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REGULAR ARTICLE

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GnRH systems of *Cichlasoma dimerus* (Perciformes, Cichlidae) revisited: a localization study with antibodies and riboprobes to GnRH-associated peptides

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Abstract The distribution of cells that express three prepro-gonadotropin-releasing hormones (GnRH), corresponding to salmon GnRH, sea bream GnRH (sbGnRH), and chicken II GnRH, was studied in the brain and pituitary of the South American cichlid fish, *Cichlasoma dimerus*. Although the ontogeny and distribution of GnRH neuronal systems have previously been examined immunohistochemically with antibodies and antisera against the various GnRH decapeptides, we have used antisera against various perciform Gn RH-associated peptides (GAPs) and riboprobes to various perciform GnRH+GAPs. The results demonstrate that: (1) the GnRH neuronal populations in the forebrain (salmon and sea bream GAPs; sGAP and sbGAP, respectively) show an overlapping pattern along the olfactory bulbs, nucleus olfacto-retinalis, ventral telencephalon, and preoptic area; (2) projections with sGAP are mainly located in the forebrain and contribute to the pituitary innervation, with projections containing chicken GAP II being mainly distributed along the mid and hindbrain and not contributing to pituitary innervation, whereas sbGAP projections are restricted to the ventral forebrain, being the most important molecular form in relation to pituitary innervation; (3) sbGnRH (GnRH I) neurons have an olfactory origin; (4) GAP antibodies and GAP riboprobes are valuable tools for the study of various GnRH systems, by avoiding the cross-reactivity problems that occur when using GnRH antibodies and Gn RH riboprobes alone.

Keywords GAP · Brain · GnRH · Cichlids · *Cichlasoma dimerus* (Telestei)

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Introduction

Gonadotropin-releasing hormone (GnRH) is a decapeptide that is synthesized primarily by neurons within the central nervous system. Its principal biological function is to induce the release of gonadotropins (follicle-stimulating hormone and luteinizing hormone) into the bloodstream, but it is also thought to act as a neuromodulator and/or neurotransmitter (Muske 1993; Eisthen et al. 2000). In addition, evidence suggests that GnRH plays a role in the release of other pituitary hormones, such as prolactin, growth hormone, and somatolactin (Marchant et al. 1989; Parhar 1997; Weber et al. 1997; Stefano et al. 1999; Vissio et al. 1999).

To date, 14 structurally distinct forms of GnRH have been identified in vertebrates (Adams et al. 2002), and many species express more than one form. In particular, several species of perciform teleosts express three different forms of GnRH in their brains: sea bream GnRH (sbGnRH or Gn RH I), chicken II GnRH (cGnRH II or GnRH II), and salmon

GnRH (sGnRH or GnRH III; Powell et al. 1994). The expression of three different GnRH forms has also been described in Characiformes (Powell et al. 1997), Clupeiformes (Carolsfeld et al. 2000), Pleuronectiformes (Andersson et al. 2001), Scorpaeniformes (Collins et al. 2001), primitive Salmoniformes (Adams et al. 2002), Synbranchiformes, Atheriniformes, and Cyprynidontiformes (Somoza et al. 2002), suggesting that this pattern of diversity is prevalent in most teleost groups. In perciforms, immunoreactive (ir)-sbGnRH fibers are abundant in the pituitary (Powell et al. 1994; Okuzawa et al. 1997), reflecting the primary hypophysiotropic role of sbGnRH, and there is a strong correlation between sbGnRH expression in the brain and gonadal activity (Senthilkumaran et al. 1999). In contrast, much less is known about the role(s) of sGnRH and cGnRH II. In particular, their brain targets and their relationships with pituitary endocrine cells remain to be determined.

The distribution patterns of sbGnRH, cGnRH II, and sGnRH in the central nervous system of various perciform species (Gothlif et al. 1996; White and Fernald 1998; Senthilkumaran et al. 1999; Pandolfi et al. 2002) suggest the existence of three distinct neuronal populations: sGnRH in the olfactory bulbs (OB), sbGnRH predominantly expressed in the preoptic area (POA), and cGnRH II in the midbrain tegmentum. Based upon these observations and on the findings from ontogenetic studies, it has been proposed that, in perciforms, sGnRH neurons originate from the olfactory placode, cGnRH II neurons originate from a mesencephalic primordium, and two possible origins have been suggested for sbGnRH neurons: the diencephalic floor or the olfactory placode (Parhar 1997; Parhar et al. 1998; Ookura et al. 1999; González-Martínez et al. 2002b; Pandolfi et al. 2002). However, the "diencephalic floor/POA hypothesis" as the origin of sbGnRH is different from that described in other vertebrate groups in which GnRH forebrain perikarya (GnRH I and III) extend in a scattered continuum, from the terminal nerve to the anterior hypothalamus, with some isolated neurons in the OBs (Silverman et al. 1994; Muske and Moore 1998; González-Martínez et al. 2001). Ontogenetic studies in amphibian, avian, and mammalian species are also in contradiction with the "diencephalic floor/POA hypothesis", because all forebrain GnRH neurons ranging from the OB to the hypothalamus seem to have a similar developmental origin from the olfactory placode (Schwanzel-Fukuda and Paff 1989; Wray et al. 1989a; Muske 1993; Norgen and Gao 1994; Schwanzel-Fukuda 1999).

In the perciform European sea bass, *Dicentrarchus labrax*, the distribution of sGnRH- and sbGnRH-expressing cells overlaps along the ventral forebrain (González-Martínez et al. 2001, 2002a), giving support to the idea that both sbGnRH (GnRH I) and sGnRH (GnRH III) originate from olfactory primordia (González-Martínez et al. 2002b). Interestingly, these studies were performed with antibodies and riboprobes for the sbGnRH-associated peptides (GAPs), which give much greater specificity than can be achieved by using antibodies and riboprobes to the smaller GnRH molecules themselves. Furthermore, GAPs represent useful markers of various GnRH neurons because they are produced from GnRH precursor molecules and are co-localized within GnRH-expressing cells (Ronchi et al. 1992; Polkowska and Przekop 1993; González-Martínez et al. 2002a). More importantly, markedly different GAPs are associated with different GnRHs. Therefore, antibodies and riboprobes to GAPs can be utilized in immunohistochemistry and in situ hybridization (ISH), respectively.

In the present study, we have studied the South American cichlid fish, *Cichlasoma dimerus*, in which the ontogeny and distribution of GnRH neuronal systems has previously been described by using antisera raised against various GnRH decapeptides (Pandolfi et al. 2002). We have used antibodies against a variety of perciform GAPs and riboprobes to different perciform GnRH+GAPs, in order: (1) to determine whether the different GnRH systems in the forebrain appear segregated or show an overlapped distribution, (2) to examine the projections from the various GnRH systems to the pituitary and to other regions of the brain, (3) to analyze the developmental origin of sbGnRH (GnRH I) neurons, and (4) to corroborate that GAP antibodies and riboprobes are valuable tools for the study of the various GnRH systems in closely related species, by avoiding the cross reactivity problems that appear when using GnRH antibodies and riboprobes alone.

Materials and methods

Animals

Fifty-nine adult fish (30 males and 29 females between 1 and 2 years old) were laboratory-reared descendants of specimens captured in Esteros del Riachuelo, Corrientes, Argentina (27°25′ S 58°15′ W). They were kept at 27±1°C, under a 12:12 h photoperiod, in large well-aerated aquaria with external filtration. The fish were fed with *Artemia* sp. larvae and commercial pellets. For developmental immunohistochemical and ISH studies, daily samples were obtained from hatching to day 15 after hatching (ah), and every 3 days from day 15 ah (6 mm length) to day 60 ah (17 mm length). Hatching took place 52 h after fertilization. At each stage, at least eight animals were sampled. Principles of laboratory animal care were followed (NIH publication no. 86–23, revised in 1985).

Western blot analysis

In order to test the specificity of *D. labrax* (sea bass) antisera against salmon GAP (sGAP), sea bream GAP (sbGAP), and chicken II GAP (cIIGAP) in the brain and pituitary of *C. dimerus*, we performed an analysis with 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot.

