Immunohistochemical Localization of Three Different prepro-GnRHs in the Brain and Pituitary of the European Sea Bass (Dicentrarchus labrax) Using Antibodies to the Corresponding GnRH-Associated Peptides

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ABSTRACT

The distribution of the cells expressing three prepro-gonadotrophin-releasing hormones (GnRH), corresponding to salmon GnRH (sGnRH), seabream GnRH (sbGnRH), and chicken GnRH-II (cGnRH-II) forms, was studied in the brain and pituitary of the sea bass (Dicentrarchus labrax) by using immunohistochemistry. To circumvent the cross-reactivity problems of antibodies raised to GnRH decapeptides, we used specific antibodies generated against the different sea bass GnRH-associated peptides (GAP): salmon GAP (sGAP), seabream GAP (sbGAP), and chicken-II GAP (cIIIGAP). The salmon GAP immunostaining was mostly detected in terminal nerve neurons but also in ventral telencephalic and preoptic perikarya. Salmon GAP-immunoreactive (ir) fibers were observed mainly in the forebrain, although sGAP-ir projections were also evident in the optic tectum, mesencephalic tegmentum, and ventral rhombencephalon. The pituitary only receives a few sGAP-ir fibers. The seabream GAP-ir cells were mainly detected in the preoptic area. Nevertheless, sbGAP-ir neurons were also found in olfactory bulbs, ventral telencephalon, and ventrolateral hypothalamus. The sbGAP-ir fibers were only observed in the ventral forebrain, innervating strongly the pituitary gland. Finally, chicken-II GAP immunoreactivity was only detected in large synencephalic cells, which are the origin of a profuse innervation reaching the telencephalon, preoptic area, hypothalamus, thalamus, pretectum, posterior tuberculum, mesencephalic tectum and tegmentum, cerebellum, and rhombencephalon. However, no cIIIGAP-ir fibers were detected in the hypophysis. These results corroborate the overlapping of sGAP- and sbGAP-expressing cells in the forebrain of the sea bass, and provide, for the first time, unambiguous information on the distribution of projections of the three different GnRH forms expressed in the brain of a single species. J. Comp. Neurol. 446:95–113, 2002.

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The decapeptide gonadotrophin-releasing hormone (GnRH) constitutes the main cerebral factor stimulating the production and secretion of the pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Breton et al., 1972; Sherwood et al., 1993). In addition to its role in releasing gonadotrophins, GnRH seems to be implicated in other functions such as nesting and sexual behavior (Muske and Moore, 1994; Yamamoto et al., 1995, 1997) and their understanding requires solid data and knowledge on the organization of these systems. However, GnRH does not represent a unique molecule. In fact, since the discovery of the mammalian GnRH form in 1971 (mGnRH, Matsuo et al., 1971), the existence of fifteen isoforms of GnRH in vertebrates and protocordates has been reported, which have received the name of the species in which they have been discovered (Powell et al., 1996; Carolsfeld et al., 2000; Okubo et al., 2000a; Montaner et al., 2001). From these 15 GnRHs, eight forms are present in the brain of different teleosts: salmon GnRH (sGnRH; Sherwood et al., 1983), mGnRH (King et al., 1990), catfish GnRH (cfGnRH: Ngamvongchon et al., 1990), chicken GnRH-II (cGnRH-II: King and Millar, 1982; Miyamoto et al., 1984), herring GnRH (hGnRH, Carolsfeld et al., 2000), medaka GnRH (mdGnRH, Okubo et al., 2000a), and pejerrey GnRH (pGnRH, Montaner et al., 2001).

In teleosts, the basic pattern of distribution of GnRH cells suggested the existence of two major GnRH systems: one system along the ventral forebrain (terminal nerve, ventral telencephalon, preoptic area, and hypothalamus) expressing systematically cGnRH-II (Muñz et al., 1981; Goos et al., 1985; Kah et al., 1986, 1991; Subbedar and Krishna, 1988; Batten et al., 1990; Amano et al., 1991; Grober and Bass, 1991; Yamamoto et al., 1995; Rodríguez-Gómez et al., 1999). The organization of these GnRH systems was mainly established via immunohistochemistry by using antibodies more or less specific for the endogenous decapeptides. However, in different teleost species, terminal nerve cells showed strong sGnRH and a weaker cGnRH-II immunoreactivity, whereas midbrain tegmentum cells exhibited strong cGnRH-II and a weaker sGnRH immunoreactivity (Yamamoto et al., 1995; Rodríguez-Gómez et al., 1999). Although colocalization of different GnRH forms in the same cell cannot be completely excluded, this observation seems to reflect the cross-reactivity of antisera with more than one GnRH form as a consequence of the high identity among the different GnRH decapeptides.

In the European sea bass, a previous study 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). 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, which also revealed the presence of cGnRH-II fibers in the pituitary (J.A. Muñoz-Cueto, unpublished). In some, but not all species of teleosts, cGnRH-II has also been detected in the pituitary (Yu et al., 1988; Schultz et al., 1993; Montero et al., 1995; Rodríguez-Gómez et al., 1999) although its origin is still uncertain.

