cDNA cloning and mRNA expression of neuropeptide Y in orange spotted grouper, *Epinephelus coioides*

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Abstract

A full-length cDNA encoding the neuropeptide Y (NPY) was cloned from the hypothalamus of orange spotted grouper (*Epinephelus coioides*) by rapid amplification of cDNA ends approaches. The NPY cDNA sequence is 688 bp long and has an open reading frame of 300 bp encoding prepro-NPY with 99 amino acids. The deduced amino acid sequences contain a 28-amino-acids signal peptide followed by a 36-amino-acids mature NPY peptide. mRNA expression of NPY was determined using semi-quantitative RT-PCR followed by Southern blot analysis. NPY mRNA was expressed in olfactory bulb, telencephalon, pituitary, hypothalamus, optic tectum–thalamus, medulla oblongata, cerebellum and spinal cord. Low levels of NPY mRNA expression were found in retina, ovary and stomach, while much lower levels of expression were detected in liver, heart, gill, skin, anterior intestine, thymus and blood. No NPY mRNA expression was observed in unfertilized eggs, newly fertilized eggs, 16-cells stage and morula stage of the embryo and lower levels of expression were detected in the blastula, gastrula and neurula stages. It was highly expressed from lens formation stage to 52-day-old larval stage. NPY might be involved in the late embryonic and larval development of the orange spotted grouper.

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Keywords: cDNA cloning; Development; mRNA expression; Neuropeptide Y; Orange spotted grouper; Reverse transcription-polymerase chain reaction; Southern blot; Tissues distribution

1. Introduction

Neuropeptide Y (NPY) is a 36-amino acid peptide belonging to the NPY family, which also includes the peptide YY (PYY), tetrapod pancreatic polypeptide (PP) and the fish pancreatic polypeptide Y (PY, Cerda-Reverter et al., 2000a). NPY is one of the most highly conserved neuroendocrine peptides (Hoyle, 1999). It is involved in the regulation of a variety of physiological functions, such as food intake, sexual behavior, blood pressure, sympathetic nervous system tone and circadian rhythms in mammals (Larhammar, 1996). NPY is widely present in the central and peripheral nervous system, especially abundant in the brain, and is also found in the peripheral tissues including eyes, thyroid gland, pancreas, ovary, kidney, skin and spleen in mammals (Dumont et al., 1992; Haefliger et al., 1999; Malmström, 2001).

In fish, it has been reported that NPY stimulates growth hormone (GH) and gonadotropin-II (GTH-II) secretion in the goldfish pituitary (Peng et al., 1993), while ovarian steroids stimulate NPY gene expression in the telencephalon–preoptic area (Peng et al., 1994). NPY was also observed to stimulate leuteinizing hormone (LH) secretion in sea bass, *Dicentrarchus labrax* (Cerdá-Reverter et al., 1999) and was shown to induce gonad reversal in the protogynous bluehead wrasses, *Thalassoma bifasciatum* (Kramer and Imbriano, 1997). Moreover, increased NPY gene expression was observed in the brain with fasting in goldfish, *Carassius auratus*, (Narnaware and Peter, 2001a).
as well as salmon, Oncorhynchus tshawytscha, (Silverstein et al., 1998). Intracerebro-ventricular (ICV) injection of NPY dose-dependently stimulated food intake in goldfish, indicating that NPY is an important factor regulating feeding behavior and food intake in fish (López-Patiño et al., 1999; Namaware and Peter, 2001b).

Recently, several studies indicated that NPY might be involved in the embryogenesis and postnatal development in mammals and fish (Takei et al., 1996; Chiba et al., 1996b,c; Mathieu et al., 2002; Neveu et al., 2002; Kurokawa and Suzuki, 2002; Grove et al., 2003). The NPY-like immunoreactivities were found in the brain, olfactory organ and retina of zebrafish, Danio rerio, during development (Mathieu et al., 2002). In the Japanese flounder, Paralichthys olivaceus, it was shown that NPY mRNA was expressed in the brain and retina during the larval stage (Kurokawa and Suzuki, 2002). The information on the changes of NPY expression in embryonic and larval development stages of fish is still limited.