Fifteen animals were anesthetized with 0.1% benzocaine and killed by decapitation. Six brains and 15 pituitaries were, respectively, homogenized in 1 ml and 700 μl 50 mM TRIS–HCl buffer, pH 7.4, with 10 μl protease inhibitor cocktail (Sigma, St Louis, Mo.) and centrifuged at 6,000*g* for 30

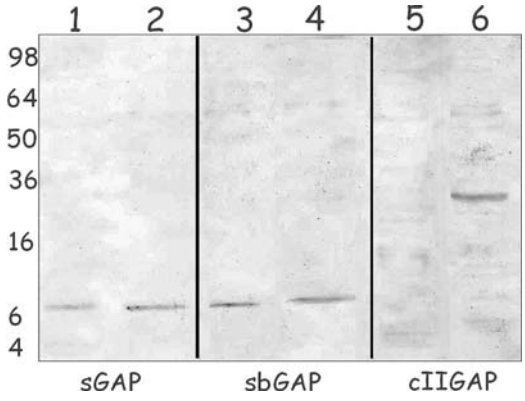

Fig. 1 Western blot analysis using brain (*lanes 2, 4* and *6*) and pituitary (*lanes 1, 3* and *5*) homogenates incubated with anti-sGAP (*lanes 1–2*), anti-sbGAP (*lanes 3–4*), and anti-cII-GAP (*lanes 5–6*). For anti-sGAP, a 7.5 kDa band was detected in the brain and pituitary; for anti-sbGAP, a 7.5 kDa band was detected both in brain and pituitary, and for anti-c-IIGAP a 32 kDa band was detected only in brain. *c-IIGAP* Chicken-II GAP, *GAP* prepro-gonadotropin-releasing hormone-associated peptides, *sGAP* salmon GAP, *sbGAP* seabream GAP

min at 4°C. Homogenate (5 μl)and 5 μl 5× sample buffer (120 mM TRIS–HCl pH 6.8, 3% dodecylsulfate, 10% glycerol or 2% glycerol, 1% β-mercaptoethanol) were heated at 95°C for 5 min before being loaded onto the 15% SDS-PAGE.

After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, UK) for 45 min at 4°C and 75 V. The membranes were washed in TBST pH 7.5 (100 mM TRIS–HCl, 0.9% NaCl, 0.1% Tween-20), blocked with TBST containing 5% nonfat dry milk for 1 h, and then incubated for 3 h at room temperature with primary antisera raised in guinea pig: anti-sea bass sGAP, anti-sea bass sbGAP, and anti-sea bass cIIGAP (González-Martínez et al. 2002a) diluted 1:2,000 in TBST. Control lanes of pituitary and brain homogenates were set up by replacing the primary antisera with TBST.

After three washes for 10 min in TBST, the membranes were incubated for 45 min with a biotinilated anti-guinea pig IgG (Vector Lab, Burlingame, Calif.) diluted 1:1,000, washed again, and incubated for 45 min in the dark with alkaline phosphatase (AP)-conjugated streptavidin (Dako, Carpinteria, Calif.) diluted 1:1,000. After the membranes had been washed, immunoreactivity was revealed with an AP-conjugate substrate kit (Bio-Rad, Hercules, Calif.). Finally, the membranes were dried and scanned, and the molecular weights were estimated by using Image Gauge software (Fuji, Japan).

Western blot analysis revealed a 7.5-kDa band corresponding to sGAP in brain and pituitary, a 7.5-kDa band corresponding to sbGAP both in brain and pituitary, and a 32-kDa band corresponding to cIIGAP in brain (Fig. 1). There were no immunoreactive bands for cIIGAP in the pituitary (Fig. 1).

Tissue processing

Forty-four adult fish and 240 larvae were anesthetized with 0.1% benzocaine and killed by decapitation. The brains with the attached pituitary (adults) and whole heads (larvae) were fixed in Bouin's solution for 24 h at 4°C. Samples where dehydrated and embedded in Paraplast (Fisherbrand, Fisher, Wash.). For immunohistochemistry, 24 animals were completely sectioned coronally at 10-μm intervals, and five others were sectioned sagitally. For isotopic ISH, the remaining 15 animals were sectioned coronally at 15-μm intervals. Various larval stages were sectioned coronally and sagitally for immunohistochemistry and ISH. Sections obtained were mounted on charged slides (Fisherbrand Superfrost/Plus, Fisher, Wash.).

Single-label immunohistochemistry

The following procedures were carried out at room temperature. Sections were deparaffinized in xylene, rehydrated through a graded ethanol series to phosphate-buffered saline (PBS, pH 7.4), treated for 15 min with a 0.75% gelatine solution and for 30 min with PBS containing 5% nonfat dry milk, incubated with a 1:500 dilution of primary guinea pig anti-sea bass sGAP, sbGAP, or cIIGAP antiserum for 16 h, washed in PBS, and finally incubated for 45 min in biotinilated anti-guinea pig IgG diluted 1:1,000. Amplification of the signal was achieved by incubating the sections with peroxidase-conjugated streptavidin (Dako) diluted 1:800 for

Table 1 Average diameter and number of immunoreactive GAP neurons in various brain zones. The brains of adult animals from the reproductive period September–March were entirely sectioned at 10 μm without losing any sections. The values expressed represent the average cell diameter (μm) ± standard error, and the average number of cells ± standard error for each cell population (*OB* olfactory bulbs, *NOR* nucleus olfacto-retinalis, *vTEL* ventral telencephalon, *POA* preoptic area, *MB* midbrain, *NE* no expression, *sGAP* salmon GAP, *sbGAP* sea bream GAP, *cIIGAP* chicken II GAP)

Brain zone	sGAP (n=8)		sbGAP (n=8)		cIIGAP (n=8)	
	Average cell diameter (μm)	Average number of cells	Average cell diameter (μm)	Average number of cells	Average cell diameter (μm)	Average number of cells
OB	16.6±2.4	8.0±1.6	4.3±1.2	4.0±1.0	NE	NE
NOR	20.5±4	115.0±10.7	6.7±2.8	7.5±2.2	NE	NE
vTel	6±1.7	21.3±6.7	15.6±3.4	30.5±7.7	NE	NE
POA	5.7±1.6	20.2±5.1	17±6.5	41±3.5	NE	NE
MB	NE	NE	NE	NE	21.2±3.7	20.6±4.9

1 h. After three washes in PBS, peroxidase activity was visualized with 0.1% 3,3′-diaminobenzidine in TRIS buffer (pH 7.6) and 0.03% H_2O_2. Sections were lightly counterstained with hematoxylin, mounted, examined with a NIKON microphot FX microscope, and digitally photographed (Coolpix 4500, Nikon).

To confirm the specificity of the immunostaining, control sections were incubated with the primary antisera preadsorbed with an excess of its respective antigen. To avoid false positives caused by the immunohistochemistry itself, replacement of primary antisera with PBS and omission of secondary antisera were also performed.

For the precise location of the various GAP cells and projections, we relied on the detailed atlases of two other perciform species, *D. labrax* (Cerdá-Reverter et al. 2001a,b) and *Haplochromis burtoni* (Fernald and Shelton 1985), and on an atlas currently being constructed for *C. dimerus* (M. Pandolfi et al., in preparation). In eight adult serially sectioned animals, the total number of immunoreactive GAP (ir-GAP) cells was counted, and 20 cells were randomly selected for measurement in order to determine the average cell diameter. As brains were sectioned at 10-μm intervals, cells larger than 10 μm were counted only if their nucleus was clearly visible (Table 1).

Double-label immunohistochemistry

In order to verify that sea bass GAP was a reliable marker of *C. dimerus* GnRH-producing cells, double-label immunofluorescence staining was performed by incubating the sections concomitantly with an anti-LRH13 (1:5,000 monoclonal antibody that recognized the three GnRH forms described in *C. dimerus* (donated by Dr. Wakabayashi, Uni-

