



Three forms of gonadotropin-releasing hormone (GnRH) in brain and pituitary of Asian seabass *Lates calcarifer* (Bloch, 1790) analysed by high performance liquid chromatography combined with time-resolved fluoroimmunoassay and immunohistochemistry

KY XUAN PHAM, MASAFUMI AMANO*, NORIKO AMIYA*, HA VIET DAO AND QUANG VAN VO

Institute of Oceanography, Vietnam Academy of Science and Technology, Cau Da - 01, Nha Trang Khanh Hoa, Vietnam

**School of Marine Biosciences, Kitasato University, Sagami-hara, Kanagawa - 252 0373, Japan
e-mail: phamkx@vnio.org.vn*

ABSTRACT

Three forms of gonadotropin-releasing hormone (GnRH) namely, salmon GnRH (sGnRH), chicken GnRH-II (cGnRH-II) and seabream GnRH (sbGnRH) were identified in the brain of Asian seabass *Lates calcarifer* using reverse-phase high performance liquid chromatography (rpHPLC) combined with time-resolved fluoroimmunoassay (TR-FIA) using rabbit polyclonal antibody raised against sGnRH, cGnRH-II and sbGnRH. An extract of *L. calcarifer* brain showed a similar retention time to that of synthetic sGnRH, cGnRH-II and sbGnRH on rpHPLC. In the brain, cGnRH-II and sGnRH levels were high, whereas sbGnRH was absolutely dominant in the pituitary. In addition, cGnRH-II, sGnRH and sbGnRH-immunoreactive (ir) cell bodies and fibers in the brain and pituitary were also examined by immunohistochemistry. sGnRH-ir cell bodies were localised in the ventromedial part of the rostral olfactory bulb, terminal nerve ganglion region, and the transitional area between the olfactory bulb and telencephalon. cGnRH-II-ir cell bodies were located only in the midbrain tegmentum and sbGnRH-ir cell bodies were evident in the preoptic area. sGnRH-ir fibers were distributed throughout the brain, especially abundant in the forebrain. cGnRH-II-ir fibers were found in many parts of the brain, being more dense in the midbrain, whereas sbGnRH-ir fibers were localised in the preoptic-hypothalamic area, innervating the pituitary. In the pituitary, sbGnRH-ir fibers were profuse in the neurohypophysis and invaded the proximal *pars distalis*. These results confirm the existence of multiple GnRH forms with different physiological roles in *L. calcarifer*. Among them, sbGnRH is considered to be the best candidate for pituitary regulation while sGnRH and cGnRH-II can function as neurotransmitters or as neuromodulators in this species.

Keywords: Brain, Fluoroimmunoassay, GnRH, HPLC, Immunohistochemistry, *Lates calcarifer*; Pituitary

Introduction

Gonadotropin-releasing hormone (GnRH) is a decapeptide regulating several physiological functions in vertebrates (Amano *et al.*, 1997; Okuzawa and Kobayashi, 1999; Lethimonier *et al.*, 2004). GnRH was first isolated from mammals (Matsuo *et al.*, 1971) and later from tetrapods (King and Millar, 1979) and teleosts (Sherwood *et al.*, 1983). Till date, eight GnRH forms have been identified in teleosts and two or three GnRHs were found in a single species. Recent reports on GnRH forms confirmed that two common forms are chicken GnRH-II (cGnRH-II) and salmon GnRH (sGnRH). There is also a third form reported as seabream GnRH (sbGnRH), herring GnRH (hrGnRH), medaka GnRH (mdGnRH), pejerrey GnRH (pjGnRH) and whitefish GnRH (wfGnRH) in fishes (Lethimonier *et al.*, 2004). The GnRH forms were named

following the species in which they were first isolated, except for the mammalian GnRH (mGnRH) (Sherwood *et al.*, 1983; Yu *et al.*, 1988; Bogerd *et al.*, 1992; Powell *et al.*, 1994; Carolsfeld *et al.*, 2000; Montaner *et al.*, 2001; Adams *et al.*, 2002). Among GnRHs, cGnRH-II is the ubiquitous form present in all teleosts and possibly the most ancient form of GnRH. The second GnRH is a species-specific form (Powell *et al.*, 1994), while the third form represents advanced evolutionary teleosts. The latter forms of GnRH may have arisen as a result of nucleotide substitutions in the gene of ancient form.

Each GnRH is distinguished from others by difference in positions of amino acid residues among ten amino acids in the chemical structure. It is generally accepted that GnRH in the brain of teleosts is involved in reproduction *via* stimulating pituitary gonadotropins

through GnRH receptors (Lethimonier *et al.*, 2004). In addition, GnRH can also induce release of other pituitary hormones, including growth hormone (Marchant *et al.*, 1989) and prolactin (Weber *et al.*, 1997). However, studies on the function of each GnRH in the brain of teleosts is complicated due to the presence of multiple GnRH forms. It is suggested that GnRHs can express different functions such as neuromodulation/neurotransmission or act as local hormones in teleosts according to their location in the brain (Kah *et al.*, 2007). More recently, it has been revealed that GnRHs can also modulate auditory processing in the fish brain (Maruska and Tricas, 2011) or function as olfactory and visual neuromodulators (Servili *et al.*, 2012).

