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Ontogenic Development of Three GnRH Systems in the Brain of a Pleuronectiform Fish, Barfin Flounder

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ABSTRACT—A pleuronectiform fish, the barfin flounder *Verasper moseri*, has three molecular forms of gonadotropin-releasing hormone (GnRH) in the brain, salmon GnRH (sGnRH), chicken GnRH-II (cGnRH-II) and seabream GnRH (sbGnRH). To elucidate the ontogenic origin of the neurons that produce these GnRH molecules, the development of three GnRH systems was examined by *in situ* hybridization and immunocytochemistry. Neuronal somata that express sGnRH mRNA were detected first in the vicinity of the olfactory epithelium 21 days after hatching (Day 21), and then in the transitional area between the olfactory nerve and olfactory bulb and the terminal nerve ganglion on Day 28. cGnRH-II mRNA-expressing neuronal somata were first identified in the midbrain tegmentum near the ventricle on Day 7. cGnRH-II-immunoreactive (ir) fibers were first found in the brain on Day 7. sbGnRH mRNA-expressing neuronal somata were first detected in the preoptic area on Day 42. sbGnRH-ir fibers were localized in the preoptic area-hypothalamus, and formed a distinctive bundle of axons projecting to the pituitary on Day 70. These results indicate that three forms of GnRH neurons have separate embryonic origins in the barfin flounder as in other perciform fish such as tilapia *Oreochromis niloticus* and red seabream *Pagrus major*: sGnRH, cGnRH-II and sbGnRH neurons derive from the olfactory placode, the midbrain tegmentum near the ventricle and the preoptic area, respectively.

Key words: ontogeny, GnRH neuron, immunocytochemistry, *in situ* hybridization, barfin flounder

INTRODUCTION

Gonadal maturation in teleost fish is primarily regulated by the brain-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) stimulates the synthesis and release of pituitary gonadotropin (GTH), and GTH stimulates the secretion of steroid hormones from the gonads. Recent study has shown that in teleosts, two or three molecular forms of GnRH exist even within the same species (see Okuzawa and Kobayashi, 1999).

Three GnRH neuronal systems exist in teleost fish judging from the location of GnRH neuronal somata and their projections (Oka, 1997); the terminal nerve (TN)-GnRH system, the midbrain tegmentum (MT)-GnRH system, and the preoptic area (POA)-GnRH system (in some species POA-GnRH neurons do not form a well-defined cell cluster, and in the present paper we define the ventral forebrain-GnRH system excluding the TN-GnRH system as the POA-GnRH system). The MT-GnRH system is the most conserved among the vertebrate species; it produces chicken GnRH-II (cGnRH-II) and is present in all the teleost fish examined to date. Among the three GnRH systems, the POA-GnRH system is considered to regulate GTH secretion, because it is the main system that projects directly to the pituitary (Oka, 1997). Perciform fish have three forms of GnRH, salmon

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GnRH (sGnRH), cGnRH-II and seabream GnRH (sbGnRH). With very few exceptions such as European sea bass *Dicentrarchus labrax* (González-Martínez *et al*., 2001), the TN-GnRH system and the POA-GnRH system are clearly distinguishable and the GnRH forms produced in these two systems are different; sGnRH is produced in the TN and sbGnRH in the POA. We have recently clarified that the medaka *Oryzias latipes* has three forms of GnRH, *i.e*., sGnRH, cGnRH-II and medaka GnRH (mdGnRH), expressed in the TN, the MT and the POA, respectively (Okubo *et al*., 2000). In contrast, in the other teleost species that have two forms of GnRH in the brain (*e.g*., salmonid fishes, eel, catfish), clear anatomical identification of the TN-GnRH system and POA-GnRH system is difficult, because the GnRH neurons located in the ventral forebrain are consecutive and the GnRH form(s) produced in these neurons are the same; sGnRH in salmonid fishes, mammalian GnRH (mGnRH) in the eel, and catfish GnRH (cfGnRH) in the catfish (see Okuzawa and Kobayashi, 1999). Recently, it has been called GnRH-I, GnRH-II and GnRH-III system instead of POA-, MT- and TN-GnRH system, respectively (Fernald and White, 1999).