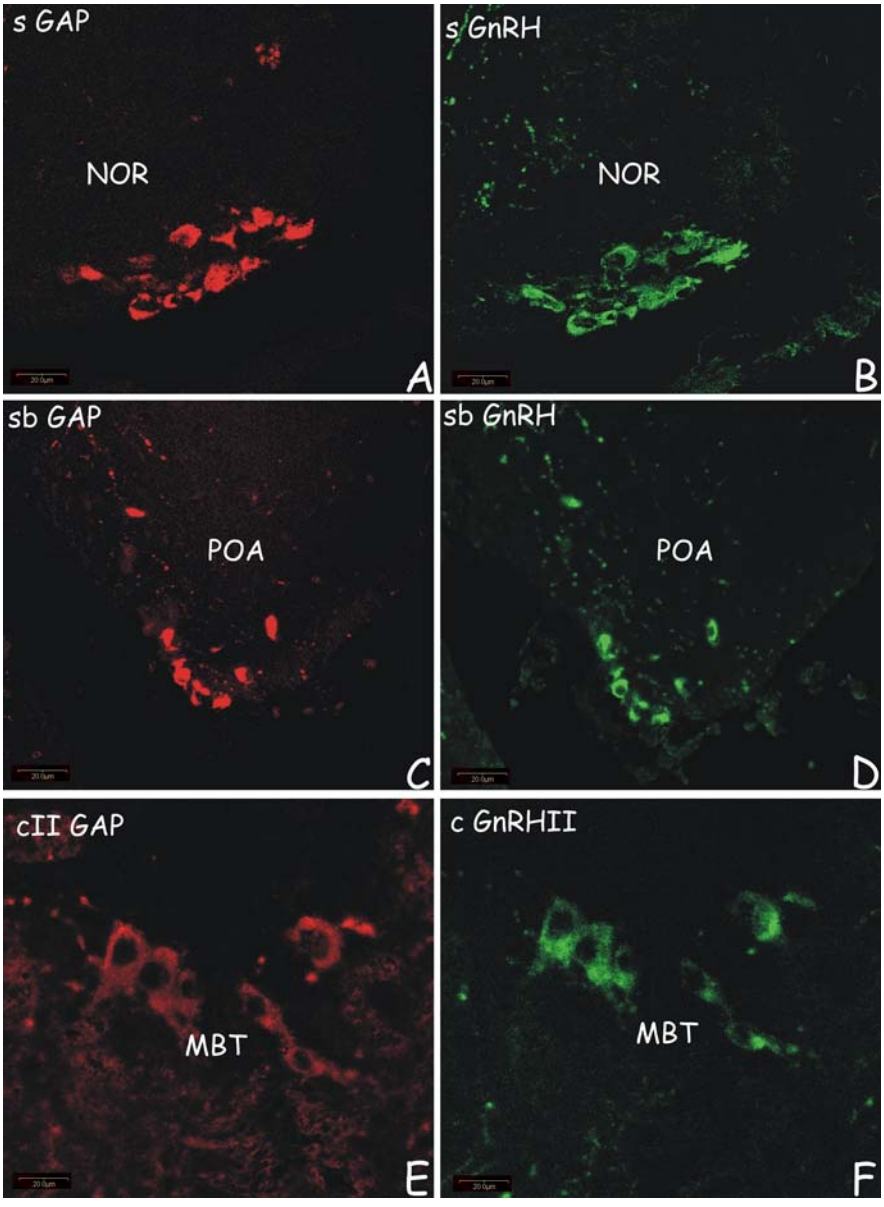

Fig. 2 Coexpression of prepro-gonadotropin-releasing hormone-associated peptides (*GAPs*, *left column*) and gonadotropin-releasing hormones (*GnRHs*, *right column*) on the same cells by using immunofluorescence methods. Compare **a** with **b**, **c** with **d**, and **e** with **f**. **a** Coronal section at the NOR level (sea bass anti-sGAP) (Rodamine). **b** Same coronal section processed with a monoclonal GnRH antibody (fluorescein isothiocyanate). **c** Coronal section at the POA level processed in the same way as **a** but using a sea bass anti-sbGAP. **d** Same coronal section processed as in **b**. **e** Coronal section at the MBT level processed in the same way as in **a** and **c** but using a sea bass anti-cIIGAP. ×400. **f** Same section processed as in **b** and **d**. *Scale bars*=20 μm. *c-IIGAP* Chicken-II GAP, *MBT* midbrain tegmentum, *NOR* nucelus olfacto retinalis, *POA* preoptic area, *sGAP* salmon GAP, *sbGAP* seabream GAP

versity of Gunma, Maebaeshi, Japan), and the corresponding GAP (1:500) in its most expressed brain area, i.e., LRH13+anti-sGAP in the nucleus olfacto-retinalis (NOR), LRH13+anti-sbGAP in the parvocellular preoptic nucleus of the POA, and LRH13+anti-cIIGAP in the dorsal midbrain tegmentum.

The ir-GnRH cells and fibers were revealed by using an antibody against mouse IgG coupled to fluorescein isothiocyanate (Sigma). The ir-GAP cells and fibers were revealed by a biotinilated anti-guinea pig IgG (Vector Lab) followed by a streptavidin coupled with Texas red (Vector Lab). Immunohistochemical sections were analyzed with a confocal microscope Olympus FV300 coupled to Fluoview Software.

Results from the double-label immunofluorescence confirmed that the various GAPs colocalized with the GnRH molecular variants in *C. dimerus* brain (Fig. 2).

Riboprobe synthesis and ISH histochemistry

Two ^{35}S-labeled antisense riboprobes were transcribed from *H. burtoni* complementary DNA that encoded the sGnRH/sGAP precursor or the sbGnRH/sbGAP precursor. The cDNAs were kindly donated by Dr. Russell Fernald (Stanford University, Stanford, Calif.). The riboprobes were previously characterized in the African cichlid, *H. burtoni* (White and Fernald 1998). ISH was performed on 15-μm-thick coronal serial or adjacent sections from ten adult animals and 27 larvae of various developmental stages. The sections were deparaffinized in xylene, rehydrated through a graded ethanol series to PBS (pH 7.4), postfixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4) for 15 min, rinsed in TRIS–ethylenediamine tetraacetate (EDTA), digested with proteinase K (10 μg/ml) in TRIS–EDTA buffer (pH 8.0; 100 mmol/l TRIS, 50 mmol/l EDTA) for 8 min, acetylated, dehydrated with ascending concentrations of ethanol, and dried under vacuum for 2 h. They were then hybridized for 18 h at 65°C with 100 μl ^{35}S-labeled antisense ripoprobe diluted to 1×10^{6} cpm/ml hybridization buffer (50 mmol/l dithiothreitol, 250 μg/ml transfer RNA, 50% formamide, 0.3 mol/l sodium chloride, 1× Denhardt's solution, 20 mmol/l TRIS pH 8.0, 1 mmol/l EDTA, and 10% dextran sulfate) under glass coverslips were affixed to the slides by using DPX mounting medium (BDH Laboratory Supplies, Poole, UK). The posthybridization procedure involved removing the coverslips after 2×30-min soakings in 4× SSC (saline–sodium citrate buffer; the 20× stock SSC solution comprised 175.3 g sodium chloride,

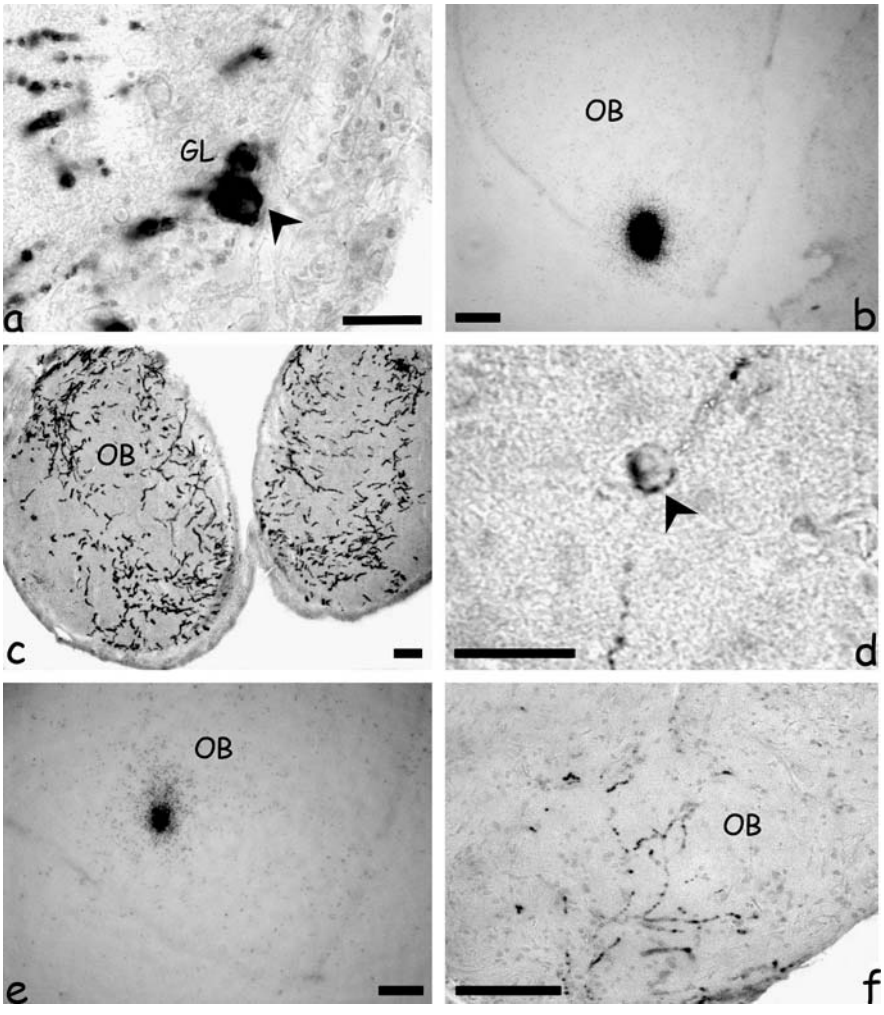

Fig. 3 Distribution of salmon and seabream prepro-gonadotropin-releasing hormone associated peptides (sGAP and sbGAP, respectively) cells and fibers in coronal sections at the OB level. **a** sGAP-ir isolated cell (*arrowhead*) in the GL of the OB. **b** sGnRH+GAP mRNA-expressing cells in the OB. **c** sGAP-ir fibers within the OB. **d** sbGAP-ir isolated cell (*arrowhead*) in the GL of the OB. **e** sbGnRH+GAP mRNA expressing cell in the OB. **f** sbGAP fibers located at ventral and central zones of the OB. *Scale bars*=20 μm. *GL* Glomerular layer, *-ir* immunoreactive, *OB* olfactory bulbs, *sGnRH* salmon gonadotropin-releasing hormone, *sbGnRH* sea bream gonadotropin-releasing hormone

88.2 g sodium citrate per liter, pH 7.0) containing 20 mmol/l dithiothreitol. The sections were then incubated in TRIS-EDTA buffer (pH 8.0; 10 mmol/l TRIS, 1 mmol/l EDTA, 0.5 mol/l sodium chloride) containing ribonuclease A (10 μg/ml) for 30 min at 37°C, followed by 2×30-min washes at room temperature with 2× SSC containing 1 mmol/l dithiothreitol. After a final 30-min wash at 70°C with 0.1× SSC containing 1 mmol/l dithiothreitol, the sections where dehydrated through ascending concentrations of ethanol containing 0.3 mol/l ammonium acetate and then air-dried for 30 min. To visualize the hybridization pattern, the sections were apposed to Hyperfilm β-max for 3 days (adults) or 5 days (larvae), dehydrated in increasing concentrations of ethanol, defatted in xylene for 1 h, dipped in photographic emulsion (NTB-2, Eastman Kodak, Rochester, N.Y.), exposed at 4°C in a light-tight box for 14 days (adults) or 18 days (larvae), processed with Kodak developer (D-19) and fixer, dehydrated with ethanol, cleared with xylene, and finally coverlipped by using DPX mounting medium.

