Developmental expression of the G protein-coupled receptor 54 and three GnRH mRNAs in the teleost fish cobia

J Shaik Mohamed, Abby D Benninghoff, G Joan Holt and Izhar A Khan

Marine Science Institute, University of Texas at Austin, 750 Channel View Drive, Port Aransas, Texas 78373, USA

(Requests for offprints should be addressed to J S Mohamed; Email: shaikm@utmsi.utexas.edu)

Abstract

The cDNAs of the G protein-coupled receptor 54 (GPR54) and three prepro-gonadotropin-releasing hormones, GnRH-I (seabream GnRH), GnRH-II (chicken GnRH-II), and GnRH-III (salmon GnRH) were isolated and cloned from the brain of the teleost fish cobia, *Rachycentron canadum*. The cobia GPR54 cDNA was 95 and 51–56% identical to those of tilapia and mammalian models respectively. The GnRH cDNA sequences of cobia showed strong identities to those of tilapia, Atlantic croaker, red drum, and the seabass and seabream species. The real-time quantitative RT-PCR methods allowed detection of all three GnRH mRNAs on the first day after hatching (DAH). The GnRH-I mRNA levels, which were the lowest among the three GnRHs, increased gradually with two distinct peaks in larvae at 3 and 4 DAH. On the other hand, GnRH-II and GnRH-III mRNAs were significantly higher in larvae at 2 and 6 DAH compared with those on the preceding days. In addition, significant peaks of all the three GnRH mRNAs were observed in the brains of 26-day-old fish. The finding of higher GnRH-I and GnRH-II mRNAs in males than females at 153 DAH may be related to early puberty observed during the first year in laboratory-reared male cobia. Moreover, this study demonstrates for the first time the expression of GPR54 mRNA during larval development in a vertebrate species. The concomitant expression patterns of GPR54 and GnRH mRNAs during different stages of larval and juvenile developments, and during early puberty in male cobia suggest a potential relationship between GPR54 and multiple GnRHs during these stages of development consistent with the role of GPR54 in controlling GnRH release in mammals. The increase in GPR54 and GnRH mRNAs observed during early puberty in cobia is consistent with a similar change reported in pubertal rats. This finding together with the localization of GPR54 mRNAs on GnRH neurons in fish and mammals suggests that the GPR54–GnRH interactions may be conserved in different vertebrate groups.

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Introduction

Considerable evidence has accumulated in the past few years demonstrating that the G protein-coupled receptor 54 (GPR54) plays a critical role in the control of gonadotropin-releasing hormone (GnRH) release at the onset of puberty in mammals. Mutations in *GPR54* gene reduce circulating luteinizing hormone (LH) levels and cause hypogonadotropic hypogonadism and delayed puberty in humans and mice (Seminara *et al.* 2004). Studies with GPR54 mutant mice have also demonstrated impairment of GnRH and LH release, which can be reversed by exogenous administration of GnRH (Colledge 2004, Seminara *et al.* 2004). Moreover, GPR54 and GnRH mRNA expressions have been co-localized in the GnRH neurons in rat and a cichlid fish, thereby providing the morphological basis for potential functional interactions between GPR54 and multiple GnRHs (Irwig *et al.* 2004, Parhar *et al.* 2004). Interestingly, significant changes in GPR54 mRNA expression during different stages of postnatal development have been demonstrated in rat (Navarro *et al.* 2004). Further, GPR54 mRNA increases in the hypothalamus of rhesus monkey

from the juvenile to mid-pubertal stage (Shahab *et al.* 2005). However, the expression of GPR54 mRNA during larval or embryonic development has not been reported for any vertebrate species.

At least two molecular forms of GnRH are present in the brains of all vertebrate species, with some teleosts expressing three different forms, i.e. seabream or whitefish GnRH (GnRH-I), chicken GnRH-II (GnRH-II), and salmon GnRH (GnRH-III). Differential distribution of the three forms of GnRH has been studied in the brains of several species belonging to the order Perciformes (White *et al.* 1995, González-Martínez *et al.* 2001, Vickers *et al.* 2004, Mohamed *et al.* 2005, Mohamed & Khan 2006). In addition, the GnRH-I and GnRH-III neuronal elements have been shown to overlap in certain anterior forebrain regions, including the preoptic area (POA) of European seabass, Atlantic croaker and red drum (González-Martínez *et al.* 2001, Mohamed *et al.* 2005, Mohamed & Khan 2006). The POA GnRH-I is considered the major hypophysiotropic hormone that controls the synthesis and release of follicle stimulating hormone (FSH) and LH in all vertebrates, and GnRH-I neurons have been shown to innervate the anterior pituitary in teleosts

(Gothilf *et al.* 1997, Amano *et al.* 2002, González-Martínez *et al.* 2002). Considering the fact that virtually all neurons in the POA of teleosts innervate the pituitary gland (Prasada Rao 1999) and that GnRH-III innervation has been detected in the European seabass pituitary (González-Martínez *et al.* 2002), it is possible that the GnRH-III neurons in the POA may also participate in hypophysiotropic functions. However, most studies to date suggest non-hypophysiotropic roles for GnRH-III (Stell *et al.* 1987, Yamamoto *et al.* 1997). In addition, GnRH-II has been implicated in a variety of non-hypophysiotropic roles such as sensory motor activity, sexual behavior, and neurotransmitter, neuromodulator and immune functions (Muske & Moore 1994, White *et al.* 1995, 1998, Rissman & Li 1998, Yu *et al.* 1998, Chen *et al.* 2002). In addition to the potential roles of the GnRHs in adult fish, their significance during larval development has been demonstrated by the knockdown of the two *GnRH* genes in zebrafish, which results in defective mid-hindbrain boundary, underdeveloped eyes and defective heart (Sherwood & Wu 2005).