Migration of GnRH neurons during ontogeny was first reported in mammals (Schwanzel-Fukuda and Pfaff, 1989; Wray *et al*., 1989): it has been suggested that mGnRH neurons are derived from the olfactory placode and migrate into the forebrain during prenatal development of the mouse. Then the ontogeny of the GnRH system has been studied in amphibian (Muske and Moore, 1990; Murakami *et al*., 1992), and avian brains (Murakami *et al*., 1991). Subsequently, it was also shown in teleost fish that sGnRH neurons are derived from olfactory placode in chum salmon *Oncorhynchus keta* (Chiba *et al*., 1994), sockeye salmon *Oncorhynchus nerka* (Parhar *et al*., 1995), masu salmon *Oncorhynchus masou* (Amano *et al*., 1998), medaka (Parhar *et al*., 1998), African cichlid *Haplochromis burtoni* (White and Fernald, 1998), tilapia *Oreochromis niloticus* (Parhar, 1999), red seabream *Pagrus major* (Okuzawa *et al*., 2002), European sea bass (González-Martínez *et al*., 2002) and cichlid fish *Cichlasoma dimerus* (Pandolfi *et al*., 2002).

However, not all forms of GnRH neurons in teleost fish originate from the olfactory placode as had originally been thought. For example, cGnRH-II neurons are reported to originate in the ventricular ependyma in the medaka (Parhar *et al*., 1998), tilapia (Parhar, 1999), African cichlid (White and Fernald, 1998), red seabream (Okuzawa *et al*., 2002), African catfish *Clarias gariepinus* (Dubois *et al*., 2001), European sea bass (González-Martínez *et al*., 2001) and cichlid fish(Pandolfi *et al*., 2002). Moreover, sbGnRH neurons are reported to have a basal diencephalic origin in tilapia (Parhar, 1999), red seabream (Okuzawa *et al*., 2002) and cichlid fish (Pandolfi *et al*., 2002). On the other hand, sbGnRH neurons are reported to migrate from the olfactory placode to the POA in African cichlid (White and Fernald, 1998) and European sea bass (González-Martínez *et al*., 2002). Thus, the origin of sbGnRH neurons is still controversial.

A pleuronectiform fish, the barfin flounder *Verasper moseri* is a large flat fish inhabiting cold sea basins around East Hokkaido, Japan. This species is promising for aquaculture and resource enhancement in Northern Japan due to its high commercial value. The barfin flounder experiences a metamorphosis early in development. We have found that the barfin flounder express three molecular forms of GnRH in the brain; sGnRH, cGnRH-II, and sbGnRH (Amano *et al*., 2002a). We have also found, using *in situ* hybridization and immunocytochemistry, that the TN-GnRH system, the MT-GnRH system and the POA-GnRH system are clearly distinguishable, as in perciform fish and medaka; sGnRH, cGnRH-II, and sbGnRH are expressed in the TN and the ventromedial part of the rostral olfactory bulbs, in the MT, and in the POA, respectively (Amano *et al*., 2002a, b). We showed that sbGnRH is physiologically the most important hypophysiotropic factor for the reproduction of the barfin flounder. This is because sbGnRH-ir fibers were localized mainly in the POA-hypothalamus-pituitary, forming a distinc-

Table 1. Sampling date, days after hatching (Days), mean total length (TL), mean body weight (BW), number of fish employed in *in situ* hybridization (ISH) and immunocytochemistry (ICC), and stage of development of barfin flounder

Sampling date	Days	TL(cm)	BW(g)	ISH			ICC			Stage of development
				sGnRH	cGnRH-II	sbGnRH	sGnRH	cGnRH-II	sbGnRH	
5 February 2001	0	0.43	0.0019	6	3	5	3	3	3	Newly hatched yolk-sac larva
12 February 2001	7	0.56	0.0022	9	3	9	4	4	4	Yolk-sac larva
19 February 2001	14	0.64	0.0025	10	3	9	4	4	4	Preflexion larva
26 February 2001	21	0.70	0.0040	10	3	9	4	4	4	Flexion larva
5 March 2001	28	0.95	0.014	9	3	9	3	3	4	Postflexion larva, onset of metamorphosis
12 March 2001	35	1.08	0.020	10	3	9	2	2	4	Postflexion larva, early metamorphosis
19 March 2001	42	1.53	0.073	0	0	12	2	2	4	Postflexion larva, late metamorphosis
26 March 2001	49	1.93	0.13	0	0	10	0	0	4	Juvenile
2 April 2001	56	2.40	0.17	0	0	10	0	0	4	Juvenile
9 April 2001	63	2.71	0.27	0	0	0	0	0	4	Juvenile
16 April 2001	70	3.39	0.42	0	0	0	0	0	4	Juvenile