As a negative control, ISH was also performed on some sections by using ^{35}S-labeled sGnRH+GAP and sbGnRH+GAP sense riboprobes.

Results

Figure 1 summarizes the results obtained by Western blot to test the specificity of sea bass anti-sGAP, anti-sbGAP, and anti-cIIGAP sera in *C. dimerus* brain and pituitary. This specificity can also be inferred by the absence of immunostaining both in Western blot and immunohistochemistry when primary antibodies were incubated with an excess of sea bass GAP antigens (data not shown).

Figure 2 demonstrates that ir-GAP cells also express GnRH peptides in *C. dimerus*. The principal cell masses are immunostained for the different GnRH forms. This coexpression is also seen in smaller ir-GAP cell populations and in the axonal pathways. All of the ir-sGnRH and ir-sbGnRH cell bodies express their corresponding GAP. However, a few of the ir-cGnRH II cells in the midbrain tegmentum do not appear to express cIIGAP (data not shown).

The number of positive cells and the average cell diameter for each antiserum was quantified in the various brain nuclei in entirely sectioned adult animals (Table 1). These results were consistent from one fish to the other, and no differences could be detected between males and females.

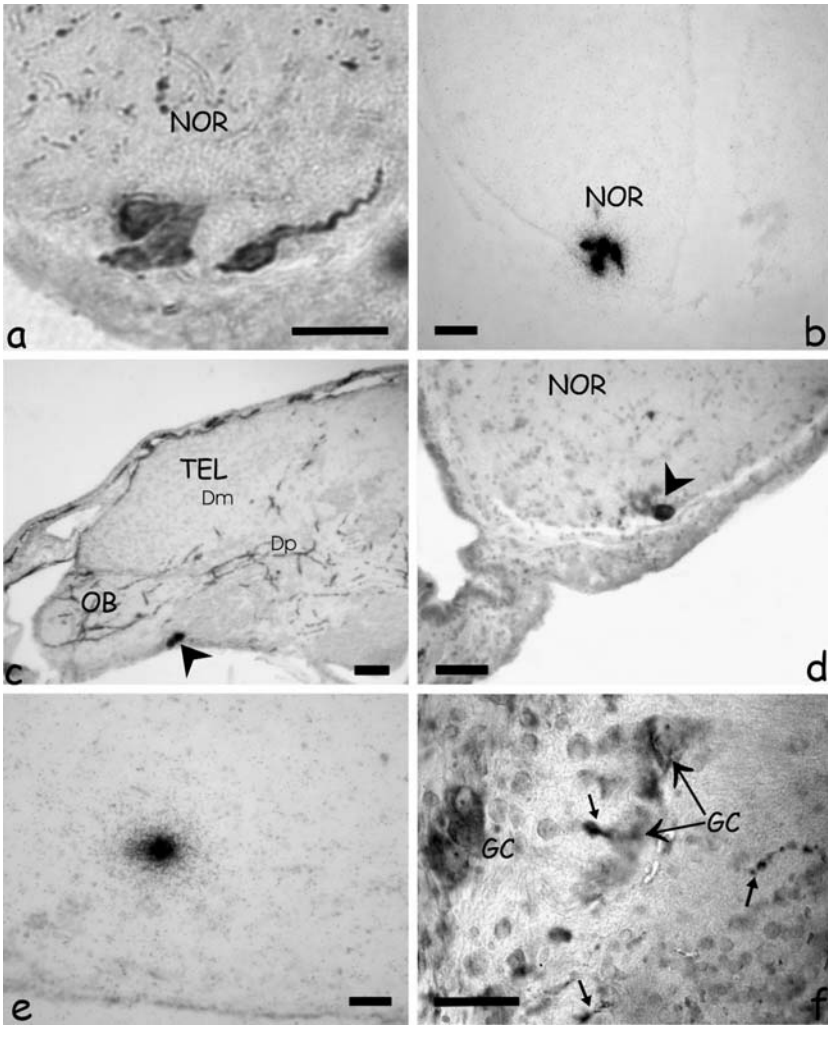

Fig. 4 Distribution of salmon and seabream prepro-gonadotropin-releasing hormone associated peptides (sGAP and sbGAP, respectively) cells and fibers at the NOR. **a** Cluster of sGAP-ir cells located at the NOR. Coronal section. **b** Coronal section at the NOR level hybridized with the sGAP antisense. **c** Sagittal section showing the innervation of the OB, NOR, and TEL by sGAP-ir fibers. *Arrowhead* indicates part of the salmon GAP-ir cell bodies in the NOR. **d** Ir-sbGAP isolated cell in the NOR. Coronal section. **e** Sagittal section at the NOR level hybridized with the sbGAP antisense. **f** Sagittal section at the NOR level showing scarce and delicate sbGAP-ir fibers (*small arrows*) in the vicinity of GC (counterstained with toluidine blue) which express sGAP. *Scale bars*=20 μm. *Dm* Medial part of the dorsal telecenephalon, *Dp* posterior part of the dorsal telencephalon, *-ir* immunoreactive, *GC* ganglionar cells, *NOR* nucleus olfacto retinalis, *OB* olfactory bulbs, *TEL* telencephalon

In terms of specificity, *H. burtoni* riboprobes recognize sGnRH+GAP and sbGnRH+GAP with no cross-reaction. This assumption is based on the finding that the oligonucleotide sequences of sGAP vs sbGAP are markedly different in all the species in which they have been sequenced. Moreover, each probe and its corresponding sense control have been systematically applied to parallel sections, the latter showing an extremely low and uniform background. Furthermore, different cell masses are recognized by each probe. In brain zones in which both sGAP and sbGAP cells can be detected, neuronal morphology and mRNA expression levels are also markedly different. Moreover, the ontogenetic appearance of each molecular form occurs at different developmental stages reinforcing this assumption of specificity.

Olfactory bulbs

Cell bodies immunoreactive for the anti-sGAP serum were detected in the glomerular layer (GL) of the OBs. Few detectable neurons were present. The immunoreactive cells were medium-sized and rounded and showed high sGAP mRNA expression (Fig. 3a, b). A strong innervation of sGAP fibers could be observed along the concentric layers of the mid-caudal zones of the OBs (Fig. 3c).

The population of ir-sbGAP cells in the OBs was small. These cells, which were located in the GL, were small in size and exhibited a lower GAP mRNA expression than the sGAP cells from the same area (Fig. 3d, e). A few ir-sbGAP fibers were detected in ventral and central zones of the OBs (Fig. 3f).

Nucleus olfacto-retinalis

The most intense immunoreactivity and mRNA expression for sGAP occurred in the NOR neurons that lay at the junction between the OBs and the ventral telencephalon (vTel). This cluster of large cells represented the largest population of ir-sGAP neurons. The immunoreactive cells were round and contained a large clear nucleus (Fig. 4a, b). sGAP fibers were observed in the OBs and the NOR and also in the vTel and dorsal telencephalon (dTel; Fig. 4c).

A few immunoreactive sbGAP cells exhibiting weak mRNA expression were also detected in the NOR (Fig. 4d, e). They were smaller in size than the ir-sGAP ganglion cells (6.7 sbGAP vs 20.5 μm sGAP) and adopted a more dorsal position (compare Fig. 3a, d). Some scarce ir-sbGAP fibers were observed in the vicinity of the large ganglion cells of the NOR; these were also immunopositive for anti-sGAP (Fig. 4f).