Several studies in fish have reported the expression of multiple GnRH mRNAs during different stages of larval development using *in situ* hybridization (ISH) techniques (González-Martínez *et al.* 2002, Wong *et al.* 2004). The results of these and similar other ISH studies show that the cells expressing GnRH mRNAs can be detected in several-day-old fish larvae. On the other hand, a recent study in gilthead seabream using a real-time RT-PCR method has demonstrated the expression of three GnRH mRNAs before the second day after hatching (Wong *et al.* 2004). These studies suggest that GnRHs may play important roles in some aspects of early larval development. However, similar information is presently lacking for other teleosts.

Cobia is an emerging aquaculture species and the only known representative of the family Rachycentridae in the order Perciformes. Cobia is a gonochoristic species with males attaining puberty within the first year under laboratory conditions, while females take 2–3 years to mature (unpublished observation). They are highly fecund with a protracted spawning season beginning from April and extending to September (Faulk & Holt 2003). Considering the importance of GnRH(s) in the control of reproduction, understanding the GnRH systems in cobia may help in the development of innovative tools for a better reproductive management of this species in aquaculture. In addition, GPR54 is considered the 'new gatekeeper of reproduction' in mammals and is directly linked to the control of puberty (Colledge 2004, Seminara *et al.* 2004). Therefore, the aims of the present study were to: (i) isolate and clone the cDNAs encoding GPR54 and three different GnRH variants in cobia, (ii) study the expression of GPR54 and GnRH mRNAs during different stages of early larval development by real-time

quantitative RT-PCR, and (iii) determine the GPR54 and GnRH mRNA levels in the brains of cobia at juvenile and early pubertal stages.

Materials and methods

Animals and sample collection

The maintenance and spawning of cobia brood stock was performed as described previously (Arnold *et al.* 2002). For initial cDNA isolation and cloning experiments, whole brains (~450 mg each) from four gonadally mature adult fish were collected in liquid nitrogen, and maintained at –80 °C until used for total RNA isolation. Eggs from a single spawn were used for subsequent experiments with larval, juvenile, and early pubertal fish. Following the evening spawn, eggs were collected the next morning and transferred to individual 150 l conical tanks for rearing at a concentration of approximately 1000 eggs per tank. Seawater salinity was adjusted to match the spawning salinity (32 ppt), and temperature was maintained at 25 °C. The photoperiod was set to a 1400 h light:1000 h darkness cycle. Cobia larvae were reared on *Brachionus plicatilis* (Muller) and *Artemia* sp. nauplii following the general procedures described in Faulk & Holt (2005).

Samples at different developmental stages were collected 1, 2, 3, 4, 6, 8, 10, 12, 14, 18, 22, 26, 30, 70, 105, and 153 days after hatching (DAH). Cobia larvae were anesthetized with 0.1% tricaine methanesulfonate (MS-222) prior to sampling. Standard length measurements were taken to the nearest 0.1 mm using a stereomicroscope with Summa Sketch III digitizing tablet (GTCO CalComp, Inc., Columbia, MD, USA) and Sigma Scan software (Jandel Corporation, San Rafael, CA, USA). For fish larger than 16 mm, standard length was determined using a vernier caliper (Fowler, Chicago, IL, USA). Larvae of similar size were collected on each sampling day in order to avoid possible variability in developmental stages. For samples collected at 1–8 DAH, 30 individual larvae were measured and the whole heads were frozen in liquid nitrogen. For each of the three biological replicates ($n=3$), ten individual larvae were pooled. For 10–18 DAH, 15 individual larvae were measured and the whole heads were pooled in groups of five each. Individual brain samples were isolated in larger fish (>20 mm) beginning with 22 DAH. All samples were stored at –80 °C until RNA extraction. Gonad samples of cobia at 153 DAH were preserved in 10% buffered formalin for histological examination after routine hematoxylin and eosin staining. Fish were killed under deep anesthesia following experimental procedures approved by the Animal Care and Use Committee of the University of Texas at Austin.

Total RNA isolation

Total cellular RNA used for the first strand cDNA synthesis was isolated from the whole brains of adult fish using TRIzol reagent (Invitrogen) as per the manufacturer's directions and stored at -80°C . Total RNA from the whole heads (1–18 DAH) and brains (22–153 DAH) was isolated using Absolutely RNA Mini Prep Kit (Stratagene, La Jolla, CA, USA) as per the manufacturer's instructions and stored at -80°C until used for one-step real-time reverse transcription-PCR (real-time RT-PCR) assays.