Recently, it has been demonstrated that perciforms express three different GnRH forms in their brains: salmon GnRH, seabream GnRH, and chicken GnRH-II (Powell et al., 1994; White et al., 1995). Furthermore, most studies performed up to now suggested the existence of a neuroanatomical segregation in the expression of these three different GnRHs (Gothilf et al., 1996; Okuzawa et al., 1997; White and Fernald, 1998). Thus, the expression of salmon GnRH appears restricted to the rostral olfactory bulbs; seabream GnRH is mainly expressed in the preoptic area, whereas chicken-II GnRH is synthesized in large cells at the diencephalic-mesencephalic transition. Based on these results, it has been proposed that sGnRH neurons differentiate from the olfactory placode, whereas sbGnRH and cGnRH-II neurons originate from preoptic and mesencephalic primordia, respectively (Parhar et al., 1997; Parhar et al., 1998; Ookura et al., 1999). Although physiological data provided evidence that the three GnRH forms can stimulate the gonadotrophin secretion (Zohar et al., 1995), sbGnRH seems to be the main hypophysiotropic hormone in perciforms (Powell et al., 1994; Holland et al., 1998), whereas the role of salmon GnRH and chicken-II GnRH in reproduction is still unclear.

Recently, the cDNAs encoding the three GnRH isoforms present in sea bass have been cloned (Zmora et al., 2002), providing information on the molecular structure of the three preproGnRHs in this species. Sea bass GnRH cDNAs contain coding sequences for a signal peptide, the decapeptide GnRH, a processing tripeptide, and a GnRH-associated peptide or GAP. These GAP sequences represent valuable tools for in situ hybridization techniques because they are longer when compared to GnRH sequences and there is a lower sequence identity among GAP sequences. By using these probes, we have recently demonstrated that, according to previous studies, each of the three GnRH prepro-mRNAs is preferentially expressed in a specific brain region. However, we have described for the first time a clear overlapping of the sGAP- and sbGAP-expressing cells in the telencephalon and diencephalon of the sea bass, suggesting that the anterior GnRH systems are not clearly segregated, at least in this species (González-Martínez et al., 2001).

One of the aims of the present study was to corroborate which brain areas express the different GnRH preproforms in the sea bass by using immunohistochemical techniques. The second aim of this study was to obtain precise information about the projections of each GnRH form into the brain and pituitary of sea bass, determining which brain areas express the different GnRH prepro-mRNAs is preferentially expressed in a specific brain region. However, because it has been reported that the distribution of immunoreactive GnRH was consistently similar to that of the corresponding immunoreactive GAP (Ronchi et al., 1992; Polkowska and Przekop, 1993).

MATERIALS AND METHODS

Animals

Adult immature male (n = 5) and female (n = 5) specimens of sea bass ranging in weight from 200 to 250 g were purchased from a local fishery (Cupimar, San Fernando, Spain) and kept in the laboratory in running seawater. Animals were treated in agreement with the European
Union regulation concerning the protection of experimental animals.

**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 (Zmora et al., 2002; González-Martínez et al., 2001). The full-length sequences of the three sea bass prepro-GnRH forms are available at the GeneBank, accession numbers AAF62899 for sb-GnRH, AAF62899 for sGnRH, and AAF62900 for cGnRH-II. Briefly, for the production of recombinant GAPs, two expression vectors were used: the His-tagged expression system for sGAP and sbGAP, and the Glutathione-S-transferase (GST) expression system for cIGAP. Analysis on 17% sodium dodecylsulfate-polyacrylamide gel electrophoresis (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 cIGAP, SDS-PAGE analysis revealed two bands: the cIGAP/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 9 female guinea pigs (5/6 weeks old; 345–365 g) obtained from CEGAV (France) and housed in laboratory cages designed for guinea pigs. Animals, three each for 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 intervals, were given intramuscularly in the posterior legs. Eight days after the fourth injection, blood was collected under anesthesia by cardiac puncture, centrifuged and the serum stored at −25°C.

**Dot blot analysis**

The specificity of the antisera used in the immunohistochemical study was assessed by dot blot analysis by using the recombinant sGAP, sbGAP, and cIGAP proteins as antigens. Briefly, 6–12 μg of recombinant sGAP, sbGAP, and cIGAP proteins were added to individual nitrocellulose filter slices. The slices were saturated for an hour in Tris buffer saline pH 7.4 (TBS) containing 5% of nonfat dry milk. Samples were then incubated for 12 hours with the primary antisera (anti-sGAP, anti-sbGAP, anti-cII-GAP) at a 1:2,000 dilution. After incubation, nonfat dry milk. Samples were then incubated for 12 hours with the primary antisera (anti-sGAP, anti-sbGAP, and anti-cIGAP antibodies (1:500 to 1:1,000 dilution in CBT 0.5% casein). Sections were washed in CBT (2 × 15 minutes) and incubated for 1.5 hours at room temperature with Biotin-sp.-Conjugated-Affinity Pure Goat Anti-Guinea Pig–IgG (Jackson Immuno Research Laboratories, West Grove, PA) diluted 1:1,000 in CBT. After washing in CBT (2 × 15 minutes), sections were incubated 1.5 hour at room temperature with peroxidase-conjugated-streptavidin complex (Jackson Immuno Research Laboratories) diluted 1:1,000 in CBT. Finally, sections were washed in CBT followed by Tris-HCl (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) and 0.01% hydrogen peroxide or 0.04% 4-chloro-1-naphthol (Sigma) and 0.01% hydrogen peroxide. To confirm the specificity of the immunostaining, controls were performed by preabsorption of primary antisera with their respective antigens, replacement of primary antisera with the corresponding preimmune sera, and omission of primary or biotinylated antisera.

To check that GAP was a reliable marker of GnRH-producing cells, double immunofluorescence staining was performed after incubating the sections concomitantly with anti-sGAP and anti-sGnRH (donated by Dr. Breton) sera, anti-sbGAP and anti-mGnRH (donated by Dr. Tramu) sera, and anti-cIGAP and anti-cGnRH-II (donated by Dr. Peute) sera. The GAP and GnRH antisera used were obtained in guinea pigs and rabbits, respectively. The immunoreactive GAP cells and fibers were revealed by incubating the sections with an antibody against guinea pig IgG coupled to fluorescein (Jackson ImmunoResearch), whereas the immunoreactive GnRH cells and fibers were evidenced by using an antibody against rabbit IgG coupled to 7-amino-4-methylcoumarin-3-acetic acid (AMCA) or Texas Red (Jackson ImmunoResearch).