Ventral telencephalon and preoptic area

The ventral part of the vTel contained a moderate number of ir-sGAP cells, which were small and rounded or pyr-

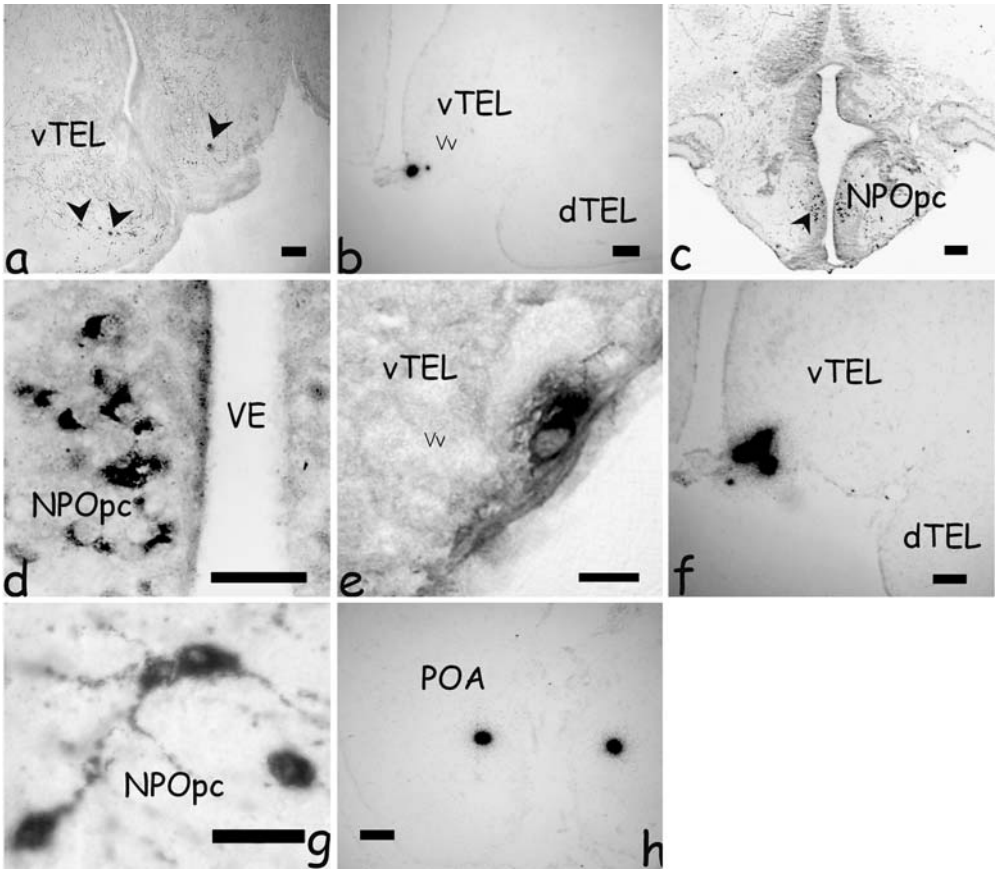

Fig. 5 Distribution of salmon and seabream prepro-gonadotropin-releasing hormone associated peptides (sGAP and sbGAP, respectively) cells and fibers in coronal sections of the vTEL and POA. **a** sGAP-ir cell bodies (*arrowheads*) at the vTEL. **b** Isolated sGAP mRNA-expressing cell in the ventral zone of the vTEL. **c** sGAP-ir cells in the NPOpc. **d** Detail of sGAP-ir cells at the NPOpc. **e** sbGAP-ir cell at the vTEL in a more ventral position than ir-sGAP expressing cells in the same area. **f** Section hybridized with the sbGAP antisense. **g** sbGAP-ir cells at the NPOpc. **h** sbGAP mRNA expressing cells in the rostral POA. *Scale bars*=20 μm. *dTEL* dorsal Telencephalon, *-ir* immunoreactive, *NPOpc* parvocellular part of the parvocellular preoptic nucleus, *POA* preoptic area, *Ve* ventricle, *vTEL* ventral telencephalon, *Vv* ventral part of the ventral telencephalon

amidal. The cell number, cell size, and mRNA expression in this telencephalic sGAP population was markedly reduced in comparison to those of the NOR sGAP group (Fig. 5a, b). Further caudally, a few small ir-sGAP cells were detected in the ventral POA, mainly associated with the parvocellular preoptic nucleus (Fig. 5c, d).

The vTel was also characterized by the presence of ir-sbGAP cell bodies that exhibited a remarkable sbGnRH+ GAP mRNA expression. This population consisted of fusiform, bipolar, and rounded cells that were larger and more numerous than the sGAP cells of the same region (Fig. 5e, f). sbGAP immunoreactivity was more evident in the fusiform, bipolar, and rounded cells that were scatterred throughout the POA, mainly in the parvocellular preoptic nucleus and the anterior periventricular nucleus, in which sbGnRH+ GAP mRNA expression was also detected (Fig. 5g, h). No ir-sbGAP cells were detected in more caudal brain regions of *C. dimerus*.

sGAP and sbGAP axonal pathways

The ir-sGAP fibers were observed mainly in the telencephalon, principally in the GL of the OBs, NOR, vTel and dTel (mainly in the posterior part of the dTel). At the diencephalic level, strong sGAP innervation was detected mainly in the ventral thalamus and parvocellular and magnocellular preoptic nuclei (Fig. 6a). The basal hypothalamus and dorsal tegmentum also received prominent sGAP innervation. The optic tectum showed ir-sGAP fibers reaching the central zone and periventricular gray zone (Fig. 6b). A few fibers were detected ventrally to the corpus of the cerebellum (Fig. 6b) and also at the ventral rhombencephalon. The pituitary gland received only a few ir-sGAP fibers at the neurohypophysis level; these contacted the proximal pars distalis (PPD) and pars intermedia (PI; Fig. 6c). This ir-sGAP innervation was also observed in the retina, in close contact with the bipolar neurons layer (Fig. 6d).

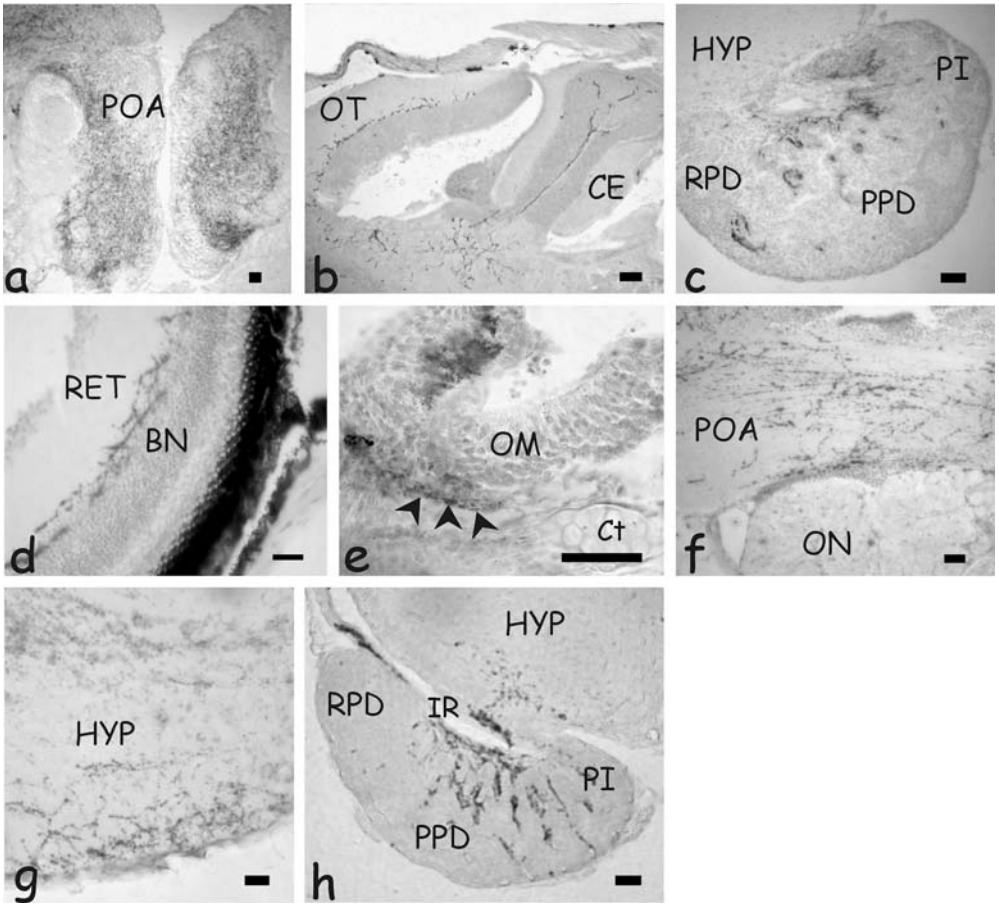

Fig. 6 Distribution of immunoreactive salmon and sea bream preprogonadotropin-releasing hormone associated peptides (sGAP and sbGAP, respectively) fibers along the brain and some extracerebral parts of Cichlasoma dimerus. **a** Strong innervation of the POA by ir-sGAP fibers. Coronal section. **b** Presence of a few ir-sGAP fibers in the OT and the corpus of the CE. Sagittal section. **c** Innervation of the pituitary gland by a short number of small ir-sGAP fibers mainly located at PPD and the external border of the PI. Sagittal section. **d** Presence of ir-sGAP fibers in the eye RET close to the BN. Sagittal section. **e** Presence of ir-sGAP fibers (*arrowheads*) in the lamina propia of the OM. Sagittal section. **f** sbGAP-ir fibers running through the ventral POA. Sagittal section. **g** sbGAP-ir fibers running through the ventral HYP. Sagittal section. **h** Strong innervation of the pituitary gland by sbGAP-ir fibers. Sagittal section. *Scale bars*=20 μm. *BN* Bipolar neuronal layer, *Ct* cartilage, *CE* cerebellum, *-ir* immunoreactive, *IR* infundibular recess, *HYP* Hypothalamus, *OM* olfactory mucosa, *ON* optic nerve, *OT* Optic Tectum, *PI* pars intermedia, *POA* preoptic area, *PPD* proximal pars distalis, *RET* retina, *RPD* rostral pars distalis

Furthermore, the olfactory nerve contained ir-sGAP fibers that extended to the underlying connective tissue of the olfactory epithelium (Fig. 6e).

