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Expression of gonadotrophin-releasing hormone binding sites in somatic tissues of the gilthead seabream (*Sparus aurata*): a quantitative autoradiographic study

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Summary. In this study, we have analysed the expression of gonadotrophin-releasing hormone (GnRH) binding sites in somatic tissues (intestine, liver, gill, skeletal muscle, ovary, heart, stomach, kidney and spleen) of the gilthead seabream, *Sparus aurata* using 3-[^{125}I]iodototyrosyl^{5}-mammalian GnRH and autoradiographic techniques. The qualitative and quantitative analysis showed the existence of a basal expression of specific GnRH binding sites in intestine, skeletal muscle, ovary, stomach and spleen. Furthermore, our data suggest that the level of expression of GnRH binding sites can be significantly enhanced by GnRH treatment in intestine, gill, heart, stomach, kidney and spleen. This study shows that GnRH can exert direct effects in both reproductive and non-reproductive somatic tissues of the gilthead seabream.

Key words: GnRH binding sites, Hormonal induction, Somatic tissues, Perciform, Autoradiography

Introduction

The gonadotropin-releasing hormone (GnRH) represents the main cerebral factor responsible for the secretion of gonadotrophin that, in turn, controls the gametogenesis and gonadal steroidogenesis. The distribution pattern of the GnRH systems has been established in many vertebrate species (Muske, 1993; Lethimonier et al., 2004). However, its presence is not restricted to the central nervous system because the expression of different GnRH forms have been detected in pituitary, skeletal muscle, heart, liver, kidney, spleen, thymus, placenta, mammary gland, ovary and testis of several vertebrate species (Kakar and Jennes, 1995; Weesner et al., 1997; White and Fernald, 1998; Nabissi et al., 2000; Uzbekova et al., 2001).

After GnRH is secreted, it is recognized by a specific membrane–associated GnRH receptor present in target cells. The GnRH receptor is part of the large rhodopsin-like G protein-coupled receptor superfamily. Upon binding to GnRH, the GnRH receptor can activate multiple signal transduction pathways, i.e. protein kinase C, protein kinase A, arachidonic acid and calmodulin (Stojilkovic et al., 1994; Kakar and Neill, 1995; Lin and Conn, 1999). In mammals, GnRH binding sites and/or GnRH receptors have been identified in pituitary and brain (Marshall et al., 1976; Jennes and Conn, 1994), gonad (Hsueh and Schaeffer, 1985), placenta (Currie et al., 1981), thymus (Marchetti et al., 1989), liver, cardiac and skeletal muscle, spleen and lung (Marshall et al., 1976; Kakar and Neill, 1995). In fish, GnRH receptors have been found in pituitary (Crim et al., 1988; Andersson et al., 1989; Weil et al., 1992; Pagelson and Zohar, 1992), but also in brain, gonad, retina, liver and kidney (Pati and Habibi, 1993; Yu et al., 1998; Madigou et al., 2000; Robison et al., 2001; Gonzalez-Martínez et al., 2004). Recently, Moncaut et al. (2005) identified five GnRH receptors in the European sea bass, which exhibit a differential expression in peripheral tissues related to reproduction (gonads), chemical senses (eye and olfactory epithelium) and osmoregulation (kidney and gill). The presence of GnRH receptors outside the brain-pituitary-gonadal axis of vertebrates suggests that this neuropeptide could also be involved in various non-reproductive functions.

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Several studies have shown that the number of pituitary GnRH receptors varies over the maturation process (Habibi and Peter, 1991; Gonzalez-Martinez et al., 2004). Furthermore, the capacity and/or expression of GnRH receptors can be regulated by GnRH (Omeljaniuk et al., 1989) and dopaminergic treatment (de Leeuw et al., 1986, 1988) but also by gonadectomy (Conn et al., 1981) and steroid hormones (Trudeau et al., 1993; Seong et al., 1998). In this study, we have analysed the qualitative and quantitative expression of gonadotrophin-releasing hormone (GnRH) binding sites in somatic tissues (intestine, liver, gill, skeletal muscle, ovary, heart, stomach, kidney and spleen) of the gilthead seabream, Sparus aurata using 3-[^{125}I]iodotyrosyl^{5}-mammalian GnRH and autoradiographic techniques. Moreover, we have studied the effects of GnRH treatment on the level of expression of GnRH binding sites in these tissues.

