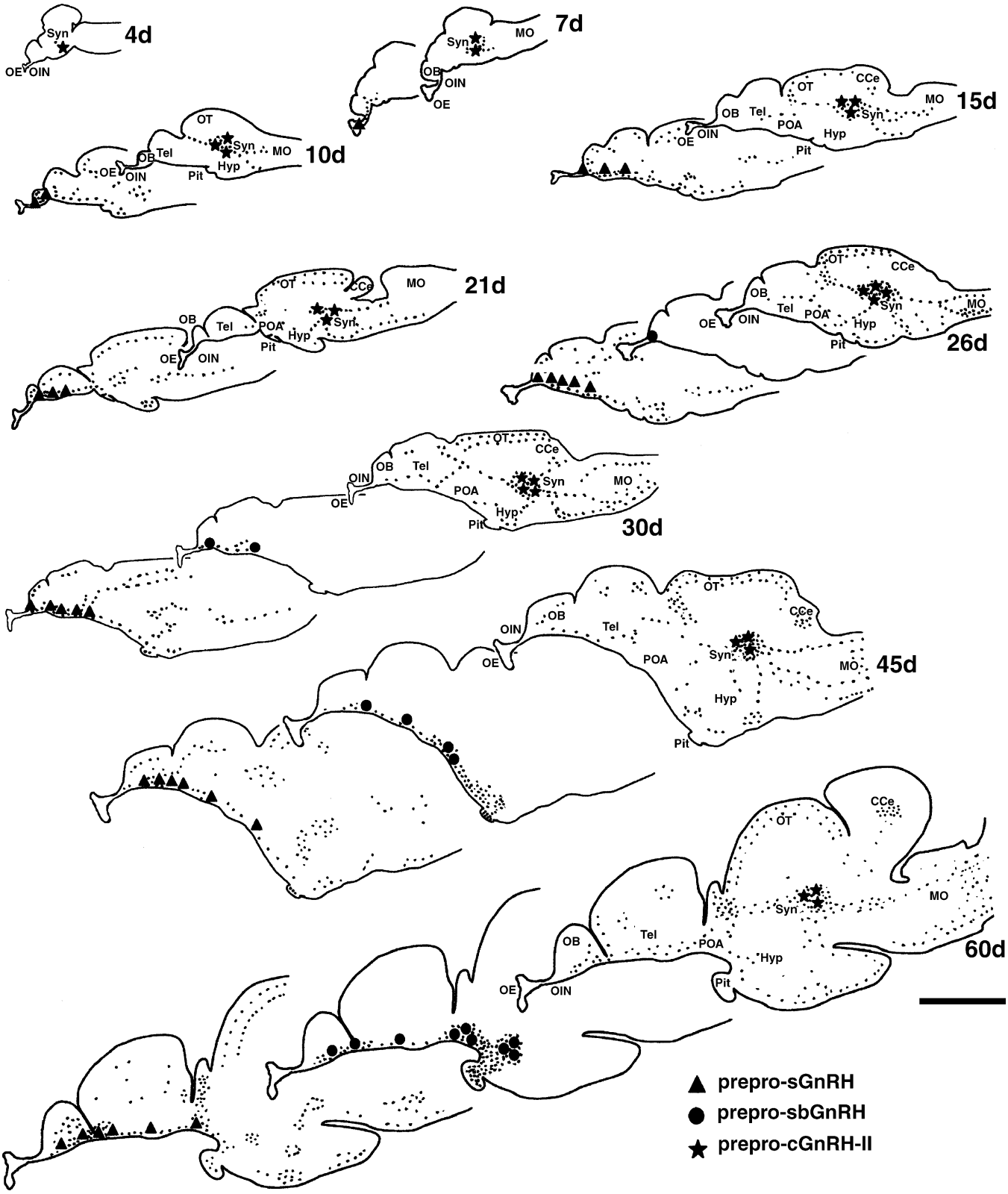

Fig. 6. Brain sagittal drawings summarizing the distribution of immunoreactive prepro-sGnRH- (triangles), prepro-sbGnRH- (circles) and prepro-cGnRH-II- (stars) cell bodies and fibers (small dots) in pre-larvae (4 days after hatching), larvae (7, 10, 15, 21, 26 and 30 days after hatching), post-larvae (45 days after hatching) and juveniles (60 days after hatching) of sea bass. Scale bar represents 500 μm. Abbreviations: CCe, corpus of the cerebellum; Hyp, hypothalamus; MO, medulla oblongata; OB, olfactory bulbs; OE, olfactory epithelium; OlN, olfactory nerve; OT, optic tectum; Pit, pituitary; POA, preoptic area; Syn, synencephalon; Tel, telencephalon.

et al., 1997; White et al., 1998; Yu et al., 1998). It has been proposed that cGnRH-II might modify the motor activity in relation to reproductive behaviour (Sakuma and Pfaff, 1980; Rissman, 1996; Fernald and White, 1999). The presence of a profuse cGnRH-II innervation in the optic tectum, cerebellum, medulla and sensory processing centers of developing (this study) and adult sea bass (González-Martínez et al., 2002a) suggests a relevant role of cGnRH-II in the control of the motor activity and integration of sensory inputs in sea bass.

Furthermore, the data obtained in this study demonstrated that both sGnRH and sbGnRH cells were first detected in the olfactory region, and migrate during early development in a rostro-caudal direction to reach their final positions along a continuum from the olfactory bulbs to the hypothalamus. These results exhibit a complete