The ir-sbGAP fibers were confined to the ventral surface of the forebrain, along the OBs, NOR, vTel, POA (Fig. 6f), and ventral hypothalamus (Fig. 6g). These ir-sbGAP fibers contributed strongly to the pituitary innervation by their deep projections into the PPD and the border of the PI (Fig. 6h).

cIIGAP: cell bodies and axonal pathways

cIIGAP-immunoreactive (cIIGAP-ir) cells appeared to be restricted to the dorsal midbrain tegmentum, close to the ventricular surface and were occasionally associated with large blood vessels. This cell population consisted of large neurons, rounded to ovoid in shape (Fig. 7a, b).

The cIIGAP-ir fibers were widely distributed throughout the brain, especially in the midbrain and hindbrain. Some fibers were observed in the forebrain, viz., in the dTel and vTEL, POA, and hypothalamus. A profuse innervation with cIIGAP fibers was observed in the midbrain tegmentum (Fig. 7c). The optic tectum exhibited a more pronounced innervation with cIIGAP fibers than with sGAP fibers (Fig. 7d). The cIIGAP fibers also extended into the cerebellar corpus and valvula (Fig. 7e) and into the medulla oblongata and spinal cord (Fig. 7f). Although cIIGAP-ir fibers were especially abundant in the hypothalamic nucleus lateralis tuberis and in the vicinity of the pituitary stalk, they did not extend into the pituitary gland.

Ontogeny of the sbGAP system

After confirming the overlapping distribution of ir-sbGAP and ir-sGAP cells along the ventral forebrain by GAP immunohistochemistry and GnRH+GAP ISH, we confirmed the origin of sGAP neurons from the olfactory placode and cIIGAP neurons from the mesencephalic primordium, as previously described in this species (Pandolfi et al. 2002).

However, remarkable differences were observed concerning the development of the sbGAP neurons. In contrast to ir-sGAP cells, ir-sbGAP cells or sbGnRH+GAP mRNA were detected neither in the olfactory placode nor in the olfactory mucosa. The first expression of sbGAP was evident in a few cells that were detected along the olfactory nerves at day 18 ah (Fig. 8a). By this stage, the sGAP system had differentiated in the NOR, in which a cluster of immunoreactive cells and a profuse innervation could be observed (Fig. 8b). At day 20 ah, ir-sbGAP cells could be seen in the NOR (Fig. 8c). This sbGnRH+GAP mRNA expression was lower in comparison with the sGnRH+GAP mRNA expression and appeared in cells adopting a more medial position in relation to the sGAP cells (compare adjacent sections in Fig. 8c, d). Overall, the distribution pattern was similar to that observed in the adults.

The vTel population of ir-sbGAP cells was first evident between days 23 and 26 ah (Fig. 8e). Finally, the largest population of ir-sbGAP cells within the POA was detectable in the parvocellular preoptic nucleus between days 25 and 28 ah (Fig. 8f).

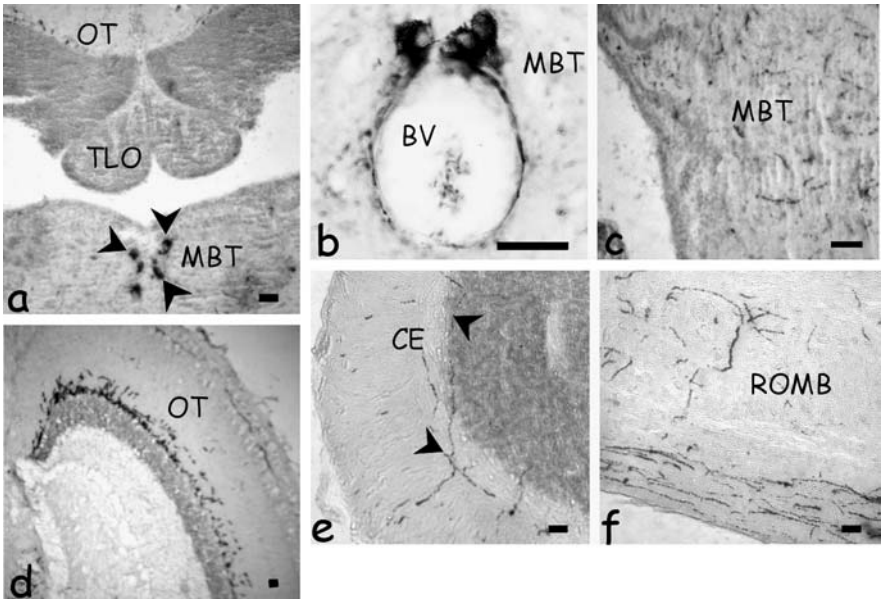

Fig. 7 Distribution of immunoreactive chicken II prepro-gonadotropin-releasing hormone associated peptides (cII-GAP) cells and fibers in *Cichlasoma dimerus* brain. **a** cII-GAP ir-cells (*arrowheads*) in the dorsal MBT. Coronal section. **b** Detail of cII-GAP cells in the MBT associated with BV. Coronal section. **c** Presence of cII-GAP-ir fibers along the MBT. Coronal section. **d** cII-GAP innervation of the OT. Horizontal section. **e** Presence of cII-GAP-ir fibers (*arrowheads*) in the corpus of the CE. Coronal section. **f** Profuse cII-GAP innervation of the ROMB. Sagittal section. *Scale bars*=20 μm. *BV* Blood vessels, *CE* cerebellum, *-ir* immunoreactive, *MBT* midbrain tegmentum, *OT* optic tectum, *ROMB* rombencephalon, *TLO* torus longitudinalis