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tive bundle of axons projecting to the pituitary, and the dominant form of GnRH in the pituitary measured by radioimmunoassay (RIA) was sbGnRH. sGnRH-immunoreactive (ir) fibers and cGnRH-II-ir fibers were observed throughout the brain but not in the pituitary gland, suggesting that they act as neuromodulators and/or neurotransmitters (Amano *et al*., 2002b; c.f. Oka, 1997). Furthermore, the amount of sbGnRH mRNA per brain increased two months before spermiation, and pituitary sbGnRH peptide content increased at spermiation (Amano *et al*., 2002c).

In the present study, we examined the ontogenic development of the three GnRH systems in the barfin flounder brain by *in situ* hybridization and immunocytochemistry.

MATERIALS AND METHODS

Fish

Barfin flounder that hatched on February 5 2001 at the Iwate Fisheries Technology Center, Iwate Prefecture, Japan were used. The fish were reared under a natural photoperiod in seawater of natural temperature. They were sampled on February 5 (Day 0), 12 (Day 7), 19 (Day 14), and 26 (Day 21), March 5 (Day 28), 12 (Day 35), 19 (Day 42), and 26 (Day 49), and April 2 (Day 56), 9 (Day 63) and 16 (Day 70), as shown in Table 1. Metamorphosis began on Day 28 and finished on Day 49.

In situ hybridization

Fish were anesthetized by immersion in 0.05% 2-phenoxyethanol. The entire head or isolated brain was fixed with 4% paraformaldehyde and 1% picric acid in 50 mM phosphate buffer (pH 7.3) at 4°C for 48 hr, and subsequently rinsed in cold 70% ethanol, dehydrated through graded ethanol and embedded in paraplast (Monoject, Sherwood Medical, St Louis, MO). Serial sagittal sections were cut at 5 or 7 μm, separated into several groups, and mounted on gelatinized slides. The hybridization, including counterstaining with cresyl violet, was done according to Amano *et al*. (2002a). Antisense oligonucleotide probes for sGnRH (5'-AGGTCTCTCTTGGGTTTGGGCACTCGCCTCCTCAGGAAGAGACA-3'), cGnRH-II (5'-CCTGAGAATGTTCCTCCTCTGGGGTCTCAAGTAGCTGCATTCCC-3'), and sbGnRH (5'-GATTCCTCTGCACCACCCAGAACACTGCAAGGCGAGTCCACACG-3') were labeled with the oligonucleotide 3'-end labeling kit (NEP-100) and [α-^{35}S]dATP (Dupont/ NEN Products, Tokyo, Japan). We applied 3.75 ng probe onto the sections. The specificity of the ISH method was previously confirmed (Amano *et al*., 2002a). The number of fish used for *in situ* hybridization is shown in Table 1. Our preliminary study suggests that sGnRH and cGnRH-II neurons first appeared before metamorphosis, while sbGnRH neurons first appeared after metamorphosis. Thus, we examined only sbGnRH neurons after metamorphosis in this study.

Immunocytochemistry

Immunocytochemistry was conducted using frozen sections according to Amano *et al*.(1991). Fish were anesthetized by immersion in 0.05% 2-phenoxyethanol. Heads or brains were fixed with a fixative that had been freshly prepared by mixing 40ml of 10% paraformaldehyde, 50ml of 0.2M phosphate buffer (pH 7.4), 7ml of saturated picric acid, and 3ml of distilled water for 48 hr at 4°C. The tissues were embedded in 15% gelatin dissolved in a 0.1M phosphate buffer (pH 7.4) and quickly frozen by immersion in powdered dry ice. Sagittal sections were cut at 50 μm on a cryostat, and all procedures were carried out using floating sections.