Materials and methods

Animals

Post-spawning adult gilthead seabream female specimens, ranging in body weight from 1750 to 2800 g were obtained from CUPIMAR (San Fernando, Cádiz, Spain) and kept in running seawater. Animals were treated according to the European Union regulations concerning the protection of experimental animals.

Hormonal treatments

Seabream specimens were anesthetized with 2-phenoxiethanol (Sigma, St. Louis, MO) and injected intraperitoneally with seabream GnRH (sbGnRH, obtained from the Laboratoire de Cristallographie et de Physique Cristalline, Talence, France, 10 mg/kg body weight, in 0.9% NaCl, n=4). The injected animals were then sacrificed 6 hours after the treatment. Untreated animals (n=4) were injected with a similar volume of 0.9% saline solution and sacrificed 6 hours after the treatment. Intestine, liver, gill, skeletal muscle, gonad, heart, stomach, kidney, spleen and pituitary samples were removed and frozen in liquid nitrogen.

Autoradiography

For autoradiography, 3-[^{125}I] iodotyrosyl^{5}-LHRH was obtained from Amersham (Amersham, UK). Specific activity of the radio labelled hormone was 2000 Ci/mmol. Non-radio labelled mammalian GnRH (Sigma, St. Louis, MO) and seabream GnRH (Laboratoire de Cristallographie et de Physique Cristalline, Talence, France) were used for control sections. To serve as a positive control of the technique, pituitary sections were used to identify GnRH binding sites in the proximal pars distalis, which is known for the presence of GnRH receptors in fish species, including seabream (Pagelson and Zohar, 1992).

Collected tissues were embedded in Einbett medium (Jung GmbH, Heidelberg). Tissue sections 16 µm-thick were obtained on a cryomicrotome and washed in 50 mM Tris-HCl, pH 7.4 buffer containing 0.2% BSA for 30 min at 4°C. Subsequently, sections were incubated in the same buffer containing 5 mM $MgCl_2$, 0.02 mM bacitracin and 35.4 pg of radioiodinated 3-[^{125}I] iodotyrosyl^{5}-LHRH/150 µl buffer for 24 h at 4°C. In order to determine the non specific binding of radio labelled GnRH, control sections of all tested tissues were also incubated with an excess of non-labelled mammalian GnRH and seabream GnRH (354 ng GnRH/150 µl buffer) and analyzed. After incubation, the medium was aspirated and the sections were washed in cold buffer and fixed in 4% paraformaldehyde for 30 min, at room temperature. The slides were coated with Hypercoat LM-1 autoradiographic emulsion (Amersham International, UK) and exposed for 3 weeks before development in Kodak D19. Sections were counterstained with nuclear red or haematoxylin-eosin.

Autoradiographic 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 5.5 and no subsequent alterations were made.

Quantification of GnRH binding sites expression

For quantitative analysis of GnRH binding sites expression in somatic tissues of seabream, at least 60 tissue sections (15 sections/animal) were selected at random, and randomly selected brightfield pictures (30-45 pictures/animal) covering 17,700 µm^2 of tissue surface were obtained in the photomicroscope with the help of the digital camera. The area occupied by autoradiographic silver grains was measured using Scion Image Beta 4.0.2 software (NIH, USA) and results were represented as mean ± standard deviation. The statistical analysis was performed wit the help of the SPSS 10.0 program. As requirements of normality and homogeneity of variances were not satisfied, the level of statistical significance of differences between mean values was assessed by the Kruskal-Wallis non-parametric analysis of variance (W), followed by the Mann-Whitney and Wilcoxon tests. Differences between mean values were considered significant at $p<0.05$.

Results

In this study, we have presented a quantitative and qualitative analysis of putative GnRH receptors in somatic tissues. The distribution of GnRH binding sites in somatic tissues (intestine, liver, gill, skeletal muscle, gonad, heart, stomach, kidney, spleen) of gilthead seabream, *Sparus aurata* was evidenced by autoradiography using a radioiodinated mammalian LHRH. Previously, in order to test if 3-[^{125}I] iodototyrosyl^{5}-LHRH used is able to bind to seabream GnRH receptors, we have analysed the presence of

GnRH binding sites in the pituitary, which represents the main target for GnRH and contains GnRH receptors in seabream (Pagelson and Zohar, 1992). As expected, radioiodinated mammalian LHRH binds to the seabream pituitary. In particular, we have found specific GnRH binding sites in the proximal pars distalis (Fig. 2A), which is known for the presence of gonadotropes and somatotropes in fish.