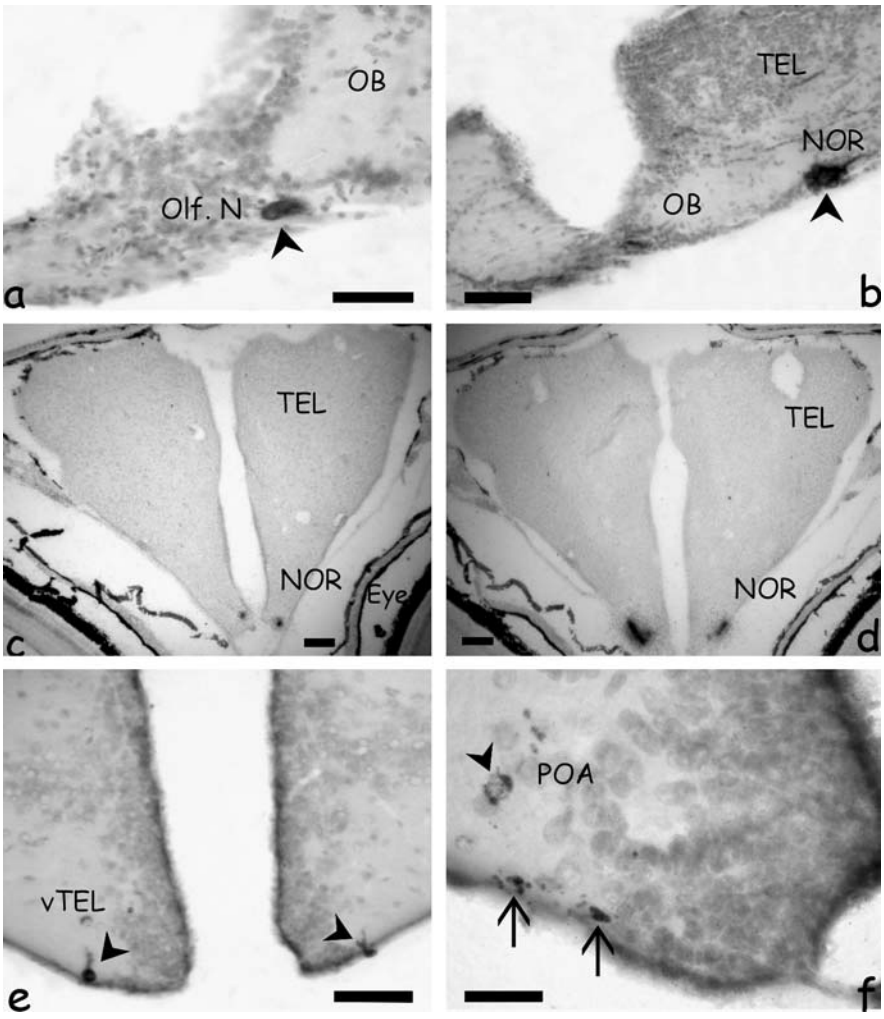

Fig. 8 Ontogeny of the seabream prepro-gonadotropin-releasing hormone associated peptides system (sbGAP). **a** Sagittal section of a day 18 larvae in which an isolated ir-sbGAP cell (*arrowhead*) can be observed in the Olf. N close to the rostral OB. **b** Sagittal section of the same day 18 larvae treated with anti-salmon GAP sera in which the cluster of cells (*arrowhead*) at NOR was already differentiated. **c** Coronal section at the NOR level of a day 20 larvae treated with the sbGAP antisense. **d** Coronal section at the NOR level of the same day 20 larvae treated with the sGAP antisense, showing a stronger mRNA expression. **e** Coronal section of a day 24 larvae in which sbGAP-ir cells (*arrowheads*) were detected at the ventral part of the ventral telencephalon (vTEL). **f** Coronal section of a day 28 larvae showing the presence of sbGAP-ir cells (*arrowhead*) and fibers (*arrows*) at the POA level. *Scale bars*=20 μm. *OB* Olfactory bulbs, *Olf. N* olfactory nerve, *NOR* nucleus olfacto retinalis, *POA* preoptic area, *TEL* Telencephalon

Discussion

The brain distribution pattern of the different prepro-GnRH cell bodies and fibers, based on the expression of the different GAPs, is summarized in Fig. 9. The distribution of the three most conspicuous populations (sGAP in the NOR, sbGAP in the POA, and cIIGAP in the midbrain) is consistent with the distribution previously reported for other perciform species (Powell et al. 1994; White et al. 1995; Gothlif et al. 1996; Okuzawa et al. 1997; White and Fernald 1998; Pandolfi et al. 2002). However, as in the European sea bass *D. labrax* (González-Martínez et al. 2001, 2002a,b), there appears to be an overlapping distribution of prepro-sGnRHs and prepro-sbGnRHs along the ventral forebrain of the South American cichlid fish *C. dimerus*. Moreover, in *C. dimerus*, prepro-sbGnRH cells are first detected in the olfactory region, in which prepro-sGnRH cells have also been found. This early expression of sbGn RH in the olfactory region has also been demonstrated in a perciform species, *D. labrax* (González-Martínez et al. 2002b), but not in those perciform species in which the "diencephalic floor/POA hypothesis" has been proposed to explain the origin of sbGnRH (GnRH I) neurons (Parhar 1997; Ookura et al. 1999).

Although we have used heterologus antisera (*D. labrax*) and riboprobes (*H. burtoni*) in order to localize the various *C. dimerus* prepro-GnRH cell populations, their specificity is not in doubt in our species given the absence of cross-reactivity. This assumption is based on several findings. The large ganglion sGAP cells of the NOR (20.5 μm) do not immunostain with anti-sbGAP antisera. Conversely, anti-sGAP serum does not immunostain the smaller sbGAP cells of the NOR (6.7 μm), nor the larger ir-sbGAP POA cells. The fiber pathway of sbGAP is restricted to the OBs, the vTel, and the ventral floor of the POA and hypothalamus, which contrasts markedly with the profuse innervation shown by ir-sGAP fibers in the forebrain, midbrain, and hindbrain. The specificity of the sea bass antibodies used in this study is reinforced by the results from Western blot analysis and also by the use of antibody controls. Similarly, the specificity of each of the previously characterized *H. burtoni* riboprobes (White and Fernald 1998) in *C. dimerus* is supported by the differential hybridization patterns obtained by using sGnRH+GAP and sbGnRH+GAP riboprobes in adjacent sections, by the use of stringent hybridization and washing conditions, by the low and uniform background observed in sense control sections, and by the consistency of the ISH and immunohistochem-

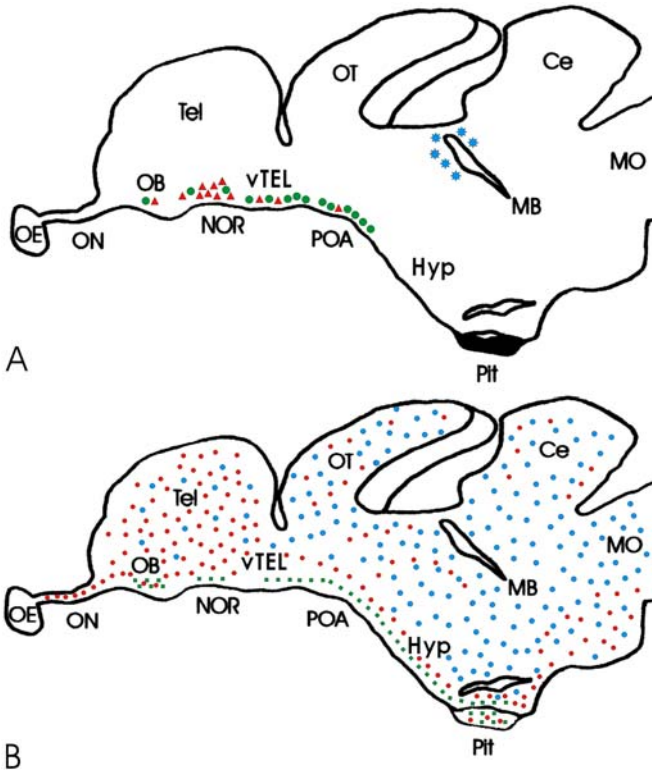

Fig. 9 Sagittal drawing of *Cichlasoma dimerus* brain summarizing: **a** Distribution of sGAP (*red triangles*), sbGAP (*green circles*), and cIIGAP (*blue stars*) cells. **b** Distribution of sGAP (*red dots*), sbGAP (*green squares*), and cIIGAP (*blue dots*) fibers in transverse sections. *Ce* Cerebellum, *Hyp* Hypothalamus, *MO* medulla oblongata, *MB* midbrain, *NOR* nucleus olfacto retinalis, *OB* olfactory bulb, *OE* olfactory epithelium, *ON* olfactory nerve, *OT* optic tectum, *Pit* pituitary, *POA* preoptic area, *Tel* Telencephalon, *vTEL* ventral telencephalon

ical results. Furthermore, the appearance of the three GnRH systems at different developmental stages reinforces the specificity of the tools used in this study.

Zamora et al. (2002) have demonstrated that the sea bass cDNAs encoding for the precursors of sGnRH and cGnRH II have 87%–99% sequence identity with the corresponding sequences from other perciforms, viz., striped bass, sea bream, and African cichlid. In turn, the sbGnRH precursor sequence identity is approximately 90% similar to that of the striped bass and 40%–60% similar to that of the sea bream and African cichlid. Both sea bass GAP antiserum and *H. burtoni* GAP riboprobes seem to recognize the proteins and mRNA sequences of *C. dimerus* with high specificity, because they show a clear unique band in Western blot, because they recognize small and scarce cell populations, and because they colocalize with their corresponding GnRH in every ir-GAP cell mass. Thus, the employed heterologous GAP probes and antisera, raised in phylogenetically close species, have been revealed as valuable tools for the study of the different GnRH systems in *C. dimerus*.

Analysis by 15% SDS-PAGE followed by Western blot recognized one specific band for each GAP in the brain (sGAP, sbGAP, and cIIGAP) and pituitary (sGAP and sbGAP). For sGAPs and sbGAPs, the estimated molecular weights are 7.5 kDa, viz., 1.5 kDa higher than the sea bass sGAPs and sbGAPs (González-Martínez et al. 2002a). For cIIGAP, *C. dimerus* expresses a 34-kDa band in brain and no band in pituitary. Although the expected size for the cIIGAP is about 7 kDa (based on the amino acid sequence of cGnRH-II precursors in other species), the specificity of the sea bass cIIGAP antisera is in no doubt. This assumption is based on the finding that the double immunohistochemistry with the cIIGAP and cGnRH II antisera immunostains cIIGnRH cells only. Furthermore, the absence of immunostaining in Western blot lanes incubated with a sea bass cIIGAP antisera with an excess of sea bass cIIGAP antigen reinforces the assumption of the specificity of this reaction.