Antibodies to sGnRH (Lot no. 2), cGnRH-II (aCII6), and sbGnRH (AS-691) were used. Antibodies to sGnRH, cGnRH-II, and

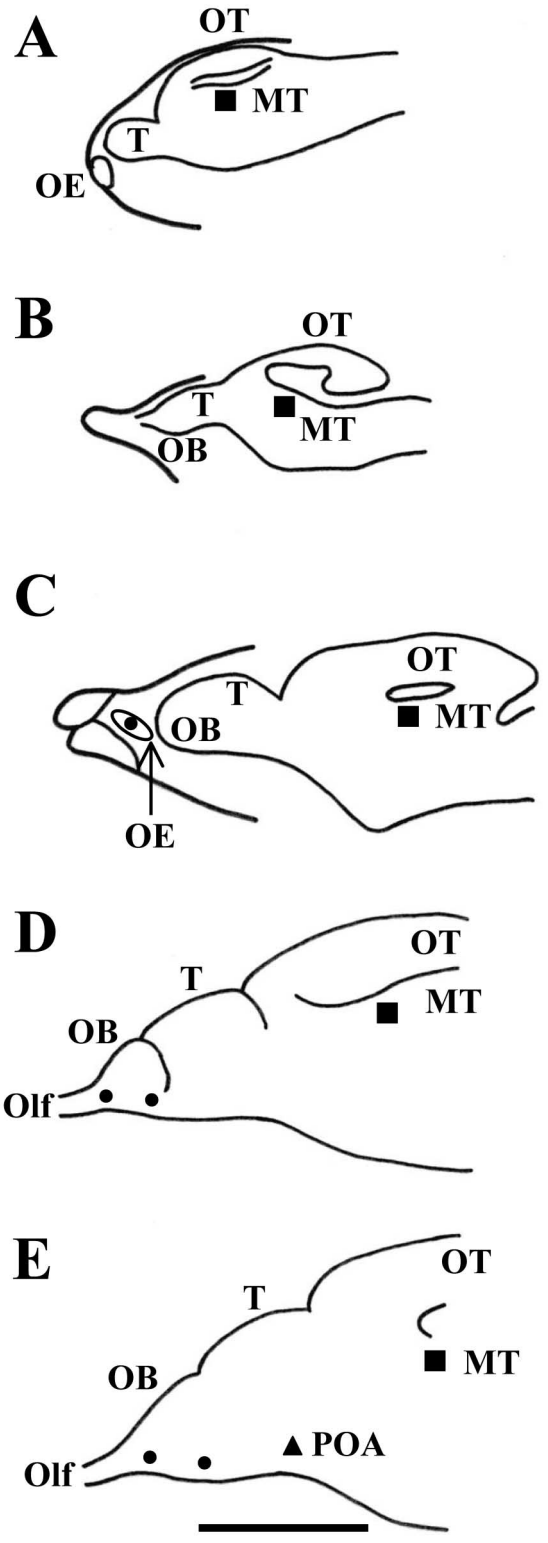

Fig. 1. Microprojections of a series of saggital sections through whole head of barfin flounder on Days 7 (A), 14 (B), 21 (C), 28 (D) and 42 (E), showing the distribution of neuronal somata expressing sGnRH (solid circle), cGnRH-II (solid square) and sbGnRH (solid triangle); MT midbrain tegmentum, OB olfactory bulb, OE olfactory epithelium, Olf olfactory nerve, OT optic tectum, POA preoptic area, T telencephalon. Bar indicates 0.5 mm.