Rapid amplification of cDNA ends (RACE)

First-strand cDNAs were synthesized from 5 µg total RNA using the RNA ligase mediated (RLM)-RACE system (GeneRacer kit, Invitrogen) according to the manufacturer's protocol and stored at -20°C until used for PCRs. The primers used in all the PCR reactions are shown in Table 1. Gene-specific primers (GSPs) for 5'RACE and full length cDNAs were synthesized based on the sequence information of the 3'RACE and 5'RACE PCR products. All PCRs were performed using a PCR master mix (Promega) in a Mastercycler gradient (Eppendorf Scientific Inc., Westbury, NY, USA) thermocycler using a temperature cycle profile of 94°C for 2 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min followed by 72°C for 7 min. The final holding temperature was 4°C .

Cloning and sequencing of PCR products

All PCR products were purified from 1.2% agarose gel using MinElute Gel Extraction Kit as per the manufacturer's instructions (Qiagen, Valencia, CA, USA). The purified PCR products were cloned into pGEM-T Easy vector plasmid and transformed into *Escherichia coli* JM 109 competent cells (Promega). The plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen). Both strands of the plasmid DNA containing candidate GPR54 and GnRH cDNA inserts were sequenced by T7 and SP6 universal primers. The homology search of GPR54 and GnRH cDNAs of cobia was performed by basic local alignment search tool (<http://www.ncbi.nlm.nih.gov/BLAST>). The sequences of GPR54 and three GnRH cDNAs were submitted to the GenBank (Accession numbers: GPR54, DQ790001; GnRH-I, AY677175; GnRH-II, AY677174; GnRH-III, AY677173).

Phylogenetic analyses

Sequences encoding different vertebrate GPR54 and GnRH cDNAs were obtained from GenBank. The sequence identities of GPR54 and GnRH cDNAs of cobia and other vertebrates were confirmed by CLUSTAL

Table 1 Nucleotide sequence of primers

Primers ^a	GnRH-I	GnRH-II	GnRH-III	Usage
DP1	5'-CARCAYTGGTCITAYGGICTIAG-3' (257)	5'-CARCAYTGGTCITAYGGITGGTA-3' (362)	5'-CARCAYTGGTCITAYGGITGGYT-3' (212)	3'RACE
DP2	5'-TAYGGICTIAGCCGIGG-3' (245)	5'-CAYGGITGGTAYCCGIGG-3' (350)	5'-TAYGGITGGYTICGIGG-3' (200)	3'RACE
GSP1	5'-CAGTGTGTCTGAAGGCTGTCCAGTTC-3' (191)	5'-GAAGGCTTGAACGCTGCTACCTTCTCC-3' (402)	5'-TTGGTTTGGGCACTCGCCTCTTCA-3' (227)	5'RACE
GSP2	5'-TCTGAAGGCTGTCCAGTTCCTCTTC-3' (184)	5'-TCTGGCTAAGGATCCAAAGAAATG-3' (367)	5'-GGGTTTGGGCACTCGCCTCTTCA-3' (225)	5'RACE
GSP3	5'-GAAACAAAGGACTGAAGACGAAC-3' (382)	5'-ACAGTAATAGCCCACTCTG-3' (539)	5'-TGCTAACAGGCAATACAGAGC-3' (308)	Full length
GSP4	5'-CAAAGGACTGAAGACGAACAAATC-3' (377)	5'-CAGTAATAGCCCACTCTGTA-3' (538)	5'-GCTAACAGGCAATACAGAGCT-3' (307)	Full length
GSP5	5'-CTGGTGTGACGACTGGTCAT-3' (207)	5'-GCTACTTGAGACCGCAGAGG-3' (217)	5'-GCAGAGTGTGGTGCAGGTA-3' (122)	qRT-PCR
GSP6	5'-CGTCCATTTTCCCTGTCTGT-3'	5'-AGGCTTGAACGCTGTCACTT-3'	5'-GATGGTTGCTTCCAGCTCTC-3'	qRT-PCR
GPR54-F	5'-GCATTTGGATCGGTTCTTCG-3' (442)			Partial
GPR54-R	5'-TAGGACATGCAGTTGCCAC-3'			Partial
GPR54 GSP-1	5'-CCAGTTCATTGCTGCCTACC-3' (520)			3'RACE
GPR54 GSP-2	5'-TTCACTGCTGCTACCTGCTCCT-3' (688)			qRT-PCR
GPR54 GSP-3	5'-CCAGTTCATTGCTGCCTACC-3' (165)			qRT-PCR
GPR54 GSP-4	5'-ATCTTGGGGACTTGTCTCCT-3'			qRT-PCR
Actin-F	5'-GAGCTGGCTACTCTCTTCAAC-3' (233)			qRT-PCR
Actin-R	5'-CAGGACTCCATACCGAGGAA-3'			qRT-PCR

^aDP, degenerate primer; GSP, gene specific primer; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; F, forward; R, reverse; R = A or G; Y = C or T; I = A, T, C or G; number within the parenthesis indicates size of the PCR product.

W alignment program with default settings (<http://www.ebi.ac.uk/clusterw/index.html>).