In situ hybridization and Northern blot studies have shown that NPY mRNA is mainly expressed in the brain of adult fish, particularly in the telencephalon—preoptic (TEL—POA), optic tectum—thalamus (OT—THAL) and hypothalamus (HYP) of goldfish (Peng et al., 1994; Namaware and Peter, 2002), chinook salmon (Silverstein et al., 1998), sea bass (Cerdá-Reverter et al., 2000b), zebrafish (Soderberg et al., 2000), rainbow trout, Oncorhynchus mykiss (Doyon et al., 2003), and river lamprey, Lampetra fluviatilis (Söderberg et al., 1993). The NPY expression seems to be confined to the neural tissues of the sea bass (Cerdá-Reverter et al., 2001). NPY is widely distributed in the central nervous system of fish. Immunohistochemical studies have identified NPY-like immunoreactivity in the brain of several fish species (Pontet et al., 1989; Danger et al., 1991; Chiba and Honma, 1992, 1994; Chiba et al., 1996a; Chiba, 1997; Vallarino et al., 1998; Marchetti et al., 2000; Pirone et al., 2004; Traverso et al., 2003; Gaikwad et al., 2004). NPY-immunoreactive cells were also detected in the stomach and intestine of the sea bass (Visus et al., 1998) and in the anterior intestine of five species of Osteoglossomorpha (Al-Mahrouki and Youson, 1998). However, there is limited information on NPY expression in peripheral tissues or neural tissues in adult fish.

The orange spotted grouper, Epinephelus coioides, a protogynous hermaphroditic fish, is a commercially important marine teleost and popular cultured fish in Southern China. To accelerate the reproduction and improve the growth of grouper, we used the orange spotted grouper as an experimental model and studied the genes related to reproduction and growth of this fish. For the purpose of investigating the role of NPY in the grouper, in the present study, the full-length cDNA encoding orange spotted grouper NPY was cloned and sequenced. Subsequently, the presence of NPY mRNA was examined in the embryonic and larval stages and the distribution of NPY mRNA was determined in the central nervous system and peripheral tissues from 2-year-old orange spotted groupers.

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2. Materials and methods

2.1. Sample preparation

Female 2-year-old orange spotted groupers, E. coioides, ranging from 550 to 650 g body mass, 29–32 cm body long and with 0.015–0.018% gonadal somatic index, were obtained from Guangdong Daya Bay Fishery Development Center. Unfertilized eggs, embryos and larval groupers from 1- to 52-day-old were obtained from May to June 2003, during reproductive season. Tissue samples for NPY mRNA distribution were obtained from 2-year-old groupers. These samples were frozen immediately in liquid nitrogen and stored at −80 °C before RNA extraction.
2.2. NPY cDNA cloning

2.2.1. Total RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from the orange spotted grouper hypothalamus with Trizol® reagent (RNA Extraction Kit, Invitrogen, USA). The concentration of the total RNA was estimated by measuring the absorbance at 260 nm. Five micrograms of isolated RNA were reverse transcribed using GeneRacer® Kit (Invitrogen).

2.2.2. Rapid amplification of cDNA end (RACE)

To obtain the sequence of full-length NPY cDNA from the orange spotted grouper, nested 3′ and 5′ RACE PCR was performed.

The 3′-end of grouper NPY cDNA was amplified by 3′ RACE PCR. Two degenerate sense primers (Y001-F and Y002-F, Table 1) and anti-sense primers (GeneRacer® Kit 3′ primers, Table 1) for nested PCR reaction were used. The design of the two degenerate sense primers based on the conserved NPY cDNA sequence from the known NPY of sea bass (GenBank accession number: AJ005381), zebrafish (D. rerio) (NP571149), carp (Cyprinus carpio, Q9DGK7) and goldfish (C. auratus) (P28672).