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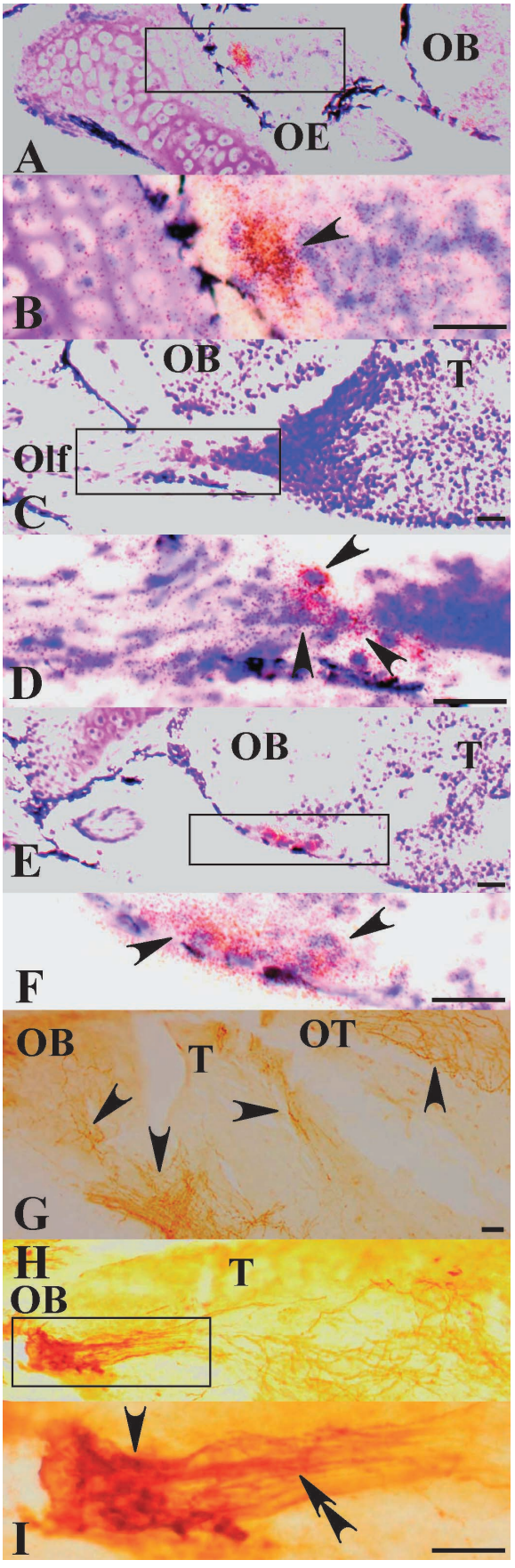

sbGnRH were diluted 10000, 5000, and 10000-fold, respectively, with 0.1M phosphate buffer (pH 7.4) containing 0.75% NaCl and 0.3% Triton X-100. For immunocytochemical reactions, a Histofine immunostaining kit (Nichirei, Tokyo, Japan) was used. The specificity of the immunoreaction was previously confirmed (Amano *et al.*, 2002b). The number of fish used for immunocytochemistry is shown in Table 1.

RESULTS

sGnRH system

A schematic drawing of the distribution of sGnRH neurons is shown in Fig. 1. Neuronal somata expressing sGnRH mRNA were first detected in the vicinity of the olfactory epithelium on Day 21 (Fig. 2A, B). They were then observed in the transitional area between the olfactory nerve and the olfactory bulb (Fig. 2C, D) and in the terminal nerve ganglion (Fig. 2E, F) on Day 28. On Day 21, sGnRH-ir fibers were observed to run caudally from the vicinity of olfactory epithelium to the various brain regions such as telencephalon, optic tectum-thalamus and hypothalamus (Fig. 2G). On Day 28, sGnRH-ir neuronal somata were detected in the terminal nerve ganglion and were observed to send fibers to all brain regions except the pituitary, such as optic tectum, hypothalamus, midbrain, cerebellum and medulla oblongata (Fig. 2H, I).

cGnRH-II system

A schematic drawing of the distribution of cGnRH-II neurons is shown in Fig. 1. On Day 7, neuronal somata expressing cGnRH-II mRNA were first detected in the midbrain tegmentum near the ventricle (Fig. 3A, B), and cGnRH-II-ir fibers were running caudally in the brain (Fig. 3C). Afterwards, cGnRH-II neuronal somata were observed in the midbrain tegmentum (Fig. 3D, E).

sbGnRH system

A schematic drawing of the distribution of sbGnRH neurons is shown in Fig. 1. sbGnRH mRNA-expressing neuronal somata (Fig. 4A, B) and sbGnRH-ir neuronal somata (Fig. 4C) were first detected in the preoptic area on Day 42