**Immunohistochemistry**

Animals were anesthetized with 2-phenoxethanol (0.3 ml/liter of water; Sigma) and perfused via the aortic bulb with Bouin’s fixative (4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4). Brains with the pituitary attached, were then removed and postfixed in the same fixative for 5–7 hours in the dark at 4°C. After cryoprotection overnight in 0.1 M phosphate buffer containing 15% sucrose, brains were embedded in Tissue-Tek, frozen in cold isopentane and serial coronal and sagittal sections of 16-μm-thick were obtained with a cryostat.

Immunohistochemical staining was performed by using a streptavidin-biotin-peroxidase complex method. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in Coons buffer pH 7.4 (CBT, 0.01 M Veronal, 0.15 M NaCl) containing 0.1–0.2% Triton X-100 for 30 minutes. Before immunostaining, sections were transferred for 5 minutes to CBT and then saturated in CBT containing 0.5% casein for 30 minutes. Sections were incubated overnight in a moist chamber at room temperature with anti-sGAP, anti-sbGAP, and anti-cIGAP antisera (1:500 to 1:1,000 dilution in CBT 0.5% casein). Sections were washed in CBT (2 × 15 minutes) and incubated for 1.5 hours at room temperature with peroxidase-conjugated-streptavidin complex (Jackson Immuno Research Laboratories) diluted 1:1,000 in CBT. Finally, sections were washed in CBT followed by Tris-HCl (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) and 0.01% hydrogen peroxide or 0.04% 4-chloro-1-naphthol (Sigma) and 0.01% hydrogen peroxide. To confirm the specificity of the immunostaining, controls were performed by preabsorption of primary antisera with their respective antigens, replacement of primary antisera with the corresponding preimmune sera, and omission of primary or biotinylated antisera.

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Immunohistochemical sections were analyzed on a Leica photomicroscope and computer images were obtained with a Sony DKC-CM30 Digital Camera (Sony, Japan). The software used was Adobe Photoshop 5.0 and no subsequent alterations have been made with the exception of the color of Figure 1B, which has been altered for purposes of illustration. Ten to fifty immunoreactive cells of each nucleus were measured in order to evaluate the average cell size. For the precise localization of the different GAP cells and projections, we have used a detailed sea bass brain atlas recently developed in our laboratory (Cerdá-Reverter et al., 2001a, b).
primary and biotinylated antisera were omitted. 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 positive cells for each antisera was not quantified. The levels of transverse sections illustrated in Figure 2 are shown in Figure 1G.

Salmon GAP-immunoreactive cells

Immunoreactivity for anti-sGAP serum was mostly detected in the olfactory bulbs, and particularly in ganglion cells of the terminal nerve associated to the glomerular layer (Figs. 2A and 3A) and at the junction between the caudal olfactory bulbs and the telencephalon (Figs. 2B and 3B). These medium-sized to large cells (10–25 μm in diameter) were rounded, ovoid, or pyramidal in shape. Furthermore, sGAP-immunoreactive (sGAP-ir) cells were also detected further caudal, in the ventral telencephalon (Fig. 2C) and in the ventral preoptic area, associated to the parvocellular and anteroventral subdivisions of the parvocellular preoptic nucleus (Fig. 2D). Such cells were also round, bipolar, or pyramidal in shape but they were much smaller (4–8 μm in diameter) than in olfactory bulbs (Fig. 3D and E). More caudally, no other sGAP-ir cells could be detected in the brain of the sea bass.

The sGAP-ir fibers were observed mainly in the forebrain. These sGAP-ir fibers were more evident in olfactory bulbs (Figs. 2A,B, 3C), ventral telencephalon (ventral, dorsal, supracommissural, postcommissural, and intermediate nuclei, Figs. 2C,D, 3D), preoptic area (parvocellular and magnocellular preoptic nuclei, anterior periventricular nucleus, suprachiasmatic nucleus, Figs. 2D,E, 3F), hypothalamus (medial part of the lateral tuberal nucleus, anterior tuberal nucleus, nucleus of the lateral recess, Fig. 2F,G), ventral thalamus (ventromedial and ventrolateral thalamic nuclei, Fig. 2E), pretectum (central pretectal nucleus, Fig. 2F) and dorsal tegmentum (semincircular torus, secondary gustatory nucleus, perilemniscal nucleus, Fig. 2G,H). However, a conspicuous sGAP innervation was also observed in the dorsal telencephalon (lateralventral and lateroposterior parts, posterior part, second subdivision of the medial zone, Fig. 2C,D), optic tectum (central zone and periventricular gray zone, Figs. 2E–H, 3G), and ventral rhombencephalon (reticular formation, anterior octaval nucleus, vagal lobe, inferior olivary nucleus, Figs. 2H–J, 3H). The pituitary only receives a small number of sGAP-ir fibers (Fig. 2F). These fibers enter the neurohypophysis and reach the proximal pars distalis of the adenohypophysis (Fig. 6A).