The first round of 3′ RACE PCR reaction were performed in a total volume of 50 µL PCR mixture containing 5 µL of 10 × MBI PCR buffer with (NH₄)₂SO₄, 4 µL of 25 mM MgCl₂, 1 µL of 10 mM dNTP mix, 2.5 µL of 10 µM each primer (Y001-F, GeneRacer® Kit 3′ first primer, Table 1), 30.75 µL of sterile deionized water and 1.25 U Taq DNA Polymerase (MBI Fermentas, USA), using 4 µL of a 1 : 100 dilution of RACE-ready first strand cDNA as template. The first PCR conditions were the following: 5 min initial denaturation at 94 °C for one cycle, then 30 cycles of denaturation at 94 °C, primer annealing at 57.1 °C for 30 s and extension at 72 °C for 60 s, and finally 10 min

Fig. 1. Amplified products of RACE PCR for orange spotted grouper NPY. (A) 3′ RACE PCR production. (B) 5′ RACE PCR production. M: 100 bp DNA ladder marker; 1: first round PCR products; and 2: nested PCR products.

Fig. 2. The cDNA sequence and deduced amino acid sequence of the orange spotted grouper prepro-NPY (DNAStar). Deduced amino acid sequence of mature NPY is underlined. The translation stop codon is designated with an asterisk (*). The putative signal for polyadenylation (ATTAAT) is doubly underlined. The cDNA sequence is in the GenBank Sequence Databases under accession no.AY626561.
final extension at 72 °C for one cycle. The PCR reaction was carried out in a PTC-200 thermocycler (MJ Research, Watertown, MA, USA).

Using 4 µL of 1:100 dilution of the first PCR product as nested PCR template, the 3′RACE nested PCR reaction was performed under the same conditions except using Y002-F instead of Y001-F and GeneRacer™ Kit 3′ nested primer instead of GeneRacer™ Kit 3′ first primer (Table 1). The 3′ nested PCR product of the predicted size was gel-separated and purified with E.Z.N.A® Gel Extraction Kit (Omega BioTek, USA) and inserted into pGEM®-T Easy Vector (Promega, USA). Positive clones containing the expected size inserts were sequenced using T7 and Sp6 primers.

To extend the grouper cDNA sequence in the 5′ direction, two specific antisense primers (Y003-R and Y004-R, Table 1) based on the 3′ sequenced region were used. The first round 5′ PCR was carried out for 5 min initial denaturation at 94 °C for one cycle, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 45 °C for 30 s and extension at 72 °C for 60 s, and finally 10 min final extension at 72 °C for one cycle, using GeneRacer™ Kit 5′ first primer and primer Y003-R.

The 5′ first round PCR product was diluted for 50 times and amplified again under the same conditions except using GeneRacer™ Kit 5′ nested primer instead of GeneRacer™ Kit 5′ first primer and primer Y004-R instead of Y003-R (Table 1). The primer annealing temperature used for the nested PCR was 62 °C. The 5′ nested PCR product of the predicted size was gel-separated, purified, and inserted into a pGEM®-T Easy Vector. Then the positive clones were sequenced.

2.3. Sequence analysis

The cDNA sequence and the deduced amino acid sequence were compared with the sequences in the GenBank database using BLAST program available for the NCBI Internet website. Mutipile alignments of cDNA sequences and amino acid sequences were performed using the programs of DNAstar and Clustalx. The phylogenetic tree of prepro-NPY was constructed based on their amino acid sequences with the programs Phylip, Clustalx, Bioedit and Treeview. The cDNA sequence data encoding orange spotted grouper NPY was deposited under GenBank accession number: AY626561.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Semi-quantitative RT-PCR assays were performed to measure the level of mRNA expression of orange spotted grouper NPY. Total RNAs of embryo, larvae and 26 tissues...
were isolated, respectively, using Trizol® reagent (Invitrogen, U.S.A). Two micrograms of total RNA was incubated with DNase I and then reverse transcribed with ThermoScript™ RT-PCR System (Invitrogen, U.S.A). First strand cDNAs were used as templates for PCR with orange spotted grouper NPY specific upstream and downstream primers (Y010-F and Y004-R, Table 1), which amplified a PCR product of 252 bp.