Immunological, molecular and chromatographic analyses showed that the order Perciformes, the largest group of teleosts, with representatives such as the gilthead seabream *Sparus aurata* (Gothilf *et al.*, 1996), whitefish *Coregonus clupeaformis* (Adams *et al.*, 2002), European seabass *Dicentrarchus labrax* (Gonzalez-Martinez *et al.*, 2002), barfin flounder *Verasper moseri* (Amano *et al.*, 2002), Japanese flounder *Paralichthys olivaceus* (Pham *et al.*, 2007) and chub mackerel *Scomber japonicus* (Selvaraj *et al.*, 2009) express three GnRH forms in the brain. Among them, cGnRH-II, with the population of unique GnRH cells in the midbrain and wide distribution of fibers in different regions of the brain, is proposed to play a role in neuromodulation. sGnRH usually act as a neuromodulator and/or neurotransmitter (Soga *et al.*, 2005). cGnRH-II and sGnRH are considered to be non-hypophysiotropic forms and their physiological roles remained unclear in teleosts possessing three GnRHs. The third GnRH form, with distribution in the neuronal population of the preoptic area (POA) as well as hypothalamus and innervation of fibers into the anterior pituitary though hypothalamus is the hypophysiotropic form regulating reproduction in teleosts by stimulating gonadotropin release (Lethimonier *et al.*, 2004).

Lates calcarifer, a carnivorous fish, is distributed in the tropical and sub-tropical areas of the Western Pacific and Indian Ocean (Phillips, 1998). This fish is a highly commercial species and is important for aquaculture in several Asian countries. *L. calcarifer* is a diadromous fish exhibiting complex sexuality making it an interesting specimen for academic studies (Moore, 1979). Under natural condition, fish spend most its life in river mouths, lakes or lagoons and spawning event occurs in brackishwater estuaries (Rimmer and Russell, 1998). Under captive condition, gonadal maturation in mature fish can be induced by pellet implantation of luteinising hormone-releasing hormone analogue [(D-Ala⁶; Pro⁹-NEt) LHRH], [D-Ala⁶; Pro⁹-ethylamide] mGnRH and [D-Arg⁶; Pro⁹-ethylamide] sGnRH and 17 α -methyltestosterone.

Spawning in mature female could be stimulated by injection of LHRH and GnRH-analogues and the fish can spawn repeatedly in batches for several days (Almendras *et al.*, 1988; Garcia, 1989). However, endocrine profile and mechanism of hormones regulating reproduction have not been fully studied in this species. Until now, only preprocGnRH-II precursor sequences in the brain of this species have been identified and analysed (Tan *et al.*, 2008). To better understand the possible role and physiological relevance of hormones in *L. calcarifer*, we analysed GnRH forms, the anatomical localisation and distribution of these hormones in the brain and pituitary in this species.

Materials and methods

Fish sampling

Wild *L. calcarifer* were caught in Nha Phu Lagoon, Khanh Hoa Province, Vietnam employing gillnet or fish hook with baits. Live fishes were kept in a floating net cage in the sea for 1-2 days. The fish were then transported to Oceanography Institute, Nha Trang, Vietnam with oxygen supplementation within 1 h. The fishes were anaesthetised by immersion in ice water. Total length (TL) and body weight (BW) were measured, ovaries were removed and weighed to calculate gonadosomatic index (GSI). The fish were treated in accordance with the guidelines stipulated by the world society for the protection of animals in Vietnam.

Tissue preparation

For high performance liquid chromatography (HPLC) and time-resolved fluoroimmunoassay (TR-FIA) analyses, the brains and pituitaries were removed quickly from the decapitated fish and placed on dry ice. The samples were then stored at -80°C until GnRH peptide extraction. Deeply frozen brain tissues in dry ice were air lifted to Japan and analyses were carried out in Kitasato University. The TL, BW and GSI of the five individuals used for this experiment were 40.1 - 50.2 cm, 3.5 - 4.7 kg and 2.4 - 3.50% respectively.

For immunohistochemical analysis, chondrocranium including brain were fixed in Bouin's solution at 4°C for 24 h. The TL, BW and GSI of the individuals used for the experiment were 50.2 - 115 cm, 5.0 - 16.74 kg and 2.70 - 11.15% respectively.