The present study shows that, in *C. dimerus*, sGAP and sbGAP are expressed with two distinct gradients. sGAP immunoreactivity follows an anterior–posterior gradient, the most conspicuous immunoreactivity being in the NOR and to a lesser extent in more posterior locations, i.e., vTel and POA. In contrast, sbGAP cells show a posterior–anterior gradient with the most conspicuous immunoreactivity occurring in the POA and weak immunoreactivity in the NOR and OBs. The present results confirm previous findings in other species, based on immunohistochemistry showing that GnRH neurons have a widespread distribution in the ventral forebrain (Kah et al. 1986; Oka and Ichikawa 1990; Amano et al. 1991; Montero et al. 1994; Kim et al. 1995). These results are also consistent with a localization study performed in *D. labrax* with the same antibodies (González-Martínez et al. 2002a). However, a comparison between these previous results and those obtained in the present study reveals some differences that may reflect species-specific variations. Thus, although a few small and fusiform ir-sbGAP cells were detected in the hypothalamus of sea bass, none were found in the hypothalamus of *C. dimerus*. Recently, Vickers et al. (2004) have demonstrated, in the salmonid *Coregonus clupeaformis*, that GnRH III (sGnRH) and GnRH I (wfGnRH) are expressed in the same anterior brain areas, but not in the same cells. Therefore, in all teleosts (including perciforms) and in other vertebrates groups, GnRH forebrain perikarya (GnRH I and III) probably extend in a scattered continuum, from the terminal nerve to the anterior hypothalamus with some isolated neurons in the OBs (Silverman et al. 1994; Muske and Moore 1998; González-Martínez et al. 2001). The problem with detecting ir-GnRH cells outside the main nucleus (NOR, POA, midbrain) may be related to their small size and number in perciform species.

cIIGAP-ir cells were exclusively located in the dorsal midbrain tegmentum of *C. dimerus*, and no immunoreactivity was detected in any other cell body of the forebrain. The presence of ir-GnRH neurons in this area was first reported in platyfish (Münz et al. 1981) and subsequently confirmed in the goldfish (Kah et al. 1986). In the Masu salmon, those cells express the cGnRH-II form of the decapeptide (Amano et al. 1991). The presence of cGnRH-II neurons has now been reported in all of the vertebrate classes, with the exception of lampreys (Bennis et al. 1989; van Gils et al. 1993; Lescheid et al. 1997; Urbanski et al. 1999). The functions and sites of the projections of these midbrain neurons are still unclear. In some teleost species,

cGnRH-II has been detected in the pituitary (Yu et al. 1988; Schulz et al. 1993; Montero et al. 1994). However, in other teleost species, cGnRH-II-ir fibers do not reach the pituitary, despite the presence of cGnRH-II-ir cells in the dorsal midbrain tegmentum (González-Martínez et al. 2002a; Montero et al. 1994). These different patterns may be a reflection of species-specific differences. In *C. dimerus*, the pituitary gland appears to be devoid of cGnRH-II-ir fibers, although profuse innervation in the hypothalamic nucleus lateralis tuberis and in the vicinity of the pituitary stalks has been detected. Of interest, a small proportion of cGnRH-II-ir cells could not be immunolabeled with the sea bass anti-cIIGAP sera. This may be attributable to the lower specificity of the sea bass cIIGAP antiserum compared to the monoclonal anti-LRH13 that was used for the identification of cGnRH-II-ir cells in this area. In addition, some midbrain tegmentum cGnRH-II-ir cells may not express GAP or may express a different form of GAP, with some point mutations or post-transductional modifications that render it unrecognizable by the antiserum used.

In addition to showing the overlapping distribution of prepro-sGnRH and prepro-sbGnRH along the ventral forebrain and the restriction of prepro-cGnRH-II in the dorsal midbrain tegmentum, we have obtained information concerning the distribution of the various immunoreactive fibers. The ir-sGAP fibers showed a marked expression in the forebrain (OBs, NOR, vTel and dTel, parvocellular and magnocellular preoptic nuclei, ventral thalamus, and hypothalamus). Most posterior brain regions are also innervated by these fibers (dorsal tegmentum, central zone and periventricular grey zone of the optic tectum, corpus of the cerebellum, and rhombencephalon). The pituitary gland also receives some small projections that reach the proximal pars distalis and pars intermedia. Some ir-sGAP fibers extend to the retina and are in close contact with the bipolar neuronal layer and the olfactory epithelium. These morphological observations are consistent with some electrophysiological studies that have reported neuromodulatory effects (control of the excitability of target cells) of GnRH in the goldfish retina (Walker and Stell 1986; Umino and Dowling 1991) and in olfactory receptor cells of the mudpuppy (Eisthen et al. 2000). sGnRH cells have also been shown to be involved in the control of the motivation for nest building initiation but not in the performance of reproductive behavior in the dwarf gourami (Yamamoto et al. 1997). The ir-sbGAP fibers have been observed only at the ventral surface of the forebrain, associated with the POA and ventral hypothalamus. Axons of the ir-sbGAP preoptic cells gather to enter a fiber tract (preoptico-hypophysial tract) that runs through the pituitary. A strong presence of ir-sbGAP fibers has been detected in the proximal pars distalis and pars intermedia of the pituitary gland. No fibers have been found to extend from this tract toward other brain regions. However, a few ir-sbGAP fibers project locally in the vTel, NOR, and OBs. These fibers may originate in the ir-sbGAP populations outside the POA. Finally, cIIGAP-ir axons are widely distributed throughout the brain (especially in the midbrain and hindbrain) and the spinal cord of *C. dimerus*. Based on their widespread projections, these GnRH cells may function as a neuromodulatory system, similar to that suggested for ir-sGAP cells. Although the physiological role of this GnRH variant is unclear, the stringent conservation of cGnRH-II during a million of years of vertebrate evolution indicates that its function is essential for reproduction and/or neuromodulation in vertebrate species.

The present ontogenetic studies demonstrate that, in *C. dimerus*, prepro-sGnRH neurons have their origin in an olfactory primordium, whereas prepro-cGnRH-II cells develop from a mesencephalic primordium. These results confirm previous findings in this species based on the use of antibodies against different molecular forms of GnRH (Pandolfi et al. 2002). However, the present results, obtained with antibodies and riboprobes that recognize the various GAPs, show that both the forebrain GnRH systems (i.e., sGnRH–GnRH III and sbGnRH–GnRH I) first arise in rostral olfactory regions. These findings contradict those obtained in several other perciform species (Gothlif et al. 1996; Okuzawa et al. 1997; Parhar 1997; Parhar et al. 1998; White and Fernald 1998; Ookura et al. 1999; Senthilkumaran et al. 1999; Pandolfi et al. 2002) in which the forebrain populations appear to be neuroanatomically segregated, and an origin of sbGnRH neurons in a preoptic primordium located at the diencephalic ventricular wall has been hypothesized. Nevertheless, our results agree with those obtained in another perciform, the sea bass (González-Martínez et al. 2002b), and in amphibian, avian, and mammalian species, in which all forebrain GnRH neurons (from the OBs to the hypothalamus) seem to have a similar developmental origin in an olfactory primordium (Schwanzel-Fukuda and Pfaff 1989; Wray et al. 1989b; Murakami et al. 1992; Muske 1993; Muske and Moore 1994; Norgren and Gao 1994; Schwanzel-Fukuda 1999; Fiorentino et al. 2001). Further evidence in support of the idea that these cells originate from the olfactory region is the observation that sbGAP-expressing cells become detectable in the POA between days 23 and 26, whereas immunoreactivity to sbGAP antisera and expression of sbGnRH+GAP mRNA can be detected in the olfactory nerves on day 18. If a preoptic primordium is the origin of this population of GnRH cells, then mRNA or sbGAP peptides should be first detected in this region, but this is not the case. The chronological appearance from anterior to most posterior locations (olfactory nerves–olfactory bulbs–nucleus olfacto-retinalis–ventral telencephalon–preoptic area) during development also reinforces this assumption. Interestingly, in the sea bass, a small sbGAP population has been detected in the ventral hypothalamus (González-Martínez et al. 2002b). González-Martínez et al. (2002b) postulate that, if sbGnRH cells originated in the PO, then ir-sbGAP neurons presumably migrate in two different directions during development, viz., rostrally to reach the vTel and OBs and then caudally to arrive at the ventral hypothalamus; this seems improbable. In *C. dimerus*, we have been unable to detect this hypothalamic population by immunohistochemistry or ISH, either in adults or in various developmental stages. Although this may be a reflection of species-specific difference between members of different families of the perciform group,

we may have failed to detect these cells because they are present in only one or two histological sections. Recently, Okubo et al. (2004), using transgenic medaka lines in which the green fluorescent protein reporter had been genetically targeted to forebrain GnRH neurons, have demonstrated that GnRH neurons of both the preoptic and the terminal nerve originate in the nasal-forebrain junction and migrate to their final positions during embryonic development. Taken together, these results support the olfactory origin hypothesis for the sbGnRH neurons (GnRH I).

In conclusion, the results of the present study demonstrate that both GnRH populations in *C. dimerus* forebrain (sGnRH–GnRH III and sbGnRH–GnRH I) have an overlapping distribution pattern along the OBs, NOR, vTel, and POA. They also clearly demonstrate the distribution pattern of fibers immunoreactive for the three different GnRH forms. Furthermore, morphological evidence is presented in support of an olfactory origin of sbGnRH–GnRH I neurons. Finally, the study further emphasizes the great value that GAP antibodies and riboprobes have as tools for studying the different GnRH systems, thereby avoiding cross-reactivity problems even in phylogenetically related species.

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