The quantitative analysis in control animals (Fig. 1) revealed the highest specific expression of GnRH binding sites in the gastro-intestinal tract (stomach, 290.58±156, W=42, $p<0.005$; intestine, 218.71±58, W=43, $p<0.007$). Intestinal GnRH binding sites were evident in microridges (Fig. 2B) but also in the external serosa layer marking the boundary between the intestine and the peritoneal cavity (Fig. 2C). Specific GnRH binding sites were also expressed in ovary (153.18±60, W=40, $p<0.002$, Fig. 2D), skeletal muscle (194.27±21, W=39, $p<0.001$, Fig. 2E) and spleen (101.71±58, W=44, $p<0.01$). In the ovary, GnRH binding sites were detected in oogonia but no specific binding was observed in previtellogenic or vitellogenic oocytes (Fig. 2D). The analysis of the basal level of expression in control tissues (Fig. 1) did not reveal significant specific GnRH binding in liver (306.75±85, W=52, $p<0.056$, Fig. 2F), gill (153.13±89, W=63, $p<0.645$), heart (147.90±50, W=58, $p<0.328$) and kidney (51.63±20, W=59, $p<0.382$).

Furthermore, our data suggest that the level of expression of GnRH binding sites can be significantly enhanced by GnRH treatment in several somatic tissues (Figs. 1-3). Thus, GnRH treatment significantly induced the expression of GnRH binding sites in stomach (717.49±407, W=47, $p<0.028$), intestine (674.15±279, W=38, $p<0.001$), spleen (607.51±355, W=37, $p<0.001$), gill (403.04±150, W=45, $p<0.015$, Fig. 3A), heart (372.67±145, W=39, $p<0.001$, Fig. 3C) and kidney (259.12±150, W=36, $p<0.001$, Fig. 3E). In gill, specific