Reverse-phase HPLC separation and TR-FIA detection of GnRH

GnRH peptides from frozen brains and pituitaries were extracted as per the method described by Okuzawa *et al.* (1993) with slight modification. The extracts after removing lipids with petroleum ether were

passed through a 0.22 µm filter (Millipore Corp, Bedford, MA). The filtered GnRH extracts were separated by reverse phase (rp) HPLC to separate GnRH forms as described by Amano *et al.* (2009). In brief, the filtrates (800-900 µl) were injected through a 1 ml injection loop onto an ODS-120T (0.46 x 25 cm, Tosoh, Tokyo, Japan). Liquid chromatography was programmed at a flow of 1 ml per min in a high-pressure gradient system with two pumps (980-PU, JASCO, Tokyo, Japan) which used acetonitrile containing 0.1% trifluoroacetic acid (TFA). The percentage of acetonitrile was increased linearly from 10% (0 min) to 50% (50 min). Fractions were collected every 30 sec from the 18th to 36th min. A standard solution containing 1 µg each of cGnRH-II, lamprey (l) GnRH-II, octopus (oct) GnRH, sGnRH and sbGnRH in 200 µl of 0.1% TFA was also injected and fractionated as explained above. All fractions collected from the samples and standard were freeze-dried in a vacuum centrifuge concentrator. Dried residues were dissolved in 500 µl of TR-FIA assay buffer (20 mM sodium phosphate buffer, 0.9% NaCl, 0.1% BSA, 20 µM diethylenetriamine-N,N,N',N'',N''-pentaacetic acid, 0.01% Tween-40; pH 7.2) and were checked for cGnRH-II, sGnRH and sbGnRH by TR-FIA according to Amano *et al.* (2002). Antibodies to sGnRH (lot 2), cGnRH-II (acII 6), and sbGnRH (AS-691) were used in these assays.

Immunohistochemistry for GnRHs

The whole brains in Bouin's solution were removed from the chondrocranium and post-fixed in the same fluid at 4°C for 12-24 h. The tissues then were rinsed in cold 70% ethanol, subsequently dehydrated through a graded series of ethanol concentrations up to 100% and finally embedded in paraplast. Saggital sections at a thickness of 10 µm were cut and mounted on aminopropyltriethoxysilane (APS) coated slides (Matsunami, Osaka, Japan). Immunohistochemistry for GnRHs was performed according to Amano *et al.* (2002) and Pham *et al.* (2007). The immunohistochemical reaction used was based on a streptavidin-biotin-peroxidase complex method using a Histofine immunostaining kit (Nichirei, Tokyo, Japan).

In these experiments, antibodies to sGnRH, cGnRH-II and sbGnRH were diluted into 2000-fold, 500-fold and 10000-fold, respectively, in 0.1M phosphate buffer (pH 7.4) containing 0.75% NaCl and 0.3% Triton X-100 (PBST). The specificity of the immunoreactions was tested with pre-absorbed antibodies with the corresponding synthetic peptides of GnRH and the possibility of non-specific immunoreactions was also checked using the pre-absorbed antibodies with the non-corresponding synthetic peptides in the adjacent sections as described by Pham *et al.* (2007).

Histology

Histology of nuclear boundaries was identified in the adjacent sections stained with cresyl violet. The terminology for brain nuclei was according to Stefano *et al.* (2000) and Soga *et al.* (2005). All stained sections were examined under a microscope and digitally photographed. Figures of brain sections describing the distribution of GnRH-ir cell bodies and fibers were drawn using a *camera lucida*.

Results and discussion

HPLC and TR-FIA analysis

In the brain extract of the fish, three chromatographic and immunological peptides corresponding to fractions 32, 26 and 23 were identical to sGnRH (Fig. 1a), cGnRH-II (Fig. 1b) and sbGnRH (Fig. 1c) respectively. Retention time of each GnRH-ir form was similar to the elution positions of the synthetic sGnRH, cGnRH-II and sbGnRH standards. The extract from pituitaries of seabass showed dominance of sbGnRH, corresponding to the fractions 23 and 24 (Fig. 1d).

The presence of multiple GnRH forms (sGnRH, cGnRH-II, and sbGnRH) in *L. calcarifer* brain was confirmed in the present study as reported in a number of modern teleosts (Powell *et al.*, 1994; Senthilkumaran *et al.*, 1999; Montaner *et al.*, 2001; Amano *et al.*, 2002; Pham *et al.*, 2007; Selvaraj *et al.*, 2009). The results also revealed that sbGnRH was the dominant form in the pituitary of *L. calcarifer*. This study, together with the above-mentioned reports, confirmed that the third GnRH form in the brain of most perciform fish was sbGnRH. The data obtained from the present work showed three populations of GnRH-ir cell bodies expressed in distinct regions of *L. calcarifer* brain: sGnRH in the anterior brain, sbGnRH in the POA and cGnRH-II in the midbrain. Localisations of sGnRH and sbGnRH-ir cell bodies were differential in the forebrain of *L. calcarifer*. Distribution of GnRH-ir cell bodies in *L. calcarifer* brain was similar to *D. labrax* (Gonzalez-Martinez *et al.*, 2002) and *S. japonicus* (Selvaraj *et al.*, 2009), which possessed sGnRH, cGnRH-II, and sbGnRH.