Synthesis of cRNAs

Synthesis of sense GPR54 and GnRH cRNAs was performed using Riboprobe *in vitro* transcription systems (Promega) as described previously (Mohamed *et al.* 2005). The plasmid DNA from the transcriptional reaction was removed by digestion with TURBO DNase I (Ambion, Austin, TX, USA) for 15 min at 37 °C. The cRNAs were purified by MEGA clear kit (Ambion) and stored at -80 °C until used for real-time RT-PCR. The cRNA concentrations were estimated using spectrophotometer (SPECTRAMax 190, Molecular Devices, Sunnyvale, CA, USA) at 260 nm and on 1.4% denatured formaldehyde agarose gel with a known RNA weight marker. The weight of a single molecule of each cRNA was calculated by dividing the molecular weight with 6.022×10^{23} (Avogadro's number).

Real-time RT-PCR

The GPR54 and GnRH mRNA levels were estimated using the Mx3000P quantitative PCR system (Stratagene). After optimization of each of the qRT-PCR primer pairs (Table 1) for both cRNA and sample RNA, reactions were assayed using Brilliant SYBR Green QRT-PCR Master Mix Kit (Stratagene) in a 25 µl reaction mixture containing 12.5 µl 2× SYBR-QRT-PCR master mix, 50 nM of each primer, 0.375 µl diluted reference dye (ROX, 1:500), 0.0625 µl StrataScript RT/RNase block enzyme mixture, and 100 ng total RNA or 5 µl cRNA. The thermocycle profile was of 50 °C for 30 min, 95 °C for 10 min, and 40 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 30 s. Melting curve analysis was also included at one cycle of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. After every assay, the RT-PCR products were checked on 2% agarose gel to verify the size of the amplified products. A set of standards were run in every plate and sample RNAs normalized with β-actin. Copy number of the target mRNA in the unknown sample was determined by comparing average C_t (threshold cycles) values of unknown samples to the specific standard.

Six tenfold serially diluted GnRH-I and GnRH-II (10^3 – 10^8) and seven tenfold serially diluted GPR54 and GnRH-III (10^2 – 10^8) cRNAs were used for the standard curves to estimate the GPR54 and GnRH mRNA levels by real-time RT-PCR (Fig. 1). A strong linear relationship with a correlation coefficient of $R^2 > 0.999$ between the fractional cycle number and the starting copy number was demonstrated for GPR54 and three GnRH cRNA standard curves. The PCR efficiencies for GPR54, GnRH-I, GnRH-II, and GnRH-III were 100.6 ± 1.2 , 91.3 ± 1.9 , 90 ± 1.2 , and $102.5 \pm 2.4\%$ respectively.

Figure 1 Standard curves of GPR54 and three GnRH cRNAs plotted as log starting copy number versus threshold cycles (C_t) to estimate mRNA levels by real-time quantitative RT-PCR.

Data analyses

Data were presented as mean $\pm$ s.e.m. One-way ANOVA followed by Tukey's HSD post-test was performed for statistical analysis of GPR54 and GnRH mRNA expressions at each developmental stage. Student's *t*-test was performed to compare mRNA levels between males and females at 153 DAH. Analyses were performed using GraphPad Prism and SigmaPlot computer software.

Results

Cloning of GPR54 and GnRH cDNAs

The nucleotide sequences of primers for GPR54 and GnRHs used in conventional and real-time RT-PCRs are shown in Table 1. A 442 bp sequence of partial cobia GPR54 cDNA was amplified initially by GPR54-F and GPR54-R primers. Subsequently, the GPR54 gene-specific primers (GSP) 1 and 2, and GR (GeneRacer kit primers) amplified 520 bp C-terminal and 688 bp N-terminal regions respectively of GPR54 cDNA. The overlapping of partial and C- and N-terminal regions of GPR54 cDNA sequences provided 1159 bp long cDNA (excluding poly A tail). The complete 1159 bp cobia GPR54 cDNA sequence consists of a 14 bp 5'UTR, seven transmembrane (TM) regions, (TM1–TM7), a stop codon (TGA), and a 32 bp 3'UTR region. The deduced amino acid (AA) sequence of GPR54 cDNA consists of a 377 AA polypeptide.

A 245 bp C-terminal region of cobia GnRH-I cDNA was amplified by GnRH-I DP1 and GnRH-I DP2 with GR3'/nested primers. Similarly, 350 and 200 bp C-terminal regions of cobia GnRH-II and GnRH-III cDNAs were amplified by GnRH-II DPs and GnRH-III DPs with GR3'/primers. GSP1 and GSP2 amplified 184 bp GnRH-I, 367 bp GnRH-II, and 225 bp GnRH-III cDNAs of the N-terminal regions. The overlapping