RT-PCR were conducted in a 10 µL total reaction mixture containing 1 µL of 10× MBI PCR buffer with (NH₄)₂SO₄, 0.6 µL of 25 mM MgCl₂, 0.2 µL of 10 mM dNTP mix, 0.2 µL of 10 µM each primer, 6.75 µL of sterile deionized water, 0.25 U Taq DNA polymerase (MBI Fermentas, USA) and 1 µL of each first strand cDNA. The PCR conditions were similar to those described for cloning of the orange spotted grouper NPY cDNA except that of 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s and extension at 72 °C for 45 s. An internal control PCR reaction was performed in a separate tube, using two specific 18S ribosomal RNA primers (18S-U and 18S-D, Table 1). The PCR condition for 18S ribosomal RNA was the same as the grouper NPY PCR, except for using the 30 cycles instead of 35 cycles. The specific primers for 18S ribosomal RNA amplified a PCR product of 250 bp in all of the tissues examined.

Each RT-PCR analysis was repeated three times with independently extracted RNA samples from different orange spotted groupers. The RT-PCR products were separated on 2.0% agarose gels, followed by ethidium bromide staining, then detected under ultraviolet light with Gel Doc 2000 (BioRAD, U.S.A). The cDNAs band densities were measured using Quantity one software. The NPY mRNA levels were expressed as the ratio between NPY mRNA and the mRNA of 18S ribosomal RNA (internal control) with the same sample. Data are represented as means±S.E.M. (n=3).

2.5. Southern blot analysis

In order to confirm the authenticity of the mRNA expression of the orange spotted grouper NPY, Southern blot analysis of RT-PCR products was conducted. The cDNA probe was labeled with digoxigenin (PCR DIG Probe Synthesis Kit, Roche, Germany), using Y010-F and Y004-R as primers (Table 1).

RT-PCR products were separated on 2.0% agarose gels, then transferred to a positive charged nylon membrane (Roche, Germany) and crosslinked for 5 min in a UV crosslinker (UVP, U.S.A). After being prehybridized for 2 h, hybridization with a Dig-labeled orange spotted grouper NPY cDNA probe was carried out overnight in a total volume of 10 mL hybridization buffer containing 2.5 mL of 20× SSC, 0.1 mL of 10% N-lauroyl sarcosine, 1 mL of 10× blocking solution, 5 mL of deionized formamide, 20 µL of 10% SDS and 1.38 mL of sterile deionized water at 50 °C. After that, the membrane was washed twice with 2× SSC/0.1% SDS at room temperature for 5 min, twice with 0.5× SSC/0.1% SDS at 68 °C for no longer than 15 min, and once with washing buffer/Tween 20 at room temperature for 5 min. Afterwards, the membrane was blocked for 90 min with 1% blocking solution in washing buffer/Tween 20. For signal detection, the membrane was

Table 2

Amino acid sequence percent identity of orange spotted grouper prepro-NPY compared to other vertebrates

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The identity was calculated with MegAlign of DNAStar soft.
placed in detection buffer, shaken at room temperature for 5 min, and placed on a sheet of filter paper. The membrane was air-dried briefly and applied with diluted 1:100 CDP star (Roche). Signal was captured in Syngene Genenome (Syngene, England).

3. Results

3.1. Cloning and characterization of the orange spotted grouper NPY cDNA

To obtain the sequence of full-length NPY cDNA from the orange spotted grouper, RACE PCR was performed in the 3′ and 5′ directions. The 3′ RACE PCR generated a single cDNA fragment of 448 bp, including partial of ORF (open reading frame) and full part of 3′-untranslated region (Fig. 1A). The 5′ RACE PCR extended the cDNA sequence in the 5′ direction with a single cDNA fragment of 334 bp and provided the complete peptide precursor sequence (Fig. 1B).