Immunohistochemical localisation of sGnRH-ir cell bodies and fibers

The schematic illustration of sGnRH-ir cell bodies and fibers is summarised in Fig. 2(a-c). sGnRH-ir cell bodies were located along the ventral medial part of the rostral olfactory bulb (OB), the terminal nerve ganglion (TNG)- the transitional area between the olfactory bulb and telencephalon (Fig. 3 a-d). These cell bodies were strongly immunostained and were clearly visible. sGnRH-ir fibers

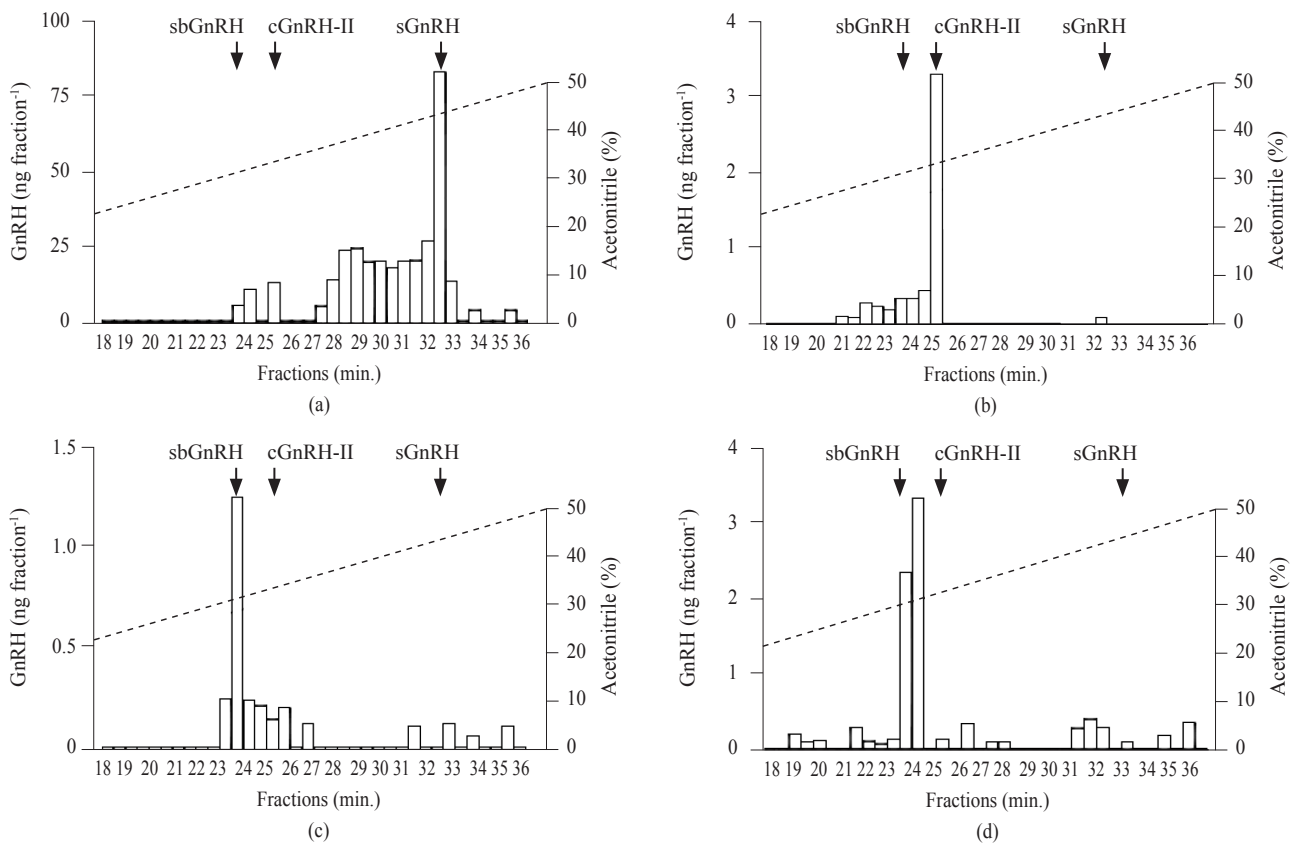


Fig. 1. Reverse-phase HPLC of GnRH immunoreactivity in the brain and pituitary extracts of *L. calcarifer*. Chromatograms show the elution position (minutes) and concentration of immunoreactive GnRH material (ng fraction^{-1}) detected by specific antisera to three GnRH forms. Arrows at the top of each chromatogram indicate the elution positions of synthetic GnRH standards. (a) Immunoreactive GnRH in the brain detected by sGnRH antiserum, (b) Immunoreactive GnRH in the brain detected by cGnRH-II antiserum, (c) Immunoreactive GnRH in the brain detected by sbGnRH antiserum, (d) Immunoreactive GnRH in the pituitary detected by sGnRH antiserum. The dotted lines represent percentage of CH_3CN (acetonitrile) containing 0.1% trifluoroacetic acid in the mobile phase