of 5' and 3' RACE sequences provided 382 (GnRH-I), 557 (GnRH-II), and 311 (GnRH-III) bp long cDNAs (excluding poly A tails). The primers for full length cDNAs (GSPs-3 and 4) yielded full length GnRH cDNAs which completely matched with their partial cDNAs obtained by 3' RACE and 5' RACE. The 412 bp GnRH-I cDNA consists of a 59 bp 5' UTR, a 285 bp ORF, a stop codon (TGA), and a 35 bp 3' UTR. Similarly, the 557 bp GnRH-II (or 311 bp GnRH-III) cDNA consists of a 128 bp (32 bp) 5' UTR, a 255 bp (270) ORF, a stop codon (TGA), and a 173 bp (8 bp) 3' UTR. These GnRH cDNAs have a 285 (GnRH-I), 255 (GnRH-II), and 270 (GnRH-III) bp open reading frame including 5' and 3' untranslated regions (UTR). The deduced amino acid sequences of GnRH-I cDNA consists of a 95 AA polypeptide comprising a 22 AA signal peptide (SP), GnRH decapeptide (GnRH-DP), 3 AA signal processing site (GKR) and 60 AA GnRH associated peptide (GAP). The deduced amino acid sequences of GnRH-II (or GnRH-III) cDNA consist of an 85 (90) AA polypeptide comprising a 23 AA SP, GnRH-DP, 3 AA GKR, and 49 (57) AA GAP.

The complete nucleotide and deduced amino acid sequences of GPR54 are shown in Fig. 2. The cobia GPR54 cDNA had 95% identity with that of tilapia and 51–56% identity with those of rat, mouse, and human GPR54 cDNAs (Table 2). Amino acid sequences of GnRH cDNAs in cobia and other closely related representatives of teleosts are shown in Fig. 3. The GnRH-I cDNA of cobia showed 71% identity with that of striped seabass, followed by 70% with barfin flounder, and 68% each with those of Atlantic croaker and red drum. The cobia GnRH-II cDNA had highest identity with the GnRH-II cDNAs of tilapia (94%), gilthead seabream, red drum, European seabass (92% each), Atlantic croaker, striped bass (91% each), and barfin flounder (90%). The highest identity of cobia GnRH-III cDNA (>90%) was observed with the GnRH-III cDNAs of gilthead seabream, red drum, Atlantic croaker, red seabream, European seabass, and tilapia.

Developmental expression of GPR54 and GnRH mRNAs

The expression profiles of the GPR54 and three GnRH mRNAs were evaluated during different stages of development. A low expression of GPR54 mRNA was detected in larvae at 1 DAH, which increased subsequently at 2 and 3 DAH and remained more or less stable until 18 DAH except for the two troughs at 4 and 12 DAH (Fig. 4A). A distinct peak of GPR54 mRNA level was noticed in the brains of juveniles at 26 DAH (Fig. 4B). At 30 DAH, the level of GPR54 mRNA was significantly lower than that in fish at 26 DAH, and remained unchanged thereafter until 105 DAH.

Low levels of GnRH-I mRNA were detected in larvae at 1 and 2 DAH, increased subsequently at 3 and 4 DAH, and remained more or less stable except for a significant drop at 12 DAH (Fig. 4C). In the brains of juveniles, GnRH-I mRNA levels were significantly higher at 26 and 70 DAH when compared with the preceding sampling dates (Fig. 4D). Overall, the expression of GnRH-I mRNA was relatively lower compared with those of the other two GnRH mRNAs throughout the developmental periods examined. The GnRH-II mRNA was detected in larvae at 1 DAH, and then increased gradually with two significant peaks at 2 and 6 DAH and a trough at 12 DAH. Similar to GnRH-I, expression of GnRH-II mRNA was significantly higher in juveniles at 26 and 70 DAH. GnRH-III mRNA expression was also detected in larvae at 1 DAH and showed two peaks at 2 and 6 DAH similar to those of GnRH-II mRNA. Moreover, GnRH-III mRNA level increased significantly in juveniles at 26 DAH, decreased at 30 DAH and did not change significantly thereafter. Interestingly, the GPR54 and all three GnRH mRNAs showed prominent peaks at 26 DAH.

Gonadal sex of cobia could be determined by gross morphological examination at 153 DAH, thereby facilitating comparisons of selected mRNAs between males and females. At this stage, male cobia had morphologically well differentiated testes showing signs of early spermatogenesis with compact seminiferous tubules packed with spermatocytes and a few spermatids (photograph not included). In contrast, the females at the same stage of development had ovaries with oogonial nests and a few primary oocytes. When two males and two females from the same spawn maintained separately as broodstock were sampled at 320 DAH, males showed more active spermatogenesis with tubules filled with sperm, while the stage of ovaries remained unchanged from the samples at 153 DAH (photographs not included). Therefore, cobia males at 153 DAH were at early stages of puberty, whereas no sign of pubertal development was noticed in females even at 320 DAH. The GPR54, GnRH-I and GnRH-II mRNA levels were significantly higher in males than those in females at 153 DAH, whereas the GnRH-III mRNA was not significantly different between the sexes at this age (Fig. 5). The significantly higher mRNA levels in males versus females were a reflection of the three- (GPR54), two- (GnRH-I), and tenfold (GnRH-II) increases in 153-day-old males undergoing early puberty when compared with those in fish of unknown sex at 105 DAH (Figs 4 and 5).