correspondence with the in situ hybridization data we had previously obtained in this species (González-Martínez et al., 2002b). A similar embryonic origin of "telencephalic" (GnRH-3 or sGnRH) and "hypothalamic" (GnRH-1 or sbGnRH) GnRH forms in the African cichlid has been suggested by White and Fernald (1998b). Evidences obtained in salmonids, amphibians, avians and mammals showed that all forebrain GnRH neurons originate in an olfactory primordium and then migrate into the basal forebrain across the terminal/olfactory nerves (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989a,b; Murakami et al., 1992; Muske, 1993; Chiba et al., 1994; Muske and Moore, 1994; Norgren and Gao, 1994; Schwanzel-Fukuda, 1999). However, these results are in contrast with those obtained in tilapia (Parhar, 1997), medaka (Parhar et al., 1998), eel (Chiba et al., 1999) and South American cichlid fish *Cichlasoma dimerus* (Pandolfi et al., 2002) suggesting that olfactory bulb and preoptic GnRH cells have different embryonic origins in proliferative olfactory placodes and diencephalic zones, respectively. It should be noted that sbGnRH is an orthologue of the tetrapod GnRH-I gene which is expressed in hypophysiotrophic neurons and differentiates from the olfactory region (Lethimonier et al., 2004). Thus, it is dubious that sbGnRH cells migrate from a preoptic primordium, unless preoptic GnRH neurons are shown to express proliferation markers. Furthermore, it seems unlikely that these sbGnRH cells migrate both rostral and caudally from the preoptic region during development.