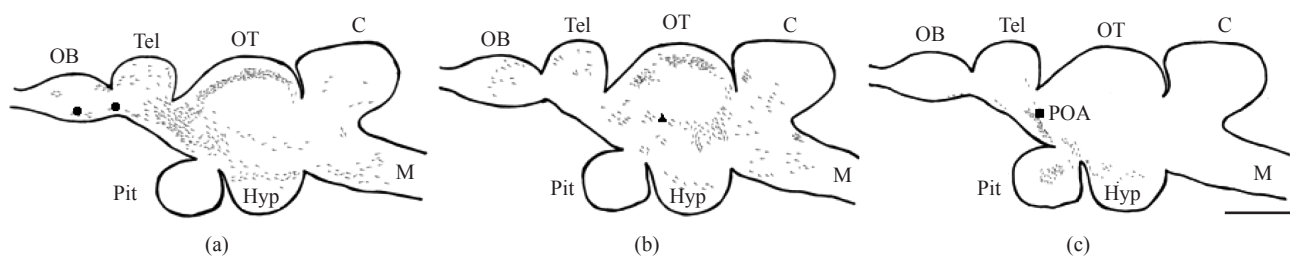


Fig. 2. Camera lucida drawing of the distribution of cell bodies (closed circles) and fibers (lines) in sagittal section for: (a) sGnRH-ir, (b) cGnRH-II-ir, (c) sbGnRH-ir. C: Cerebellum, Hyp: hypothalamus, M: medulla oblongata, MT: midbrain tegmentum, OB: olfactory bulb, OT: optic tectum, Pit: pituitary, POA: preoptic area, Tel: telencephalon, TNG: terminal nerve ganglion. Bars = 1 mm.

were distributed throughout the brain and were concentrated mainly in the anterior part of the brain. They were evident in the OB, telencephalon, POA and hypothalamus (Fig. 2a, 3c). In addition, long sGnRH-ir fibers were observed in the anterior POA, running caudal to the thalamus and hypothalamus with no clear destination (Fig. 3d). In the posterior brain, some sGnRH-ir fibers in the medulla oblongata and few in the cerebellum were observed.

Wide distribution of sGnRH-ir fibers in the brain suggested that sGnRH can function as a neuromodulator and/or neurotransmitter in *L. calcarifer* as presumed in several teleosts. However, the role of sGnRH is complicated and may be different among fish class. In fish with two GnRH forms, *i.e.*, sGnRH and cGnRH-II, besides being a neuromodulator, sGnRH also acts as a regulator of pituitary gonadotropin secretion (Amano *et al.*, 1991;