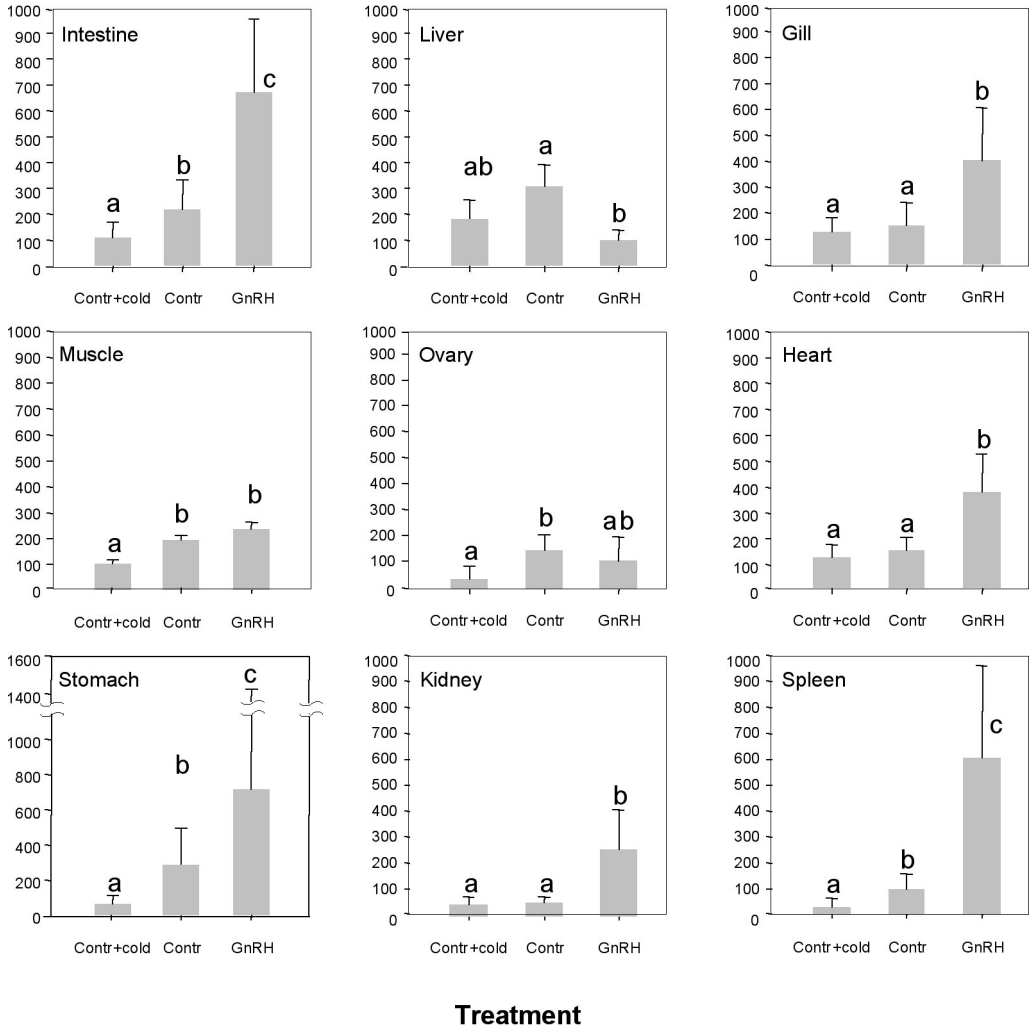

Fig. 1. Quantitative analysis of GnRH binding sites in different somatic tissues of the gilthead seabream. Values are expressed as mean area occupied by silver autoradiographic grains in $\mu m^2/8500\ \mu m^2$ of tissue section±standard deviation. *Contr + cold*: values from tissue sections of control animals incubated with radioidodinated mGnRH and an excess of cold mGnRH. *Contr:* values from tissue sections of control animals incubated with radioidodinated mGnRH. *GnRH*: values from tissue sections of GnRH-treated animals incubated with radioidodinated mGnRH. Different letters indicate significant differences between mean values from different conditions ($p<0.05$).

radiolabelled cells appeared in secondary lamellae (Fig. 3A). In heart, GnRH treatment induced the expression of specific GnRH binding sites in the cardiac muscle and vascular system (Fig. 3C). In kidney, radiollabelling was evident in renal tubules (Fig. 3E). Control tissue sections from GnRH-treated animals incubated with an excess of non-labelled GnRH showed a consistently lower autoradiographic labelling in gill (Fig. 3B), heart (Fig. 3D) and kidney (Fig. 3F).

Discussion

In this study, we have analyzed the quantitative and qualitative expression of GnRH binding sites in different somatic tissues of the gilthead seabream using a radioiodinated mammalian GnRH (mGnRH) as ligand. Three molecular forms of GnRH, which are seabream type, salmon type and chicken-II type, have been identified in gildthead seabream (Powell et al., 1994;