Seabream GAP-immunoreactive cells

The sbGAP immunoreactivity was more evident in cells of the preoptic area associated with the parvocellular preoptic nucleus (Figs. 2D, 4E) and the anterior periventricular nucleus (Fig. 2E), but were also slightly more rostral, in the ventral nucleus of the ventral telencephalon (Figs. 2C, 4D). These seabream GAP-immunoreactive (sbGAP-ir) cells were small or medium-sized (5–20 μm in diameter) and exhibited fusiform, bipolar, rounded, or ovoid shapes. However, sbGAP immunoreactivity was not restricted to these areas and some sbGAP-ir cells also appeared in the olfactory bulbs, associated with the glomerular layer (Figs. 2A, 4A) and the terminal nerve area (Figs. 2B, 4B), where the sbGAP-ir cells were found. This result did not represent a cross-reaction because labelling with anti-sbGAP serum was not evident in large sbGAP-ir cells of the terminal nerve (Fig. 4B). Furthermore,
Figure 1.
sbGAP-ir cells of the olfactory bulbs were less numerous and clearly smaller than sGAP-ir cells of the same region (4–10 μm in diameter versus 10–25 μm in diameter). There were also some sbGAP-ir cells, further caudally, in the ventrolateral hypothalamus (Figs. 2F, 4G), in which only one to two small and rounded immunolabelled cells (4–9 μm in diameter) could be observed per section. The sbGAP-ir fibers were observed only in the ventral surface of the forebrain, along the ventral telencephalon, the preoptic area, and the ventral hypothalamus, innervating strongly the pituitary gland of sea bass (Figs. 2C–F, 4F–H). Nevertheless, some varicose immunoreactive fibers originating in sbGAP-ir cells of the olfactory bulbs did not run to the pituitary and remained in the olfactory area (Fig. 4C), in the vicinity of large ganglion cells of the terminal nerve, which appeared immunostained by using the anti-sGAP serum (Fig. 3B) but not appear immunostained with anti-sbGAP serum (Fig. 4C). Running rostrocaudally from the ventral telencephalon to the pituitary stalk, there was clearly a major tract of sbGAP-ir fibers along which sbGAP-ir cell bodies could be detected. Furthermore, another tract of sbGAP-ir fibers originating in the hypothalamus arched in the ventrolateral surface of the brain, running caudorostrally to reach the pituitary of sea bass. Both fiber tracts appeared more evident in sagittal sections (Fig. 4P,H) than on transverse sections (Fig. 4D,G). These sbGAP-ir fibers entered the neurohypophysis and invaded the proximal pars distalis of the adenohypophysis (Figs. 2F, 6B), and the border of the pars intermedia.

Chicken-II GAP-immunoreactive cells

Finally, chicken-II GAP-immunoreactive (cIIGAP-ir) cells appeared restricted to the medial zone of the dorsal telencephalon, lying close to the fibers of the medial longitudinal fascicle in a periventricular position (Figs. 2G, 5A). These large cells (15–23 μm in diameter), rounded and ovoid in shape, appeared just caudal to the posterior commissure and rostral to the mesencephalic tegmentum.

In turn, cIIGAP-ir fibers were profusely distributed in the brain of sea bass, being especially evident in the midbrain and hindbrain. This innervation was conspicuous in the dorsal telencephalon (medial, lateroventral, and posterior parts, Fig. 2C,D), ventral telencephalon (ventral, dorsal, supracommissural, central, and postcommissural nuclei, Fig. 2C,D), periventricular preoptic area (parvocellular and magnocellular preoptic nuclei, anterior periventricular nucleus, Fig. 2D,E), and hypothalamus (lateral tuberal nucleus, anterior tuberal nucleus, nucleus of the lateral recess, diffuse nucleus of the inferior lobe, Fig. 2F–H), dorsal and ventral thalamus (Fig. 2E,F), pretectum (superficial and central pretectal nuclei, Fig. 2F), posterior tuberculum (posterior tuberal nucleus, periventricular nucleus, preglobular nuclear complex, Fig. 2F,G), mesencephalic tectum (central zone, periventricular gray zone, superficial white and gray zone, longitudinal torus, Figs. 2E–H, 5B) and tegmentum (semitricular torus, oculomotor and trochlear nuclei, secondary gustatory nucleus, locus coeruleus, Figs. 2G,H, 5C), granular layer of the valvula, corpus and eminentia granularis of the cerebellum (Figs. 2G–I, 5D,E), medulla oblongata (reticular formation, interpeduncular nucleus, octavolateral area, visceromotor nuclei, vagal lobe, inferior olive, Figs. 2H–J, 5F) and spinal cord. Although cIIGAP-ir fibers could be observed in the ventromedial hypothalamus, in the vicinity of the pituitary stalk, no cIIGAP-ir axons were detected in sea bass hypophysis (Fig. 6C).

**DISCUSSION**

It is considered that the presence of three GnRH forms in the brain is not restricted to perciform fish (Powell et al., 1994; White et al., 1995; Gothilf et al., 1996; González-Martínez et al., 2001) 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; Yahalom et al., 1999). These findings raise the question on the precise roles of multiple GnRH forms in the control of reproduction and other physiological processes. Emerging data also point to a multiplicity of GnRH receptors, because two GnRH receptor subtypes have been evidenced in the goldfish (Illy et al., 1999) and three in the bullfrog, a diploid species (Wang et al., 2001). One approach towards clarifying this puzzle is to clearly identify the brain nuclei expressing the different GnRH variants and their respective projections into the brain and pituitary.

In this study, we have presented the distribution of immunoreactive prepro-GnRH cells in the brain of the European sea bass, *Dicentrarchus labrax*, by using specific antisera directed against the different sea bass GAP peptides. The results permit us to demonstrate that, in sea bass, GAP and GnRH peptides are coexpressed in the same cells, as described in mammals (Ronchi et al., 1992; Polkowska and Przekop, 1993), and confirm that GAL antisera represent valuable tools to localize specifically the cells expressing the different GnRH forms, avoiding the problem of cross-reactivity of antibodies directed against the GnRH decapetides.