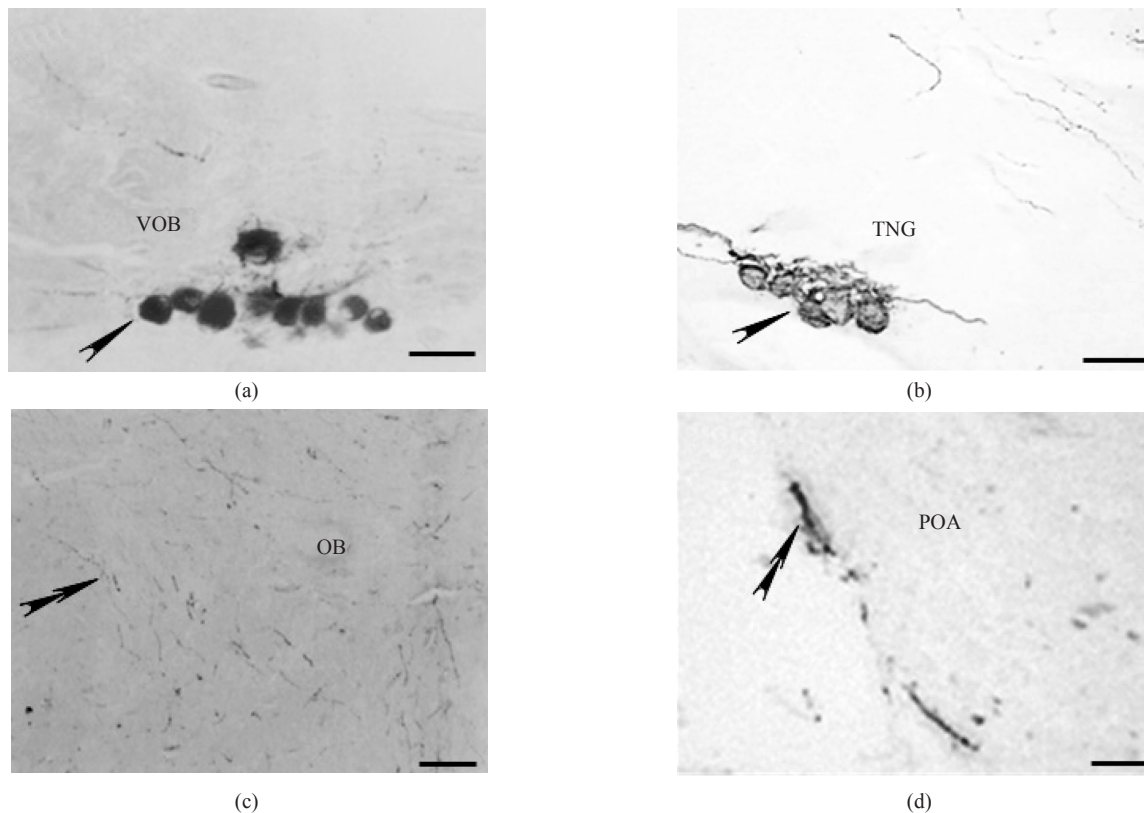


Fig. 3. Distribution of sGnRH-ir cell bodies (arrowheads) and fibers (double arrowheads) in sagittal sections. (a) sGnRH-ir cell bodies in the ventral olfactory bulb (VOB), (b) sGnRH-ir cell bodies in the TNG, (c) sGnRH-ir fibers in the OB and (d) sGnRH-ir fibers running parallel in the POA. Bars, 100 μm

Kim *et al.*, 1995) and other pituitary hormones (Marchant *et al.*, 1989; Weber *et al.*, 1997). For fishes with three different GnRH forms, especially multiple-spawners, the physiological functions of sGnRH have still been obscure. Similar to *V. moseri*, *P. olivaceus* and *D. labrax*, a certain sGnRH amount detected in the pituitary of *L. calcarifer* revealed the possible role of this hormone in regulating pituitary function in this species. In addition dense sGnRH-ir fibers in the anterior brain of *L. calcarifer* suggested that these fibers might have originated from the TN ir-cell population in the forebrain as found in other teleosts (Oka and Matshishima, 1993; Yamamoto *et al.*, 1995). It was found that sGnRH from the TN appears to serve complex functions such as regulating sexual behaviour in the goldfish *Carassius auratus* (Kobayashi *et al.*, 1994), controlling nest-building initiation in the dwarf gourami *Colisa lalia* (Yamamoto *et al.*, 1997) or coordinating sensory, motivational and visual system in the African cichlid *Haplochromis burtoni* (White *et al.*, 1995). Recently, it has also been shown that in *D. labrax*, retinopetal projection in the TN area suggests that TN-GnRH modulates dopaminergic cell activities and retinal function (Servili *et al.*, 2012). Moreover, the occurrence of the parallel sGnRH-ir fibers and

sbGnRH-ir fibers in the POA without entering the pituitary area is similar to that reported in *S. japonicus* (Selvaraj *et al.*, 2009). In *D. labrax* (Gonzalez-Martinez *et al.*, 2001) and Atlantic croaker *Micropogonias undulates* (Mohamed *et al.*, 2005), overlapping of sGnRH and sbGnRH mRNA expression patterns in the ventral telencephalon and POA has also been reported. Thus, it is necessary to investigate GnRH mRNA expression by using *in situ* hybridisation and GnRH receptor expression in GnRH neurons for clarifying the overlapping of sGnRH and sbGnRH-ir fibers in the POA in order to better understand the activity of these hormones in regulating physiological functions in *L. calcarifer*. Therefore, as in the above mentioned fishes, possible roles of sGnRH should be clarified in *L. calcarifer* too.

Immunohistochemical localisation of cGnRH-II-ir cell bodies and fibers

The schematic illustration of cGnRH-II-ir cell bodies and fibers is summarised in Fig. 2b. cGnRH-II-ir cell bodies were localised only in the midbrain tegmentum, lying close to the medial longitudinal fascicle (nMLF) (Fig. 4 a). cGnRH-II-fibers were distributed in many areas from the OB of the anterior part to the caudal medulla oblongata of