Discussion

The present study reports the novel finding of GPR54 mRNA expression during early larval development in a

-14 GGTAACGTTTCGCA

M Y S T K E L W N S T E Q V W I N G S G 20
 ATGTA CTCCACCAAGGAGCTGTGGA ACTCCACCGAGCAGGTCTGGATCAACGGATCCGGA 60
 T N F S L G R R C N D E E E E G Q Q H P 40
 ACAA ACTTCTCTCTAGGAAGACGTGGCAACGATGAGCAGGAGGAAGGACAGCAGCACCC 120
 F L T D A W L V P L F F S L I M L V G L 60
 TTCTCACAGACGCCTGGCTGGTCCCGCTCTTCTTCTCGCTCATCATGCTGGTGGGACTC 180
 V G N S R V I Y V I S K H R Q M R T A T 80
 GTGGCAACTCTCGGGTTATTTATGTCATTTCCAAACACAGACAGATGAGGACGGCAACT 240
 N F Y I A N L A A T D I I F L V C C V P 100
 AACTTCTACATAGCAAACCTGGCCGCACTGACATCATCTTCTTGGTGTGCTGCGTCC 300
 F T A T L Y P L P G W I F G N F M C K F 120
 TTCACCGCCACCCCTCTATCCTCTCCCTGGATGGATCTTTGGCAACTTCATGTGCAAATTT 360
 V A F L Q Q V T V Q A T C I T L T A M S 140
 GTTGCCCTTCTGACAGCAGGTGACGGTGCAAGCCACCTGCATCACCTGACTGCTATGAGT 420
 G D R C Y V T V Y P L K S L R H R T P R 160
 GGAGACCGCTGTTACGTCACAGTCTATCCTCTGAAGTCCCTGCCGCCACCGCACACCAAG 480
 V A M I V S V C I W I G S F I L S T P I 180
 GTGGCCATGATCGTCAGCGTCTGCATCTGGATTGGCTCCTTCATCCTGTCCACGCCGATT 540
 L M Y Q R I E E G Y W Y G P R Q Y C M E 200
 TTAATGTACCAGCGTATAGAGGAGGCTACTGCTACGGCCCGAGGCAATACTGCATGGAG 600
 R F P S K T H E R A F I L Y Q F I A A Y 220
 AGATTTCCTCTAAGACACACGAGAGGGCTTTCATCCTCTACCAGTTCATTGCTGCCTAC 660
 L L P V L T I S F C Y T L M V K R V G Q 240
 CTGCTGCCCTGCTCCTCACTATTTCTTCTGCTACACTCTGATGGTGAAGAGGGTGGGACAG 720
 P T V E P V D N N Y Q V N L L S E R T I 260
 CCTACAGTCCAACCTGTAGACAATAATTATCAGGTGAATCTCCTGTCTGAGAGAACAATC 780
 S I R S K V S K M V V V I V L L F A V C 280
 AGCATCCGGAGCAAAGTCTCCAAGATGGTGGTGGTGCATCGTCTCCTCTTCGCCGTCTGC 840
 W G P I Q I F A L F Q S F Y P N Y Q H N 300
 TGGGGTCCCATCCAGATCTTCGCCCTTTTTCAGTCTTTCTACCCAAACTACCAGCACAAC 900
 Y A T Y K I K T W A N C M S Y A N S S V 320
 TACGCCACGTACAAGATCAAGACTTGGCCAACTGCATGTCTACGCCAACTCTTCTGTC 960
 N P I V Y G F M G A S F Q K S F R K T F 340
 AACCCTATCGTTTATGGCTTCATGGGAGCTAGTTTCCAAAAGTCTTTCAGGAAAATTTT 1020
 P F L F K H K V R D S S M A S R T A N A 360
 CCGTTCCTGTTCAGCACAAAGTCAGAGACAGCAGCATGGCTTCAAGGACTGCCAAGCT 1080
 E I K F V A A E E G N N N N A M N * 377
 GAGATCAAGTTTGTGCTGCAGAGGAAGGCAACAATAATAATGCAATGAATTGAATGTGT 1140
 CTATTACTCCTATTAGCCGTACGTTCTGCAAAAAAAAAAAAAAAAAA 1150

Figure 2 Nucleotide and deduced amino acid sequences of GPR54 cDNA in cobia. Numbers on the right denote the nucleotide and amino acid sequences. Predicted transmembrane regions (TM1–TM7) are marked by lines. The stop codon is indicated by an asterisk.

Table 2 Amino acid sequence identity (%) between cobia GPR54 and those of other vertebrates

Species	Sequence identity (%)	GenBank accession number
Tilapia	95	AB162143 ^a
Human	56	NM032551 ^b
Rat	54	NM023992 ^c
Mouse	51	NM053244 ^d

^aParhar *et al.* (2004).^bClements *et al.* (2001).^cLee *et al.* (1999).^dOhtaki *et al.* (2001).

vertebrate species. In addition, the observation of remarkably similar expression patterns of the GPR54 and three GnRH mRNAs throughout the early larval and juvenile periods in cobia demonstrates a close association between GPR54 and multiple GnRHs during these developmental stages. The recent co-localization of GPR54 mRNA within the neurons expressing the three GnRHs in tilapia (Parhar *et al.* 2004) further supports the possibility of interactions between GPR54 and multiple GnRHs. Moreover, the relatively higher expression of GnRH-II and GnRH-III mRNAs when compared with that of GnRH-I mRNA in the early larval and juvenile periods may suggest a more prominent role for GnRH-II and GnRH-III during these stages. However, a more detailed examination of the GnRHs in relation to different developmental stages is necessary in order to understand their precise physiological roles. The higher expression of GPR54 and GnRH-I mRNAs observed in male cobia undergoing puberty is consistent with similar observations in pubertal rats, whereas

an increase in GnRH-II mRNA expression similar to that in the early pubertal cobia has not been reported for any other vertebrate species. Overall, the present study provides the baseline data on the expression patterns of GPR54 and multiple GnRH mRNAs during the larval, juvenile, and early pubertal development in cobia for future investigations of their possible interactions and functions.