Fig. 2. (A) Sagittal section through the olfactory epithelium on Day 21. (B) Higher magnification of A. Neuronal somata expressing sGnRH mRNA are observed in the vicinity of the olfactory epithelium (single arrowhead). (C) Sagittal section through the olfactory nerve and olfactory bulb on Day 28. (D) Higher magnification of C. Neuronal somata expressing sGnRH mRNA are observed in the transitional area between the olfactory nerve and olfactory bulb (single arrowheads). (E) Sagittal section through the terminal nerve ganglion on Day 28. (F) Higher magnification of E. Neuronal somata expressing sGnRH mRNA are observed in the terminal nerve ganglion (single arrowheads). (G) Sagittal section through the brain on Day 21. sGnRH-ir fibers (arrowheads) are observed. (H) Sagittal section through the terminal nerve ganglion on Day 28. (I) Higher magnification of H. sGnRH-ir neuronal somata (arrowheads) and fibers (double arrowheads) are observed. OB olfactory bulb, OE olfactory epithelium, Olf olfactory nerve, OT optic tectum-thalamus, T telencephalon. Bars indicate 20 μm.

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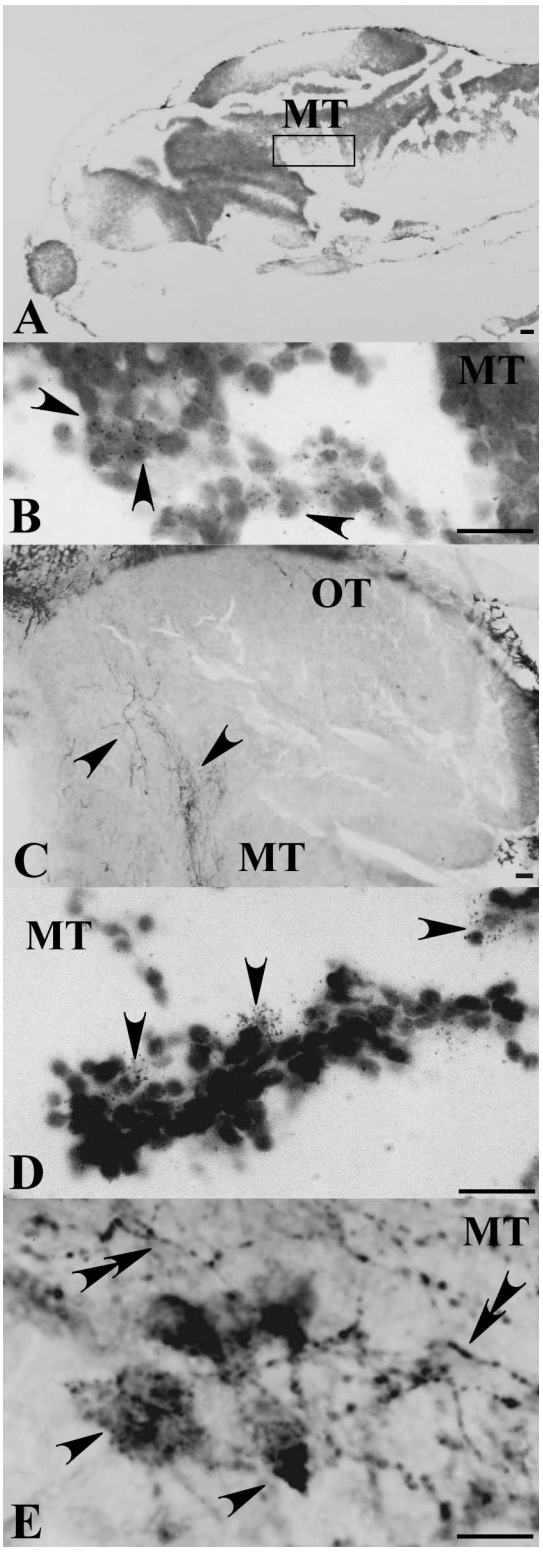