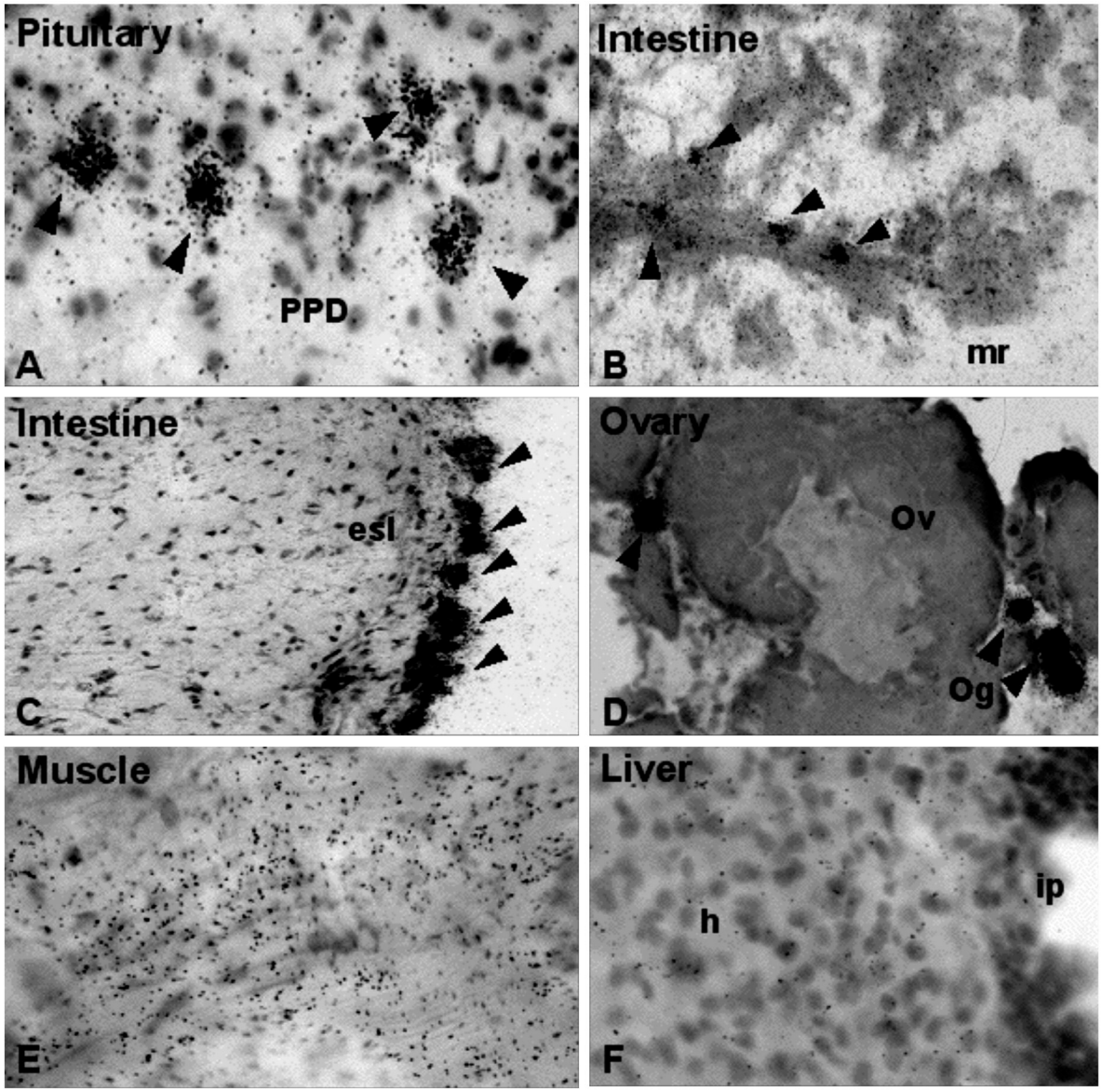

Fig. 2. Autoradiographic photomicrographs of different seabream somatic tissues. **A:** presence of GnRH binding sites in cells of the proximal pars distalis (PPD) of the seabream pituitary (arrowheads). x 1200. **B:** intestine section showing positive labelling (arrowheads) in microridges (mr) from a GnRH-treated animal. x 470. **C:** GnRH binding sites in intestinal cells (arrowheads) of the external serosa layer (esl) from a GnRH-treated animal. x 200. **D:** ovary section from a non-treated (control) animal showing intense radiolabelling in oogonia (Og, arrowhead) but not in oocytes (Ov). x 380. **E:** Muscle section from a non-treated (control) animal showing specific radiolabelling. x 500. **F:** Liver section from a non-treated (control) animal showing the absence of specific radiolabelling in hepatocytes (h) and intrahepatic pancreas (ip). x 600

Zohar et al., 1995). The mGnRH (Arg8-GnRH) isoform differs in only one aminoacid substitution in respect of the seabream GnRH form (sbGnRH = Ser8-mGnRH) that represents the main hypophysiotrophic hormone in perciforms (Zohar et al., 1995; Holland et al., 1998; Gonzalez-Martinez et al., 2002). It has been clearly demonstrated that the mGnRH isoform has a high biological activity in teleosts (King and Millar, 1995) and that radioiodinated mGnRH is able to bind specifically to fish GnRH receptors (Habibi et al., 1989a;