The immunohistochemical localization of sGAP, sbGAP, and cIIGAP cell bodies totally agrees with results obtained in the same species by using in situ hybridization techniques (González-Martínez et al., 2001). Thus, sGAP-ir cells are predominant in the olfactory bulbs, whereas sbGAP-ir cells were more abundant in the caudal telencephalon and preoptic area, and cIIGAP-ir cells ap-
Fig. 2. A–J: Series of schematic transverse sections through the brain of sea bass showing the distribution of immunoreactive salmon prepro-gonadotrophin-releasing hormone-associated peptides (sGAP; triangles), seabream GAP (sbGAP; circles), and chicken-II (cII-GAP; stars) perikarya and fibers (small dots). Thick lines represent fibers en passage. A constitutes the most rostral section and J the most caudal one. For abbreviations, see list. Scale bars = 1 mm.
Figure 2 (Continued)
Figure 2 (Continued)
Figure 4
peared restricted to the synencephalon. This study demonstrates that the overlapping of the sGAP and sbGAP from the olfactory bulbs to the preoptic region obtained at the mRNA level (González-Martínez et al., 2001) is also verified at the protein level. This overlapping did not represent a cross-reaction of antibodies because sbGAP-ir cells in olfactory bulbs were clearly smaller than sGAP-ir cells. Labelling with anti-sbGAP sera was never evident in large sGAP-ir cells, and conversely, anti-sGAP sera did not immunostain smaller sbGAP-ir cells. Furthermore, as it was observed by using in situ hybridization (González-Martínez et al., 2001), the ventrolateral hypothalamus of sea bass only contained sbGAP-ir neurons and appeared devoid of sGAP immunoreactivity on cell bodies. Finally, dot blot analysis and controls performed reinforce the specificity of antisera used in this study.

The overlapping distribution of sGAP- and sbGAP-expressing cells from the olfactory bulbs to the preoptic region obtained by using both immunohistochemical (this study) and in situ hybridization techniques (González-Martínez et al., 2001) in sea bass brain is in contradiction to previous studies in perciforms showing a neuroanatomical segregation in the expression of forebrain GnRH systems, viz. sGnRH in terminal nerve and sbGnRH in preoptic area (White et al., 1995; Gothilf et al., 1996; Okuzawa et al., 1997; White and Fernald, 1998; Parhar et al., 1998). Based on this expression pattern and ontogenic studies, several authors proposed that sGnRH cells develop from the olfactory placode, whereas sbGnRH neurons originate from the preoptic area (Parhar, 1997, 1999; Parhar et al., 1998; Ookura et al., 1999). This is in contrast with evidence obtained in other vertebrates, in which all forebrain GnRH neurons seem to have similar developmental origins. In amphibian, avian, and mammals, neurons destined to produce the preoptic/hypothalamic GnRH form develop from 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., 1989; Muske, 1993; Muske and Moore, 1994; Norgren and Gao, 1999; Swanson et al., 1999). Recently, in addition to the mGnRH and cGnRH-II isoforms (White et al., 1998; Urbanski et al., 1999), the existence of a third form of GnRH which seems to correspond with sGnRH has been referred to in mammals, including humans (Yahalom et al., 1999). Furthermore, there is growing evidence on the existence of sGnRH in amphibian (Sherwood et al., 1986), reptilian (Powell et al., 1985), and eutherian mammals (Montaner et al., 1998, 1999). In calves and humans, sGnRH and mGnRH are localized in the same regions and the total contents of sGnRH and mGnRH in the hypothalamus are similar (Yahalom et al., 1999). In the rhesus macaque, the existence of two different populations of GnRH-immunoreactive neurons that arise during development has been demonstrated, exhibiting different morphological features (Quanbeck et al., 1997). A comparable result was obtained in sea bass, in which two distinct GnRH cell populations, i.e., sGAP- and sbGAP-ir cells, were colocalized in the same brain areas. According to these results, we have also observed morphological differences between sGAP- and sbGAP-ir cells, especially in the terminal nerve area of sea bass, sGAP-ir cells being much larger than sbGAP-ir neurons. Taken together, our present and previous (González-Martínez et al., 2001) results suggest that, as in other vertebrates, the forebrain GnRH systems of highly evolved teleosts arise from a common olfactory primordium. Furthermore, it seems improbable that sbGnRH cells in sea bass originate in the preoptic area because this would imply that they migrate in two different directions during development, rostrally towards the olfactory bulbs, and caudally up to the ventral hypothalamus.

The distribution pattern of sGAP- and sbGAP-ir cells on the same brain areas suggests that both forebrain GnRH isoforms have evolved from a common ancestor gene by duplications and mutations, probably coinciding with the genomic duplication that occurred in early teleosts (Amores et al., 1998). Furthermore, this duplication appears to have occurred more recently than the duplication that gave rise to the cGnRH-II form, which represents the most conserved GnRH form in phylogeny. The identification of three GnRH forms in a representative of an early evolving teleost group such as the herring (Carolsfeld et al., 2000) indicates that the presence of three GnRHs is not restricted to the brain of highly evolved teleosts. Considering the monophyletic hypothesis of teleostean evolution (De Pinna, 1996), all teleosts evolving after the herring should exhibit three forms of GnRH, but some uuteleosts such as salmons or catfishes have only two forms of GnRH. White and Fernald (1998) remarked on the high similarities between the genomic sequence encoding the sGnRH form in the perciform Haplochromis burtoni and the presumed releasing form of GnRH in salmonids, and suggested that the gene encoding the releasing form of GnRH in salmonids might not yet be described. In salmonids, specific in situ hybridization studies showed an intense sGnRH gene expression in the terminal nerve area and a weak expression in the preoptic area (Suzuki et al., 1992; Bailhache et al., 1994). A similar result was obtained by us in sea bass by using in situ hybridization with sGAP riboprobes, whereas sbGAP mRNA expression exhibited the inverse pattern, i.e., an intense labelling in the preoptic area and a lower expression in the olfactory bulbs (González-Martínez et al., 2001). Nevertheless, immunocytochemical studies using anti-sGnRH sera in salmonids revealed an intense labelling in preoptic GnRH cells (Amano et al., 1991; Navas et al., 1995). In the sea bass, a study performed before the characterization of sbGnRH and based on the use of antibodies to sGnRH also showed the presence of numerous GnRH-ir neurons in the anterior olfactory bulbs, terminal