Recent studies in mammals have strongly implicated GPR54 and its ligand, Kisspeptin, in the control of reproduction and fertility by stimulating GnRH release (Irwig *et al.* 2004, Navarro *et al.* 2004, Seminara *et al.* 2004). The impairment of GnRH and LH release in GPR54 mutant mice can be reversed by GnRH treatments (Colledge 2004, Seminara *et al.* 2004), thereby demonstrating the centrality of the normal development of GPR54 in the control of mammalian reproduction. Although similar evidence is presently lacking for non-mammalian vertebrates, the present study provides initial data on the developmental patterns of GPR54 mRNA during the larval, juvenile, and early pubertal development in a fish model.

The more or less similar expression patterns of the GPR54 and GnRH mRNAs during the developmental stages of cobia examined in this study suggest a potential relationship between GPR54 and multiple GnRHs. For example, concomitant increases in the GPR54 and one or more GnRH mRNAs were observed in cobia at 2, 3, and 26 DAH and in early pubertal males. The increase in GPR54 mRNA in cobia undergoing early puberty is consistent with similar increases in pubertal rat and rhesus monkey (Navarro *et al.* 2004, Shahab *et al.* 2005). A gradual increase in GnRH-I mRNA levels during postnatal development with

	Signal Peptide	GnRH-I DP	GnRH Associated Peptide	
Cobia	---MGVKTALALLFVGTLPV---	QHWCHQWSYGLSPGGKRELD	SLSDTLANNVEGVPHVDS	PCSLRCAEDSPFPKIYRMKGLG
Striped seabass	---APQTFALW..LV.TLLG---	QGC.....	E..GL.E.LGNQI.GGF.H.ET..	RVLGCAYESP.FKI..M..F.DAVTD.EN.P..YKK- 95 (71)
Barfin flounder	MHRK.AVKTLTSLV..LV.TLVP---	QHC.....	Q..SL.Q.LGN-V.EEF.R.DS..	SVLGAEESP.AGI..M..F.DAVTD.EN.N..YKK- 99 (70)
Atlantic croaker	---APQTSAML..LV.SVLWC---	HGC.....	D..GR.D---M.DSF.H.DS..	SVLSCVEE---PKI..M..F.DGVTD.EN.R..FKK- 90 (68)
Red drum	---APQTSAML..LV.SVLWC---	HGC.....	D..GR.D---I.DSF.H.DS..	SVLSCVED---PKI..M..F.DGVTD.EN.R..FKK- 90 (68)
	Signal Peptide	GnRH-II DP	GnRH Associated Peptide	
Cobia	MCVSRLLVLLGLLVCVGAQLS	NAQHWSHGUYPGGKRELD	SFSTSEISEEIKLCEAGE	CSYLRPQRRSILRNILLDALARELQRRK 85
Nile tilapia	..V...A...L...L.V..Q..FA.....	G.....	R....	SI..N.L.....K.. 85 (94)
Gilthead seabream	..V...V...L...L.V..Q..NG.....	G.....	T....	SV..N.L.....K.. 85 (92)
Red drum	..V...V...L...L.V..Q..NA.....	G.....	R....	GV..S.L.....K.. 85 (92)
European seabass	..V...V...F...L.V..Q..NA.....	G.....	R....	SV..N.I.....K.. 85 (92)
Atlantic croaker	..V...V...L...L.A..Q..NA.....	G.....	R....	GV..S.L.....K.. 85 (91)
Striped seabass	..V...V...F...L.V..Q..NA.....	G.....	R....	NV..N.I.....K.. 85 (91)
Barfin flounder	..A...V...L...L.V..H..SG.....	G.....	R....	NI..N.L.....K.. 85 (90)
	Signal Peptide	GnRH-III DP	GnRH Associated Peptide	
Cobia	MEAGSRVHVQVLLALLLVQVTL	LSQHWSYGULPGGKRSVGELEATIR	MMGTGGVVLPEEASAO	QTERLPYNNVINDDPESHFDKRFPHN 90
Gilthead seabream	..AS...TVQ.....	S.....	G..S..E..S..T.....	V.K..S.P.....RF.-- 88 (93)
Red drum	..VS...MVQ.....	A.....	G..S..E..S..T.....	V.N..S.H.....RF.NN 90 (93)
Atlantic croaker	..VS...MVQ.....	A.....	G..S..E..S..T.....	V.N..S.H.....KF.NN 90 (92)
Red seabream	..AS...TVQ.....	S.....	G..S..E..S..T.....	V.K..D.H.....RF.NK 90 (92)
European seabass	..AN...MVR.....	S.....	E..S..E..S..T.....	V.N..S.H.....RS.NK 90 (91)
Nile tilapia	..AG...IMQ.....	S.....	G..S..D..N..T.....	I.N..S.H.....RF.NN 90 (91)

Figure 3 Amino acid alignments of three GnRH cDNAs between cobia and closely related teleost species. The entire open reading frames of amino acid sequences were aligned. Amino acids identical to those comprising the cobia GnRHs are indicated by dots. Numbers on the right denote the total number of amino acids with their percent identities to cobia GnRH precursors in parentheses. DP, decapeptide.