The NPY cDNA obtained from the orange spotted grouper is 688 bp long and has an ORF of 300 bp long encoding prepro-NPY with 99 amino acids (Fig. 2). The signal peptide consists of 28 amino acids followed by the 36 amino acids of mature NPY. The proteolytic processing site Gly-Lys-Arg is followed by 32 amino acids that comprise the carboxy-terminal extension denoted CPON (for C-terminal peptide of NPY). The 5′-untranslated region is 63 bp long and the 3′-untranslated region is 325 bp long with a single consensus ATTAAT polyadenylation signal.

3.2. Comparison of NPY amino acid sequences in vertebrates

The homology analysis based on the amino acid sequences revealed that the predicted orange spotted grouper prepro-NPY displayed significant identities to other vertebrates (Fig. 3, Table 2). It showed the highest homology of 97% to both of the prepro-NPY from flounder and sea bass, while it had lower identities to carp (64.6%), zebrafish (63.5%), goldfish (60.4%), electric ray, Torpedo marmorata, (56.1%), catfish, Ictalurus punctatus, (51.6%) and lamprey (35.4%). The orange spotted grouper prepro-NPY is 64.9%, 63.9%, 62.9%, 62.2%, and 61.9% homologous to the prepro-NPY from

![Phylogenetic tree](image)

Fig. 4. Phylogenetic tree of vertebrate prepro-NPY obtained using Phylip software by maximum parsimony method. The tree was generated by ClustalX1.8 and depicted visually by TreeView1.6.6. Numbers indicate bootstrap values from 100 replications. The lamprey NPY was used as outgroup.
sheep, *Ovis aries*, mouse, *Mus musculus*, monkey, *Macaca mulatta*, rat, *Rattus norvegicus*, and human, *Homo sapiens*, respectively, and 64.9% to chicken, *Gallus gallus*, 62.9% to *Typhlonectes natans*, and 59.8% to frog, *Xenopus laevis*. The predicted mature orange spotted grouper NPY peptide differed at only one amino acid from the flounder NPY sequence, two amino acids from the sea bass, and five amino acids from the human. Twenty-four amino acids of the mature NPY peptide are conserved among the vertebrate species aligned in Fig. 3.

Fig. 4 shows the phylogenetic analysis of prepro-NPY in vertebrates. The tree is rooted with the prepro-NPY sequence of lamprey, a cyclostome fish. The orange spotted grouper NPY belongs to the cluster of teleostean NPY and was closely related to sea bass NPY.

### 3.3. Tissue distribution of the orange spotted grouper NPY gene transcript

To examine the distribution of NPY mRNA in the orange spotted grouper central nervous system and in the peripheral tissues, its expression level was analyzed in 26 dissected tissues by RT-PCR followed by Southern blot analysis.
Different areas of the grouper brain, including the olfactory bulb, telencephalon, pituitary, hypothalamus, optic tectum–thalamus, medulla oblongata, cerebellum and spinal cord, were dissected. As shown in Figs. 5 and 7A, NPY mRNA was detectable in all these areas. The strongest signal was observed in the hypothalamus, less intense amplification signals were found in the olfactory bulb, telencephalon, medulla oblongata and cerebellum, and much lower levels of expression were detected in the pituitary, optic tectum–thalamus and spinal cord.

In the peripheral tissues, low levels of NPY mRNA expression were found in the retina, ovary and stomach, while much lower levels of expression were detected in the liver, heart, gill, skin, anterior intestine, thymus and blood. No NPY mRNA was detected in the spleen, kidney, red muscle, white muscle, middle intestine, posterior intestine and fat (Figs. 6 7B).

The level of 18S ribosomal RNA transcript was similar among the 26 different tissues (Figs. 5 and 6). No PCR product was detected in the negative control (PCR without DNA template).

3.4. Expression of NPY mRNA during the orange spotted grouper embryonic and larval development

The presence of NPY mRNA was examined in grouper embryos and larval stages, using RT-PCR followed by Southern blot analysis. No NPY mRNA expression was observed in the unfertilized eggs, the newly fertilized eggs, 16-cells stage and morula stage of the grouper embryo, while low levels of expression were detected in the blastula, gastrula and neurula stages (Figs. 8 and 10A). The gene was highly expressed from lens formation stage to 52-day-old larval stage (Figs. 8–10). The 18S ribosomal RNA of the orange spotted grouper was amplified as the internal control by RT-PCR. This RT-PCR analysis showed that the signal intensity of the grouper 18S ribosomal RNA transcript was present in the grouper embryonic and larval stages at similar levels (Figs. 8,9).