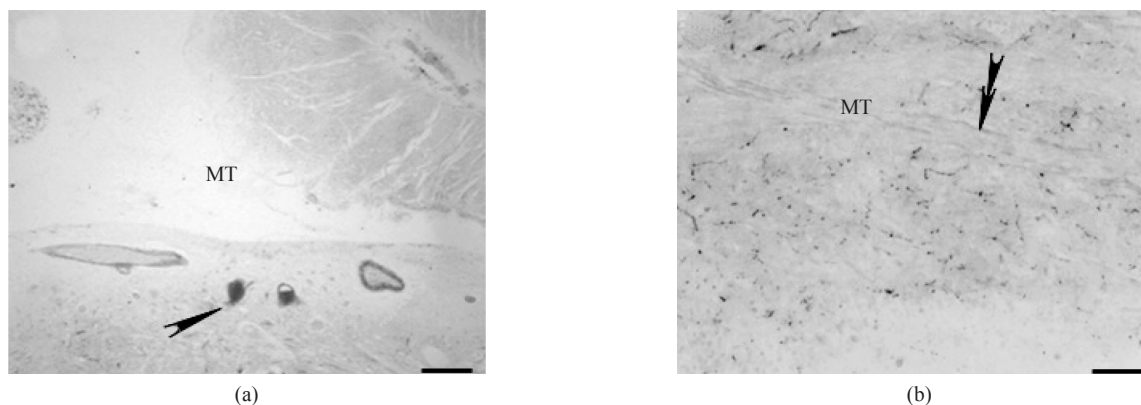


Fig. 4. Distribution of cGnRH-II-ir cell bodies (arrowhead) and fibers (double arrowhead) in sagittal sections. (a) cGnRH-II-ir cell bodies in the MT, (b) cGnRH-II-ir fibers in the midbrain. Bars, 100 μ m

the posterior part of the brain. They were conspicuous in the OB, dorsal telecephalon, hypothalamus, optic tectum thalamus and medulla oblongata. Density of cGnRH-II-ir fibers was higher in the midbrain than in the other regions (Fig. 4b). In the cerebellum, few cGnRH-II-ir fibers were observed.

Localisation of cGnRH-II cells in the midbrain and distribution of fibers in many parts of the brain of *L. calcarifer* are consistent with those obtained from other fishes such as the masu salmon *Oncorhynchus masou* (Amano *et al.*, 1991), *V. moseri* (Amano *et al.*, 2002), *D. labrax* (Gonzalez-Martinez *et al.*, 2001, 2002), *P. olivaceus* (Pham *et al.*, 2007) and *S. japonicus* (Selvaraj *et al.*, 2009). The widespread distribution of cGnRH-II-ir fibers within the central system in the brain of *L. calcarifer* also suggests a role of cGnRH-II in neuromodulation and or/neurotransmission as in the above-mentioned species. Moreover, the concentrated appearance of cGnRH-II fibers in the midbrain and hindbrain may also be involved in the auditory processing in the brain of this fish as found in damselfish *Abudefduf abdominalis* (Maruska and Tricas, 2011). Although cGnRH-II-ir fibers could be observed in the ventromedial hypothalamus, in the vicinity of the pituitary stalk, no cGnRH-II-ir fibers were detected in the pituitary of *L. calcarifer*. In addition, this hormone was undetectable in the pituitary. As such, these findings exclude the role of cGnRH-II in gonadotropic secretion in this species. However, cGnRH-II is the conservative form, suggesting complex roles in teleosts and vertebrates. For example, cGnRH-II is proposed to act as a regulator of pituitary function in *C. auratus* (Yu *et al.*, 1988), European eel *Anguilla anguilla* (Dufour *et al.*, 1993), tilapia *Oreochromis mossambicus* (Weber *et al.*, 1997), and striped bass *Morone saxatilis* (Chow *et al.*, 1998), or sexual behaviour in mammals, the marmoset *Callithrix jacchus* (Barnett *et al.*, 2005) and food intake in the musk shrew *Suncus murinus* (Kauffman

et al., 2005). On the other hand, in *S. aurata* (Zohar *et al.*, 1995), African catfish *Clarias gariepinus* (Goos *et al.*, 1997), and European seabass (Fornies *et al.*, 2003), cGnRH-II has the potential to stimulate GTH secretion both *in vivo* and *in vitro*. It was also found that cGnRH-II in the pituitary increased during pubertal development, showing a role of cGnRH-II in regulation of gonadal development in the striped bass (Holland *et al.*, 2001). In *D. labrax*, cGnRH-II is the modulator of pineal functions (Servili *et al.*, 2010).

Immunohistochemical localisation of sbGnRH-ir cell bodies and fibers

The schematic illustration of sbGnRH-ir cell bodies and fibers is given in Fig. 2c. sbGnRH-ir cell bodies were detected only in the POA (Fig. 5b). These cell bodies were heavily labeled. sbGnRH-ir fibers were concentrated in the POA and were projected to the pituitary. A bundle of axons from the POA running towards the pituitary through the POA and hypothalamus was observed (Fig. 5c). In the pituitary, sbGnRH-ir fibers were profuse in the neurohypophysis and invaded the proximal *pars distalis* of adenohypophysis (Fig. 5c, d).