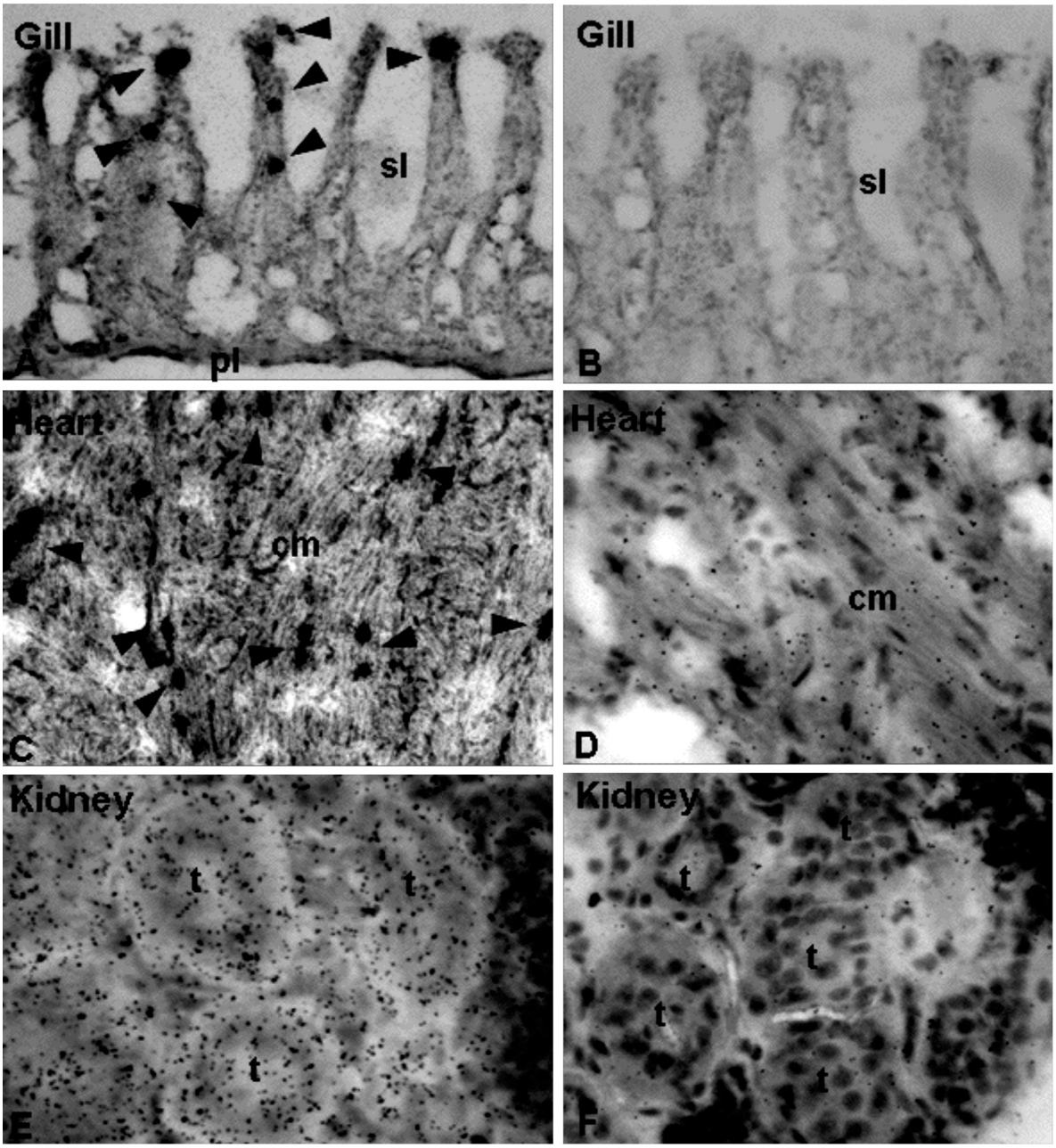

Fig. 3. Autoradiographic photomicrographs of different seabream somatic tissues. **A:** Gill section from a GnRH-treated animal showing positive labelling in secondary lamella (sl) but not in primary lamella (pl). x 240. **B:** Gill section from a GnRH-treated animal incubated with an excess of non-labelled GnRH showing a scarce non specific radiolabelling. x 310. **C:** Heart section from a GnRH-treated animal showing multiple radiolabelled cells (arrowheads) in cardiac muscle (cm). x 460. **D:** Heart section from a GnRH-treated animal incubated with an excess of non-labelled GnRH only showed a few autoradiographic grains in cardiac muscle (cm). x 620. **E:** Kidney section from a GnRH-treated animal showing intense radiolabelling in renal tubules (t). x 620. **F:** Kidney section from a GnRH-treated animal incubated with an excess of non-labelled GnRH, t: renal tubules. x 600

Huang et al., 1991). In this respect, radioiodinated mammalian GnRH binds to the seabream pituitary (see Figure 2A in this study), in which GnRH receptors have been previously characterized (Pagelson and Zohar, 1992).