4. Discussion

The full-length cDNA encoding the NPY precursor was cloned from the hypothalamus of orange spotted grouper using RACE PCR. The orange spotted grouper NPY cDNA sequence is 688 bp long and has an ORF (open reading frame) of 300 bp long encoding prepro-NPY with 99 amino acids. The deduced amino acid sequences contains a 28-amino-acids signal peptide, a 36-amino-acids mature NPY peptide, a potential amidation–proteolytic site (Gly-Lys-Arg), and a 32-amino-acids carboxy-terminal extension denoted CPON (for C-terminal peptide of NPY). It showed high amino acid identity with the NPY of other fish. Moreover, the discrimination between NPY and PYY or PY sequences can also be based on the amino acid residue present at amino acid fourteen of their mature peptides. It is an invariable proline residue in all PYY or PY sequences but displays an alanine residue in the NPY sequence (except for trout NPY which has a threonine) (Larhammar et al., 1993; Cerdá-Reverter et al., 2000a). The deduced amino acid sequence of orange spotted grouper in the present study exhibits an alanine residue, which supports its identity to the NPY sequence. Amino acid alignment and phylogenetic analysis indicate that the orange spotted grouper NPY displays highest degree of homology with flounder and sea bass. The present study on the tissue distribution of NPY gene transcript, using RT-PCR followed by Southern blot analysis, revealed that the orange spotted grouper NPY mRNA was expressed in all areas of central nervous system examined, including the olfactory bulb, telencephalon, hypothalamus, optic tectum–thalamus, medulla oblongata, cerebellum and spinal cord. The expression pattern of the NPY gene in the olfactory bulb, telencephalon, hypothalamus and optic tectum–thalamus is similar to that reported for goldfish (Peng et al., 1994), salmon (Silverstein et al., 1998), sea bass (Cerdá-Reverter et al., 2000b), zebrafish (Soderberg et al., 2000) and rainbow trout (Doyon et al., 2000).
The NPY mRNA had also been detected in the medulla oblongata and cerebellum of sea bass (Cerdá-Reverter et al., 2000b) and rainbow trout (Doyon et al., 2003).

NPY mRNA was not detected in the pituitary of goldfish (Peng et al., 1994) and rainbow trout (Doyon et al., 2003), using Northern blot analysis and ribonuclease protection assay, respectively. However, the NPY-like immunoreactivities were found in the pituitary of goldfish (Pontet et al., 1989), cloudy dogfish, Scyliorhinus torazame (Chiba and Honma, 1992), white sturgeon, Acipenser transmontanus (Chiba and Honma, 1994), bichir, Polypterus senegalus (Chiba, 1997), juvenile zebrafish (Mathieu et al., 2002) and catfish (Gaikwad et al., 2004). The presence of NPY mRNA and immunoreactivity in rat pituitary cells has been reported (Jones et al., 1989; O’halloran et al., 1990; O’Connor et al., 1995) and it has been suggested that NPY may exert an autocrine/paracrine effect in controlling pituitary hormone secretion in rat (Peng et al., 1994). In the present study, low levels of NPY mRNA were observed in the pituitary and spinal cord of the orange spotted grouper. Although several studies have identified that NPY-containing cells and NPY mRNA expressing neurons were present within the spinal cord in rat (Marti et al., 1992; Rowan et al., 1993; Minson et al., 2001), the presence of NPY mRNA in the spinal cord has not been reported previously in fish. Our study is the first example to demonstrate the expression of NPY mRNA in the pituitary and spinal cord of fish. NPY in the central nervous system was suggested to be involved in the regulation of food intake and controlling pituitary hormone secretion in fish (Peng et al., 1993; Cerdá-Reverter et al., 1999; López-Patiño et al., 1999; Lin et al., 2000; Namaware and Peter, 2001a, b). However, further investigation and more information are required for understanding the NPY expression pattern in the central nervous system and pituitary of the orange spotted grouper.