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nerve area, ventral telencephalon, ventrolateral preoptic region, and ventrolateral hypothalamus along a conspicuous tract of immunoreactive fibers running to the pituitary (Kah et al., 1991). The present study fully confirms this distribution but indicates that the sGnRH antibodies used in this pioneer study probably recognizes both sGnRH and sbGnRH of sea bass because labelling with anti-sGnRH serum fits almost perfectly with the addition of sGAP and sbGAP immunoreactivity. In addition, an in situ hybridization and immunohistochemical study performed in catfish by using specific riboprobes and antibodies against the catfish GAP isoform revealed the distribution of catfish GnRH-expressing cells in this species (Zandbergen et al., 1995). This distribution coincides al-

Fig. 5. Distribution of immunoreactive chicken-II prepro-gonadotrophin-releasing hormone-associated peptide (cIIIGAP) cells and fibers in the sea bass brain. A: cIIIGAP cells in the dorsal synencephalon. Transverse section. B: cIIIGAP-immunoreactive fibers in the optic tectum. Darkfield. Transverse section. C: cIIIGAP-immunoreactive fibers in the torus semicircularis of the tegmentum. Sagittal section. D: cIIIGAP-immunoreactive fibers in the corpus of the cerebellum. Darkfield. Sagittal section. E: cIIIGAP-immunoreactive fibers in the valvula of the cerebellum. Darkfield. Sagittal section. F: cIIIGAP-immunoreactive fibers in the caudal rhombencephalon. Darkfield. Sagittal section. Arrowheads in A mark cIIIGAP-immunoreactive cells. CCg, granular layer of the corpus of the cerebellum; CCm, molecular layer of the corpus of the cerebellum; RF, reticular formation; Syn, synencephalon; Teg, tegmentum; TS, torus semicircularis; VCg, granular layer of the valvula of the cerebellum; VCm, molecular layer of the valvula of the cerebellum. For abbreviations, see list. Scale bars = 100 μm in A,B, 200 μm in C–F.
most completely with the localization of sbGAP cells detected in sea bass by using both in situ hybridization (González-Martínez et al., 2001) and immunohistochemistry (this study). Furthermore, prepro-cfGnRH mRNA-expressing cells and cfGnRH-ir perikarya present in the olfactory area were scarce and small (see Figs. 2 and 5 of Zandbergen et al., 1995, respectively), resembling the sb-GAP cells located in the olfactory bulbs of the sea bass and differing notably of the large and abundant sGAP cells present in this area. Taken together, this evidence suggests that a third GnRH form probably has yet to be discovered in salmonids and catfishes.

Many researchers consider that cGnRH-II neurons in the synencephalon (or rostral midbrain tegmentum) belong to the nucleus of the medial longitudinal fascicle (nMLF), because of their similar location and spinal projections of the two neuronal populations. However, they have connectional, cytological, and immunological differences, and should not be regarded as the same neuronal population. Neurons of the nMLF project only to the spinal cord, whereas synencephalic GnRH neurons project to the telencephalon, diencephalon, mesencephalon, cerebellum, and medulla oblongata, in addition to the spinal cord (Yamamoto et al., 1995, 1998b). The nMLF neurons are immunonegative to GnRH sera, located ventrolateral to the GnRH neurons, and do not show ultrastructural features of peptidergic neurons (Miller and Kriebel, 1985, Miller and Kriebel, 1986b; Yamamoto et al., 1998b). Also importantly, these previous studies show that the synencephalic GnRH neurons are located in the subventricular zone surrounded with ependymal matrix and that nMLF neurons are present beneath the ependyma surrounded by nMLF fibers. Thus, synencephalic GnRH neurons and the nMLF neurons cannot be regarded as dorsal and ventral subnuclei of a single cell mass. Miller and Kriebel (1985) used the term “dorsal tegmental magnocellular nucleus” to distinguish this synencephalic GnRH cell mass from the nMLF.

Moreover, we have obtained, for the first time, unambiguous detailed information on the distribution of fibers immunoreactive for the three different GnRH forms expressed in the brain of a single species, which could provide valuable information on the precise functions of each GnRH form. The sGAP-ir fibers were observed mainly in the forebrain (olfactory bulbs, ventral telencephalon, preoptic area, periventricular hypothalamus, ventral thalamus, pretectum) although a conspicuous sGAP innervation was also observed in the dorsal tegmentum, optic tectum, and ventral rhombencephalon. In turn, the sbGAP-ir fibers were observed only in the ventral surface of the forebrain, associated with the ventral telencephalon, preoptic area, and hypothalamus. Furthermore, cIIGAP-ir axons were profusely distributed in the brain of sea bass, being especially evident in dorsal and ventral telencephalon, periventricular preoptic area and hypothalamus, thalamus, pretectum, posterior tuberculum, mesencephalic tectum and tegmentum, cerebellum, and the rest of the rhombencephalon. By using these specific anti-GAP sera, we have also demonstrated that the sb-GnRH neurons represent the main source of GnRH to the pituitary of sea bass. The sbGAP-ir fibers reach the proximal pars distalis and the border of the pars intermedia of the pituitary, where gonadotrophic cells and GnRH receptors are also found (González-Martínez et al., unpublished data). This result corroborates physiological evidence stating the main role of sbGnRH in the stimulation of the pituitary (B), the presence of a few sGAP-immunoreactive fibers (A), and the absence of cIIGAP immunostaining (C) in the hypophysis of sea bass (white arrowheads). NH, neurohypophysis; PPD, proximal pars distalis of the pituitary. For abbreviations, see list. Scale bars = 400 μm for A–C.