Fig. 3. (A) Sagittal section through the brain on Day 7. (B) Higher magnification of A. Neuronal somata expressing cGnRH-II mRNA are observed in the midbrain tegmentum near the ventricle (single arrowheads). (C) cGnRH-II-ir fibers running caudally in the brain on Day 7 (single arrowheads). (D) Neuronal somata expressing cGnRH-II mRNA in the midbrain tegmentum on Day 21 (single arrowheads). (E) cGnRH-II-ir neuronal somata (single arrowheads) and fibers (double arrowheads) in the midbrain tegmentum on Day 21. MT midbrain tegmentum, OT optic tectum-thalamus. Bars indicate 20 μm.

and Day 49, respectively. Few sbGnRH-ir fibers were observed on Day 49. sbGnRH-ir fibers were localized in the preoptic area-hypothalamus, forming a distinctive bundle of axons projecting to the pituitary on Day 70 (Fig. 4D, E).

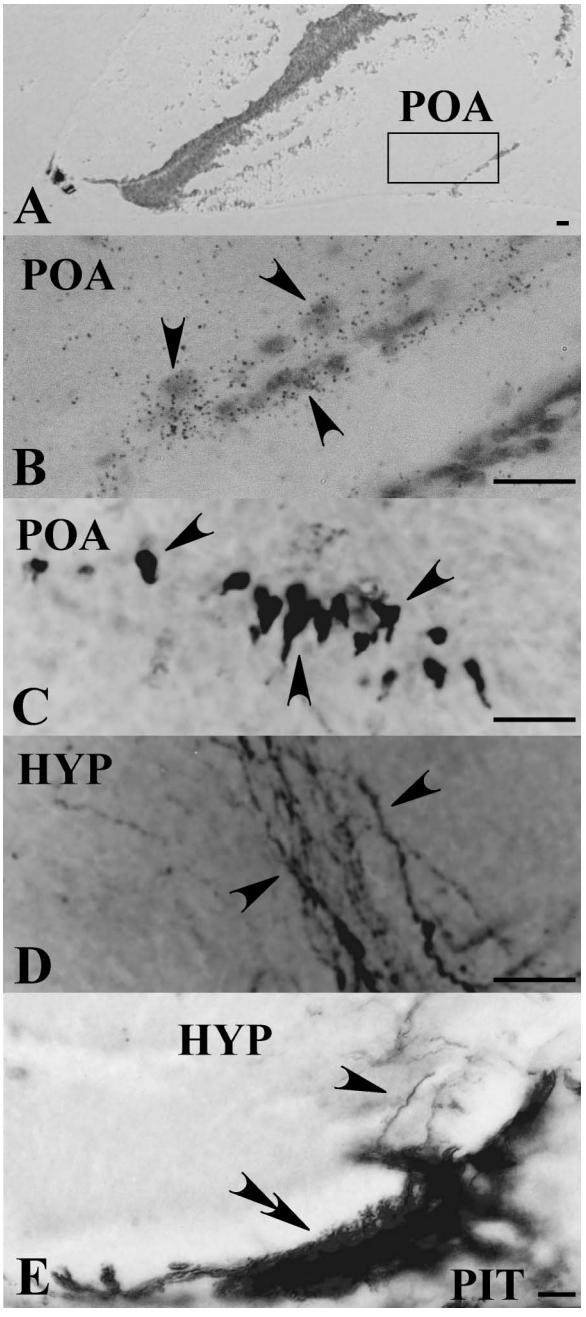

Fig. 4. (A) Sagittal section through the preoptic area on Day 42. (B) Higher magnification of A. Neurons expressing sbGnRH mRNA are observed in the preoptic area (single arrowheads). (C) sbGnRH-ir neuronal somata in the preoptic area on Day 49 (single arrowheads). (D) sbGnRH-ir fibers in the preoptic area-hypothalamus, which formed a distinctive bundle of axons projecting to the pituitary on Day 70 (single arrowheads). (E) sbGnRH-ir fibers in the hypothalamus (single arrowhead) and the pituitary (double arrowhead) on Day 70. HYP hypothalamus, PIT pituitary, POA preoptic area. Bars indicate 20 μm.