Nevertheless, some remarkable differences were observed among sGnRH and sbGnRH systems in developing sea bass. First, the detection of prepro-sGnRH-ir cells was evident at day 7 after hatching, much earlier than that of prepro-sbGnRH-ir cells, which were first detected on day 26 after hatching. In this way, prepro-sGnRH-ir cells represent early expressing GnRH cells, while prepro-sbGnRH-ir cells represent late expressing cells. Furthermore, an inverse gradient in the expression of sGnRH and sbGnRH was established during sea bass ontogeny. The number of prepro-sGnRH-ir cells was greater in the olfactory area than in the ventral telencephalon or the preoptic area, whereas prepro-sbGAP-ir cells exhibited the inverse pattern, i.e. more cells in the ventral telencephalon and preoptic area in relation to the olfactory bulbs (see Table 1). Moreover, olfactory sGnRH-ir cells were clearly larger in size than preoptic sbGnRH neurons, and prepro-sbGnRH-ir cells reached the sea bass hypothalamus whereas most caudal prepro-sGnRH-ir cells only arrived at the rostral preoptic area. In the African catfish, although all forebrain GnRH neurons produce only the catfish GnRH form, there is good evidence that two distinct GnRH cell populations exist: a terminal nerve cell group and a ventral forebrain cell population (Dubois et al., 2001). Surprisingly, our observations in a percifom species are coincident with results obtained on forebrain GnRH systems of the rhesus macaque, a representative of a distant phylogenetic group (Quanbeck et al., 1997). In this latter species, two different migrating GnRH-ir cell groups were detected, with distinct temporal expression and differences in their morphology and brain distribution: early LHRH-expressing neurons extended to the preoptic region whereas late LHRH-expressing cells reached the basal hypothalamus (Quanbeck et al., 1997).

In addition, although both sGnRH- and sbGnRH-ir fibers innervate the pituitary, the pattern of projections of both GnRH systems exhibited conspicuous differences in the brain and hypophysis of developing sea bass. This distinctive pattern of innervation could reflect the separate functions for both GnRH forms. Indeed, prepro-sGnRH-ir fibers exhibited a much more abundant distribution in the brain, while prepro-sbGnRH-ir axons were much more abundant in the pituitary of developing and adult sea bass (González-Martínez et al., 2002a). According to these results, Rodríguez et al. (2000) found that pituitary sbGnRH levels were 17-fold higher than sGnRH levels in the hypophysis of immature male sea bass. These results corroborate physiological evidences stressing out the main role of sbGnRH in the stimulation of the secretion of gonadotrophins in perciforms (Powell et al., 1994; Zohar et al., 1995; Gothilf et al., 1996, 1997; Yamamoto et al., 1998; Holland et al., 1998; Senthilkumaran et al., 1999). It should be noted that sbGnRH cells represent the most delayed GnRH form expressed in the sea bass brain during ontogeny, at 26 days after hatching, not reaching the pituitary until 45 DAH. Interestingly, gonadotrophin-immunoreactive cells were not detected in the sea bass pituitary during the first 26 days after hatching (Cambré et al., 1990) and the genital crest was not evident until 43 days after hatching (Roblin and Brusle, 1983). Thus, it seems that the expression of sbGnRH results critical for the organization and functionality of the reproductive brain-pituitary-gonadal axis. Moreover, it has been proposed a possible role of sbGnRH in the processes of puberty or sex inversion in the gilthead seabream (Holland et al., 1998).

Nevertheless, the low levels of sGnRH found in sea bass pituitary when compared with sbGnRH, should not be interpreted as a trivial role in the hypophysiotrophic actions because it has a more potent effect than sbGnRH

in inducing gonadotrophin secretion (Zohar et al., 1995). It is also possible that sGnRH plays an important role during sea bass development, notably in the differentiation of the pituitary. Prolactin-immunopositive cells become 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-ir cells were detected. Interestingly, sGnRH serves as a prolactin-releasing factor in *Oreochromis mossambicus*, a perciform teleost (Weber et al., 1997). However, prepro-sGnRH-ir fibers were not apparent in the sea bass pituitary until 30 DAH. It seems that sGnRH could also be involved in coupling the sensory information from the environment with reproduction (White et al., 1995; Carolsfeld et al., 2000). The results obtained in this developmental study and in adult sea bass (González-Martínez et al., 2002a), might support these considerations because sGAP-ir fibers were also abundant in visual and gustatory sensory brain areas.

Acknowledgements

We thank A. Santos for his excellent technical help. We are grateful to Dr. F.J. Rodríguez-Gómez and the three anonymous referees for their helpful comments and advices. We also thank A. Vidaurreta and CUPIMAR fish farm for supplying the biological material used in this study. This work has been supported by UE (FAIR CT97-3785), CICYT (CYTMAR MAR95-1888-C03-02) and CNRS.

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