Fig. 6. Serial transverse sections of the sea bass pituitary and hypothalamus immunostained with anti-salmon prepro-gonadotrophin-releasing hormone-associated peptides (sGAP; A), anti-seabream prepro-gonadotrophin-releasing hormone-associated peptides (sbGAP; B), and anti-chicken-II prepro-gonadotrophin-releasing hormone-associated peptide (cIIGAP; C) sera. Darkfield. Note the intense sbGAP innervation of the pituitary (B), the presence of a few sGAP-immunoreactive fibers (A), and the absence of cIIGAP immunostaining (C) in the hypophysis of sea bass (white arrowheads). NH, neurohypophysis; PPD, proximal pars distalis of the pituitary. For abbreviations, see list. Scale bars = 400 μm for A–C.
secretion of gonadotrophins in perciforms (Powell et al., 1994; Zohar et al., 1995; Gothilf et al., 1996, 1997; Yamamoto et al., 1997; Holland et al., 1998; Senthilkumaran et al., 1999). However, sbGnRH does not represent the unique hypophysiotrophic GnRH in sea bass. Immunohistochemical results demonstrate that sGAP-ir axons also reach the pituitary of sea bass but this innervation is strongly reduced as compared to sbGAP immunoreactivity. In contrast, no cIIGAP-ir axons were detected in the pituitary of sea bass, suggesting that the putative role of chicken-II GnRH in the control of reproduction does not involve a direct action of cerebral cGnRH-II on gonadotrophic cells, at least in this species. According to our results, no immunoreactive fibers were detected in the pituitary of catfish by using a specific antibody against cIIGAP (Zandbergen et al., 1995). Thus, the detection of cIIGnRH-ir fibers in the pituitary of sea bass by using an anti-cGnRH-II serum seems to be the consequence of the cross-reactivity of this antibody with sbGnRH and/or sGnRH axons entering the hypophysis.

Recently, Rodriguez et al. (2000) found that all three GnRH forms were present in the pituitary of male sea bass during sex differentiation and first spawning season, being sbGnRH levels 9-fold higher than cGnRH-II and 17-fold higher than sGnRH levels. The contradiction of this finding to our immunohistochemical results could reflect the existence of changes in the presence of cGnRH-II in the sea bass pituitary in relation to sex, developmental, and/or physiological stages. Furthermore, the presence of cGnRH-II in the pituitary of female sea bass could be the consequence of an intrapituitary secretion because, at least in seabream, a local pituitary synthesis of GnRH has been described (Zmora et al., 2000). However, the detection of cGnRH-II in sea bass pituitary by using enzyme-linked immunooassays could also be the result of a cross-reactivity of antisera against the different GnRH peptides, as it was observed in immunohistochemistry.

In dwarf gourami and tilapia, it has been demonstrated by using immunohistochemistry combined with biocytin tract tracing that only preoptic GnRH cells innervate the hypophysis, whereas terminal nerve and midbrain GnRH neurons do not project to the pituitary (Yamamoto et al., 1998a, b). These results are consistent with the lack of changes in the distribution of GnRH fibers in the pituitary after terminal nerve and olfactory tract lesions (Kim et al., 1993; Yamamoto et al., 1995) and the absence of labelled fibers in the pituitary after intracellular biocytin injections into terminal nerve GnRH cells (Oka and Matsushima, 1993). In other fish species, cGnRH-II-ir fibers were not detected in the pituitary despite the presence of cGnRH-II-ir cell bodies in the dorsal synencephalon (Amano et al., 1991; Montero et al., 1994; Millar and King, 1994). In agreement with these results, we have not found cIIGAP-ir axons in the sea bass pituitary. However, sGAP-ir fibers reach the sea bass pituitary, the origin of these axons remaining unsolved. It seems improbable that the projection pattern of GnRH cells markedly differs between perciform species such as tilapia and sea bass. If this is true, preoptic but not olfactory sGAP-ir cells might contribute to this pituitary innervation. However, in the goldfish, small fusiform neurons, distinct from the terminal nerve cells, in the olfactory bulbs and tracts were labelled following DiI injection in the pituitary, suggesting that a minor contingent of GnRH fibers reaching the pituitary may originate from the olfactory system (Anglade et al., 1993). In mammals, the presence of sGnRH in neuronal fibers of the median eminence has been reported, suggesting that sGnRH is transported to the pituitary gland where it may interact with pituitary cells, but sGnRH content in pituitary stalk was reduced in comparison to mGnRH levels (Yahalom et al., 1999). A comparable result was obtained in sea bass, in which we noted higher sbGAP fiber immunoreactivity compared to sGAP innervation in the pituitary.