Three types of GnRH receptors have already been identified in vertebrates (Lethimonier et al., 2004; Levavi-Sivan and Avitan, 2005) but recently, five different GnRH receptors that have been grouped in two main types, have been described in a single species (Moncaut et al., 2005). It is not clear in the present study whether radioiodinated mammalian GnRH binds specifically to a single type of GnRH receptor or to other types of GnRH receptors. However, non-mammalian GnRH receptors, especially piscine ones, are not very selective and interact well with most of the vertebrate GnRHs (Levavi-Sivan and Avitan, 2005), suggesting that radioiodinated mammalian GnRH could bind all GnRH receptors expressed in seabream.

Our quantitative analysis showed the presence of a basal expression of specific GnRH binding sites in intestine, skeletal muscle, ovary, stomach and spleen but not in liver, gill, heart and kidney. Originally, it was though that GnRH was only produced in the brain to modulate pituitary gonadotropin secretion. However, there is now considerable evidence that GnRH and/or GnRH receptors are also expressed in other reproductive and non-reproductive somatic structures of vertebrates (Marshall et al., 1976; Marchetti et al., 1989; Pati and Habibi, 1993; Jennes and Conn, 1994; Kakar and Jennes, 1995; Weesner et al., 1997; White and Fernald, 1998; Madigou et al., 2000, 2002; Nabissi et al., 2000; Huang et al., 2001; Robison et al., 2001; Uzbekova et al., 2001; Bogerd et al., 2002; Jodo et al., 2003). Furthermore, the administration of sbGnRH in seabream showed a tissue-dependent induction in the expression of GnRH binding sites. Thus, the level of expression of GnRH binding sites can be significantly enhanced by GnRH treatment in intestine, stomach, spleen, gill, heart, and kidney. Unfortunately, most of the available information in the literature concerning the hormonal induction of GnRH receptor expression in vertebrates refers to the brain and pituitary (Omeljaniuk et al., 1989; Seong et al., 1998), and data concerning the hormonal regulation of GnRH receptor expression in peripheral tissues is rather scarce.

In seabream, the highest basal and induced expression of GnRH binding sites was detected in the gastrointestinal tract. This expression was evident in microridges and in the external layer separating the gastrointestinal tract from the peritoneal cavity. Interestingly, it has been reported that exogenous GnRH can be rapidly absorbed from the intraperitoneal cavity in goldfish (Sherwood and Harvey, 1986). In rat, immunohistochemical and in situ hybridization studies revealed that GnRH and GnRH receptors were expressed in the digestive tract and it has been suggested that GnRH might act as a gastrointestinal hormone (Huang et al., 2001). GnRH has an inhibitory role on cell proliferation in the gastric epithelium (Gama and Alvares, 1996) and gastric smooth muscle (Chen et al., 2004) in rats, as well as inhibitory actions on gastric secretion in dog (Soldani et al., 1982).

The spleen of seabream also exhibited a significant basal and induced expression of GnRH binding sites. The expression of the seabream GnRH isoform (sbGnRH) has been documented in the spleen of the perciform *Haplochromis burtoni* (White and Fernald, 1998). In fish, as in other vertebrates, the spleen represents an important haemopoietic tissue involved in immune functions (Van Muiswinkel et al., 1991). GnRH is known to exert direct immunomodulatory effects in mice acting on GnRH receptors present in lymphocytes and immune cells of the spleen and thymus (Jacobson et al., 1998). Furthermore, GnRH significantly increases the expression of GnRH receptor mRNA in these immune cells (Jacobson et al., 1998). An expression of GnRH and its receptor was also referred in porcine immune system (Weesner et al., 1997).

The presence of GnRH binding sites was also evident in seabream ovary, in particular, in oogonia. There is also evidence of the presence of GnRH binding sites and/or GnRH receptors in gonads of the carp (Pati and Habibi, 1992), goldfish (Pati and Habibi, 1993), catfish (Habibi et al., 1994; Bogerd et al., 2002), seabream (Nabissi et al., 1997), lamprey (Gazourian et al., 1997), rainbow trout (Madigou et al., 2000) and masu salmon (Jodo et al., 2003), as well as in rat and human ovary (Heber et al., 1978; Kakar et al., 1992), suggesting that GnRH may act as a paracrine regulation factor in gonads in all vertebrates. In the mammalian ovary, GnRH receptors have been identified in granulosa and luteal cells (Stojilkovic et al., 1994). However, GnRH receptors were more abundant in oogonia in post-spawning seabream specimens (this study). In goldfish, the immature ovary contains a single class of low-affinity binding sites whereas two classes of binding sites, a high-affinity/low-capacity site and a low affinity/high capacity site are present in the fully mature and less mature ovary (Pati and Habibi, 1993). Direct GnRH effects on gonadal functions have been described in fish, such as the promotion of oocyte meiosis (Habibi et al., 1988), the regulation of gonadal steroidogenesis (Habibi et al., 1989b; Gazourian et al., 1997), and the stimulation of gonadal apoptosis (Andreu-Vieyra et al., 2005). Thus, the high levels of GnRH binding sites in oogonia could mediate GnRH effects in the promotion of meiotic division or in determining the rate at which primordial follicles and oogonia are lost.