Distribution of sbGnRH cell bodies in *L. calcarifer* is similar to that in perciform and pleuronectiform fishes possessing three GnRH forms such as the *S. aurata* (Gothilf *et al.*, 1996), red seabream *Pagrus major* (Senthilkumaran *et al.*, 1999), *D. labrax* (Gonzalez-Martinez *et al.*, 2002), *V. moseri* (Amano *et al.*, 2002), *P. olivaceus* (Pham *et al.*, 2007) and *S. japonicus* (Selvaraj *et al.*, 2009), where sbGnRH-ir cells were expressed only in the POA. Results of the present study show that sbGnRH-ir fibers innervate the pituitary, and that sbGnRH level is dominant in the pituitary of the *L. calcarifer*. These results are in agreement with most studies conducted in the above mentioned species, which reveal the projection of the axonal sbGnRH fibers arising from the POA to the

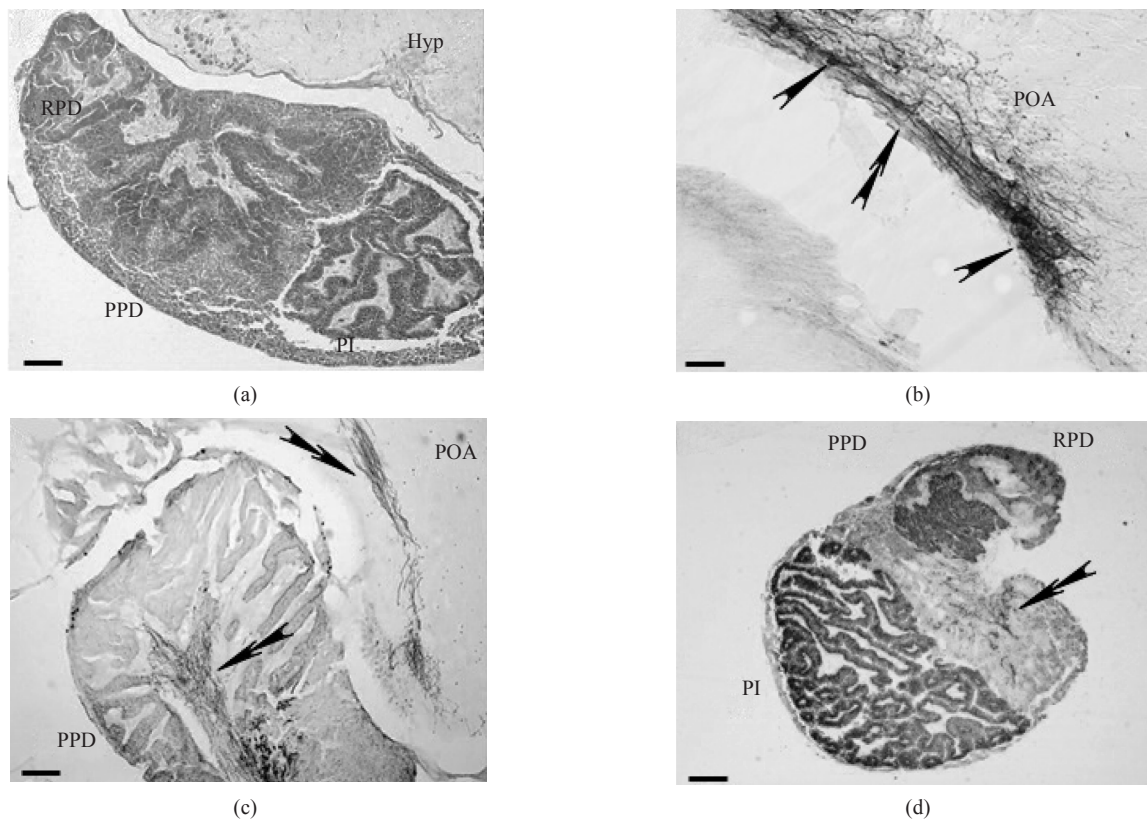


Fig. 5. Distribution of sbGnRH-ir cell bodies (arrowheads) and fibers (double arrowheads) in sagittal sections. (a) Cresyl violet stained section of the pituitary, RPD: rostral par distalis, PPD: proximal par distalis, PI: par intermedia, (b) Bundle of sbGnRH-ir fibers and sbGnRH-ir cell bodies in the POA, (c) Bundle of sbGnRH-ir fibers in the POA running toward the pituitary and sbGnRH-ir fibers in the PPD, (d) sbGnRH-ir fibers in the PPD of the pituitary. Bars, 100 µm

adenohypophysis, targeting the proximal *pars distalis* of the pituitary, and the major sbGnRH form in the pituitary. In addition, similar studies also showed that changes in the level of sbGnRH peptide and/or sbGnRH mRNA in the brains and pituitaries in these species were in accordance with ovarian development or seasonal reproductive activity. Moreover, it was demonstrated that sbGnRH cells in the POA were expressed simultaneously with the gonadal differentiation in association with maturation in *O. mossambicus* (Parhar, 1997), the cichlid *Cichlasoma dimerus* (Pandolfi *et al.*, 2002) and *S. aurata* (Wong *et al.*, 2004). All the findings suggest a role of sbGnRH in relation with reproduction by inducing gonadotropic secretion in teleosts. However, the role of sbGnRH and other GnRHs in reproductive physiology of the species should be verified by further investigations on change in GnRH peptide and/or the level of mRNA expression in the brain/pituitary during annual reproductive and spawning cycles. The origin of GnRH neuronal systems in *L. calcarifer* brain should be addressed by an ontogenic study to elucidate the precise roles of these GnRHs.

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