Should the presence of sGnRH be confirmed in higher vertebrates, this could mean that sGnRH and cGnRH-II have been submitted to a strong evolutionary pressure, in contrast with the gonadotrophin-releasing GnRH form (lamprey GnRH-III, dogfish GnRH, catfish GnRH, seabream GnRH, herring GnRH, medaka GnRH, chicken GnRH-I, guinea pig GnRH, mammalian GnRH) which exhibits considerable variation among vertebrates. This selective pressure on sGnRH and cGnRH-II structure reinforces the assumption that these neurohormones could deserve important functions in brain other than reproduction (e.g., neurotransmitter and/or neuromodulator). In this context, our recent ontogenic studies in sea bass reveal that cGnRH-II and sGnRH are expressed very early during development, much before the differentiation of gonadotrophic cells and gonads, whereas sbGnRH is expressed much later (González-Martínez et al., 2002). In turn, the diversity of hypophysiotrophic GnRHs might indicate a specialization of this GnRH towards a single function, such as the stimulation of gonadotrophin secretion. In fact, the sbGAP-ir fibers in sea bass can only be detected in the ventral forebrain running to the pituitary, sGAP-ir and cIIGAP-ir fibers being profusely distributed in other brain areas. It has been proposed that sGnRH might coordinate the sensory and motivational systems, cGnRH-II might modify motor reproductive activity, whereas sbGnRH provoked the release of gonadotrophins (White et al., 1995). The results obtained in this study might support these considerations because sGAP-ir fibers were abundant in visual and gustatory sensory areas; cIIGAP-ir fibers represented the only ir fibers detected in the cerebellum and innervated strongly the medulla oblongata and spinal cord, whereas sbGAP-ir axons constituted the main hypophysiotropic source of GnRH. Nevertheless, the presence of sGAP-ir fibers in the pars distalis of the sea bass pituitary suggests a direct effect of sGnRH on adenohypophysal functions. These effects could be exerted directly on gonadotrophic cells to regulate the gonadotrophin secretion and reproductive process. However, a direct action of sGnRH on other sea bass pituitary cells can not be neglected because sGnRH was reported to induce the release of growth hormone (Marchant et al., 1989) and prolactin (Weber et al., 1997) in teleost.

In sea bass, a previous immunohistochemical study showed the presence of varicos nerve GnRH-ir fibers contacting and surrounding GnRH-ir cell bodies and dendrites, suggesting the existence of connections between different GnRH cell groups (Kah et al., 1991). A similar observation is reported in this study, in which sGnRH and sbGnRH cells and fibers coexist in the same brain areas and seem to establish contacts. This is especially evident in the olfactory bulbs of sea bass, where varicose sbGAP-ir fibers appear surrounding the large perykaria of sGnRH cells. In mammals, the presence of GnRH endings synapsing on GnRH perykaria has been well documented (Leranth et al., 1985; Witkin and Silverman, 1985; Witkin, 1999). Although ultrastructural studies are needed before con-
including as for the existence of synaptic connections between different GnRH cells in sea bass brain, these observations may support this assumption. Whether such interactions represent some way of influencing the activity of other GnRH cells, or synchronizing the different GnRH cell populations, is not known. Nevertheless, the overlapping in the expression of sGnRH and sbGnRH cells in the same regions, including the preoptic area, the co-existence of sGAP-ir and sbGAP-ir fibers in the proximal pars distalis of the sea bass pituitary, and the putative existence of direct interactions between both GnRH cell populations, suggest that sGnRH and sbGnRH could cooperate closely in the synchronization of developmental and/or reproductive events.

Surprisingly, the hypophysiotrophic GnRH form (sb-GnRH) of perciforms is less potent than cGnRH-II and sGnRH in inducing gonadotrophin (Zohar et al., 1995) or prolactin (Weber et al., 1997) secretion. However, the high levels of sbGnRH in the pituitary may compensate for its lower bioactivity. Furthermore, its reduced effectiveness on gonadotrophin secretion could also have a physiological relevance by contributing to a significant response of pituitary to GnRH when its levels increase in a significant manner. Thus, the lower activity of sbGnRH could permit the release of eggs in batches during an extended period of time because only fully mature eggs ovulate in response to sbGnRH stimulation. The lower potency of sbGnRH may suggest a faster enzymatic breakdown and/or more rapid clearance from the circulation. Also, this evidence could be the consequence of a more recent emergence of sbGnRH and a lower affinity of this form for the pituitary GnRH receptor, which might have evolved more slowly that its ligand. In fact, GnRH receptors are very similar in eels and sea bass (80% of identity) despite the important phylogenetic distance between these two species (Okubo et al., 2000b, T. Madigou, E. Mañanos and O. Kah, unpublished data). The existence of at least three GnRHs in a particular species leads to the emerging concept of the existence of multiple GnRH receptors. Thus, two different GnRH receptor subtypes are expressed in goldfish (Illing et al., 1998), and three distinct GnRH receptors are present in several vertebrate classes (Trojskje et al., 1998; Wang et al., 2001). The elucidation of the relative potency of different GnRH forms to various GnRH receptors could be critical to understand their functions. Nevertheless, precise data on the localization of fibers containing GnRH variants compared to the distribution of GnRH receptors also represent a crucial information, because the availability of a given GnRH to a given receptor is determinant for their real physiologically relevant interactions. In this context, the GnRH receptor subtype 1 of bullfrog is expressed predominantly in the pituitary, whereas GnRH receptor subtypes 2 and 3 are expressed in the forebrain and hindbrain (Wang et al., 2001). Furthermore, it is relevant to mention that a recently cloned GnRH receptor from trout is poorly expressed in the pituitary, whereas a strong expression was detected in particular brain nuclei (Madigou et al., 2000). Current studies in sea bass aim at determining how many GnRH receptors are expressed and at clarifying their respective distributions in the brain and pituitary of this species. As we now have information on the pattern of projections of the three different GnRH forms expressed in sea bass, such information could greatly contribute to improving the knowledge on the precise roles of multiple GnRH forms and receptors in the control of reproduction and other physiological processes.

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LITERATURE CITED


GnRHs IN THE BRAIN AND PITUITARY OF SEA BASS