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DISCUSSION

In the present paper, we have shown morphological evidence indicating that neurons belonging to three different GnRH systems have separate embryonic origins in the barfin flounder: sGnRH, cGnRH-II and sbGnRH neurons derive from the olfactory placode (TN-GnRH system), the midbrain tegmentum near the ventricle (MT-GnRH system), and the preoptic area (POA-GnRH system), respectively. We have also shown here that the time of appearance differs according to the system or molecular form; cGnRH-II/MT-GnRH neurons appeared first, then sGnRH/TN-GnRH neurons were observed, and lastly sbGnRH/POA-GnRH neurons were detected.

It has been shown that the sGnRH neurons (TN-GnRH system) have a common origin among fish species. sGnRH neurons are first detected in the olfactory placode or olfactory epithelium in all the teleost fish species examined to date including barfin flounder, *e.g.*, chum salmon (Chiba *et al*., 1994), sockeye salmon (Parhar *et al*., 1995), masu salmon (Amano *et al*., 1998), medaka (Parhar *et al*., 1998), African cichlid (White and Fernald, 1998), tilapia (Parhar, 1999), red seabream (Okuzawa *et al*., 2002) and European sea bass (González-Martínez *et al*., 2002). In the present study, as in the case of masu salmon (Amano *et al*., 1998), sGnRH-ir neuronal somata could not be detected in the early developmental stages by immunocytochemistry, although immunoreactive fibers were already detectable. It should be noted that sGnRH neuronal somata were detected earlier by *in situ* hybridization than by immunocytochemistry. These results suggest that the synthesis of sGnRH begins in the early developmental stages but the synthesized peptides are immediately transported to the axons for release.

It has also been shown that the origin of cGnRH-II neurons (MT-GnRH system) is the same in all fish species. cGnRH-II neurons were first detected in the MT during ontogeny in all the teleost species examined to date including the barfin flounder; medaka (Parhar *et al*., 1998), tilapia (Parhar, 1999), African cichlid (White and Fernald, 1998), red seabream (Okuzawa *et al*., 2002), African catfish (Dubois *et al*., 2001), and European sea bass (González-Martínez *et al*., 2002).

As for sbGnRH neurons in the barfin flounder, we have shown here that they are derived from the POA, as in red seabream (Okuzawa *et al*., 2002) and tilapia (Parhar, 1999). On the other hand, it has been reported that sbGnRH neurons derive from the olfactory placode and migrate into the brain in African cichlid (White and Fernald, 1998) and European sea bass (González-Martínez *et al*., 2002). Although the possibility cannot be ruled out that sbGnRH neurons migrate without expressing sbGnRH in the barfin flounder, the origin of sbGnRH neurons (POA-GnRH system) may differ according to the fish species.

Our findings suggest that a time lag exists between sbGnRH synthesis and its release. sbGnRH-ir neuronal somata were first detected in the preoptic area, but few sbGnRH-ir fibers were observed on Day 49. On Day 70, sbGnRH-ir fibers were detected in the preoptic area-hypothalamus, forming a distinctive bundle of axons projecting to the pituitary. Morphological differentiation of the gonad into either ovary or testis beccomes distinguishable at 35 mm in total length in the barfin flounder (Goto *et al*., 1999). Thus, it is possible that sbGnRH is involved in this, because the mean total length on Day 70 was 33.9 mm. Indeed, in tilapia, it has been suggested that sbGnRH is involved in sex differentiation (Parhar, 1997).

In barfin flounder, sGnRH neurons (TN-GnRH system) and cGnRH-II neurons (MT-GnRH system) were detected at the early stages of development, suggesting that they have some physiological function in the brain during ontogeny. In adult barfin flounder, both sGnRH-ir fibers and cGnRH-II-ir fibers were distributed widely in the brain (Amano *et al*., 2002b), suggesting that they function as neuromodulators. Indeed in a perciform fish, the dwarf gourami *Colisa lalia*, the TN-GnRH (sGnRH) neurons project widely in the brain and have spontaneous oscillatory activities (Oka and Matsushima, 1993), and Oka (1997) suggests that the TN-GnRH system functions as a neuromodulator. The biological function of the MT-GnRH system is not known in teleost fish, although it has been suggested to be involved in sexual behavior in a primitive mammal, the musk shrew *Suncus murinus* (Dellovade *et al*., 1995). More precise studies are necessary to clarify the function of the MT-GnRH system in teleost fish.

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