The skeletal muscle of seabream also contains GnRH binding sites. According to our results, GnRH receptor was expressed in the muscle of striped bass (Alok et al., 2000), masu salmon (Jodo et al., 2003) and *Haplochromis burtoni* (Robison et al., 2001). However, GnRH receptors were not detected in the muscle of rainbow trout (Madigou et al., 2000) and catfish (Bogerd et al., 2002). Although the role of GnRH in fish muscle remains uncertain, it has been shown that feeding with GnRH caused a significant increase in muscle

RNA/DNA ratio in juvenile grass carp (Xiao and Lin, 2003). The expression of GnRH and GnRH receptor mRNAs in skeletal muscle has also been reported in humans using RT-PCR (Kakar and Jennes, 1995).

It should be noted that basal expression of GnRH-binding sites was not detected in heart, gill and kidney of seabream but a significant increase was induced by GnRH treatment. This expression represents a real GnRH induction of GnRH binding sites in these tissues because sections from GnRH-treated animals incubated with an excess of non-labelled GnRH only showed a few autoradiographic grains. This induction could reflect the ability of these tissues to incorporate GnRH for the modulation of different physiological functions, or just as a simple reservoir mediating metabolic clearance of this hormone. There is some controversy concerning the presence of GnRH receptors in the heart. Low affinity GnRH binding sites and/or GnRH receptors were identified in cardiac muscle of mammalian species (Marshall et al., 1976; Kakar and Jennes, 1995). In fish, GnRH receptor subtype 2 mRNA expression was described in masu salmon heart using RT-PCR techniques (Jodo et al., 2003) and both cGnRH-II ligand and GnRH receptor mRNAs were expressed in the heart of the catfish (Bogerd et al., 2002). In contrast, seabream GnRH isoform (White and Fernald, 1998) but not GnRH receptor (Robison et al., 2001) appeared expressed in the heart of *Haplochromis burtoni* and specific GnRH binding sites and/or GnRH receptors were not observed in the heart of goldfish (Habibi and Pati, 1993) and rainbow trout (Madigou et al., 2000). The physiological significance of this hormonal induction in the expression of cardiac GnRH binding sites in seabream is still unknown, but a modulation of heart activity by distinct chemical factors including GnRH has been reported previously in other vertebrates (Galoian, 1978; Strbak, 2000). These GnRH actions on cardiac function seem to be highly conserved in phylogeny because many immunoreactive GnRH fibers were detected in the heart of octopus and modulatory effects of GnRH on the contractions of the heart have been described in this invertebrate species (Iwakoshi-Ukena et al., 2004).

As in the heart, the expression of GnRH binding sites in the gill and kidney of seabream becomes significant after GnRH treatment. In goldfish, exogenous GnRH can be absorbed on gill surface, being rapidly incorporated and detected in plasma (Sherwood and Harvey, 1986; Huang et al., 1991). The kidney represents an active organ for degradation and cleavage of native GnRHs in seabream (Zohar et al., 1990) and both GnRH and GnRH receptor mRNAs were found in the kidney of *Haplochromis burtoni* (White and Fernald, 1998; Robison et al., 2001) and human (Kakar and Jennes, 1995). However, GnRH receptors were not detected in the kidney of rainbow trout (Madigou et al., 2000) and catfish (Bogerd et al., 2002).

In conclusion, our results show that GnRH binding sites are present in different somatic tissues of gilthead seabream and that their expression is regulated by GnRH in a tissue-dependent manner. The expression of GnRH outside the brain and the presence of GnRH binding sites and/or GnRH receptors in non-reproductive somatic tissues suggest that these receptors play an important role in mediating autocrine/paracrine actions of GnRH, which in most cases remain to be elucidated.

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