In order to investigate the NPY physiological functions in the grouper, the NPY mRNA expression in the different peripheral tissues was analyzed. The result showed that the NPY mRNA expressed in 10 peripheral tissues. The NPY expression pattern in the peripheral tissues in the orange spotted grouper is different from that reported previously in sea bass (Cerdá-Reverter et al., 2001) and Japanese flounder (Kurokawa and Suzuki, 2002). No NPY expression was observed in the ovary, muscle, liver, heart, spleen, intestine, fat and kidney by Northern blot analysis, while the transcript was detected in the brain of the sea bass (Cerdá-Reverter et al., 2001). In adult Japanese flounder, NPY mRNA was expressed in the brain and not expressed in the intestine and pancreas and NPY mRNA could be detected in the brain and the retina of eyes in the early larval stage of flounder using RT-PCR and in situ hybridization (Kurokawa and Suzuki, 2002). NPY expression seems to be confined to cells derived from the neural crest in sea bass (Cerdá-Reverter et al., 2001) and adult flounder (Kurokawa and Suzuki, 2002). In mammals, NPY mRNA is abundantly expressed in the central and peripheral nervous system but not restricted to the neurons. It was reported to be expressed in the astrocyte, kidney, ovary, eyes, thyroid gland, pancreas, spleen and other cells of the immune system and involved in the regulation of these tissues’ functions (Ericsson et al., 1987; Dumont et al., 1992; Barnea et al., 1998; Haefliger et al., 1999; Malmström, 2001). The present study on the distribution of NPY mRNA in peripheral tissues is consistent with the notion that NPY gene could be expressed in non-neuronal cells (Barnea et al., 1998). NPY mRNA expression were found in the retina, ovary, stomach, liver, heart, gill, skin, anterior intestine, thymus and blood. NPY might play a paracrine or autocrine role in the regulation of these 10 peripheral tissues in the orange spotted grouper. The expression pattern in tissues of the adult orange spotted grouper extends the NPY expression areas known in fish.

Previous studies in rat reported that the expression of NPY and its receptor were detected as early as the embryonic stage and increased gradually until postnatal life and NPY immunoreactivity was also present from the early stage of development (Marti et al., 1992; Tong et al., 1997; Neveu et al., 2002). It suggested an involvement of NPY in the development of mammals (Naveilhan et al., 1998; Neveu et al., 2002). In the Japanese flounder, it was shown that NPY mRNA was expressed in the brain and retina during the larval stage (Kurokawa and Suzuki, 2002) and the NPY immunoreactive system gradually increase from the ontogeny to juvenile stages and reach complete development in the mature zebrafish (Mathieu et al., 2002). NPY-immunoreactive cerebrospinal fluid-contacting neurons first appeared in the embryo at the 34 mm stage of cloudy dogfish (Chiba et al., 1996b). The present results showed that no NPY mRNA expression was observed in the unfertilized eggs, the newly fertilized eggs, 16-cells stage and morula stage of the orange spotted grouper embryo, while lower levels of expression were detected in the blastula, gastrula and neurula stages. It was highly expressed from lens formation stage to 52-day-old larval stage, which is the stage of the completion of metamorphosis of the grouper. It is indicated that NPY may be involved in the development of the orange spotted grouper. The effect of NPY on late embryonic and larval development in the orange spotted grouper remains to be further investigated.

In conclusion, the present study provides the nucleotide sequence for orange spotted grouper NPY cDNA and shows that there is a high degree of homology with other fish species at the amino acid level. The expression of NPY mRNA in different compartments of the brain and 10 different peripheral tissues of the adult orange spotted grouper suggests that NPY might act either in a paracrine or autocrine fashion. The presence of NPY mRNA at the late embryonic and larval development stages of the orange spotted grouper indicates the role of NPY as a potential regulator during development.
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