Figure 4 The GPR54 and three GnRH mRNA levels during different stages of development in cobia performed by real-time quantitative RT-PCR. (A and C) GPR54 and GnRH mRNAs in whole heads of larvae during the first 18 days of development. (B and D) GPR54 and GnRH mRNAs in whole brains of juveniles between 22 and 105 days of development. Each bar represents the mean $\pm$ S.E.M. of triplicate samples. *Significantly different from the preceding sampling times ($P < 0.05$). Day 26 is highlighted to denote the peak of GPR54 mRNA corresponding with those of the three GnRH mRNAs.

maximum level at puberty has also been shown previously in female rats (Gore *et al.* 1996). Taken together, these increases in GPR54 and GnRH-I mRNAs are most likely a precursor to the Kisspeptin-induced GnRH-I release in mammals, particularly at the time of puberty (Navarro *et al.* 2004, Seminara *et al.* 2004). Therefore, the similar patterns of GPR54 and GnRH-I expression during pubertal development in cobia and rat suggests that their interactions may be comparable in fish and mammals. Although GPR54 mRNA expression has been examined only on the GnRH-I neurons in mammals (Irwig *et al.* 2004), its expression has been demonstrated on all the three types of GnRH neurons in a teleost fish (Parhar *et al.* 2004). The latter finding provides the morphological basis for possible interactions of GPR54 with multiple GnRHs in fish, and underscores the need for a comprehensive evaluation of these interactions in both fish and mammals. Moreover, an endogenous ligand for GPR54, similar to mammalian Kisspeptin, has to be identified in fish in order to elucidate their function(s).

Figure 5 GPR54 and GnRH mRNA levels in whole brain of male and female cobia at early puberty performed by real-time quantitative RT-PCR. Each bar represents the mean $\pm$ S.E.M. of 3–6 samples. *Significantly higher than those in the brains of juveniles of unknown sex at 105 days after hatching (Fig. 4B and D). †Significantly higher in males (black bars) when compared with those in females (white bars). P values < 0.05 were considered significant.

The hypogonadism and infertility associated with Kallmann's syndrome in humans is caused by a developmental defect in the migration of GnRH-I neurons during embryogenesis, which can be reversed by the exogenous administration of GnRH (Schwanzel-Fukuda *et al.* 1989). Similarly, a mutation in the *GnRH-I* gene in mice also causes hypogonadism and infertility, and the insertion of an intact *GnRH-I* gene in these mice can restore fertility (Mason *et al.* 1986*a,b*). In a recent study in zebrafish, Sherwood & Yu (2005) have reported that knockdown of the two *GnRH* genes present in this species causes a variety of deleterious effects, including defective mid-hindbrain boundary, underdeveloped eyes and defective heart. In this study, *GnRH* gene knockdown appears to have more profound effects on normal fish development than those observed in the knockout mice studies (Mason *et al.* 1986*a,b*). Therefore, normal development of the GnRH neurons is clearly necessary for an individual to be fertile and could be critical for the very survival of fish.

The detection of GnRH-I mRNA expression at 1 DAH, and its lower abundance when compared with the levels of GnRH-II and GnRH-III mRNAs during the larval and juvenile periods of cobia are in agreement with similar findings in gilthead seabream (Wong *et al.* 2004). Cells expressing GnRH-I mRNA have been detected at 30 DAH in seabass and at 39 DAH in seabream using ISH techniques (González-Martínez *et al.* 2002, Wong *et al.* 2004). The inability of ISH techniques to detect very low mRNA levels at earlier stages of development appears to be responsible for the delayed detection of GnRH-I mRNA in these and other similar ISH studies. In addition, Wong *et al.* (2004) have reported early detection of GnRH receptor, FSH β , FSH, and LH receptors and Vasa mRNAs during the larval development of gilthead seabream using real-time RT-PCR. Therefore, these genes of the hypothalamic-pituitary-gonad (HPG) axis may potentially be active much earlier than it has been suggested based on the ISH studies (Cambré *et al.* 1990, Parhar *et al.* 1998). Furthermore, the finding of higher GnRH-I mRNA expression in male cobia undergoing puberty when compared with the juvenile females of the same age is consistent with the designation of GnRH-I as the major regulator of pituitary gonadotropins in all vertebrates.

The detection of GnRH-II and GnRH-III mRNAs at 1 DAH in cobia is also more or less similar to their detection at 1 and 1.5 DAH respectively in seabream (Wong *et al.* 2004). The relatively higher GnRH-II and GnRH-III mRNA levels compared with that of GnRH-I mRNA observed in cobia and seabream (Wong *et al.* 2004) suggests that GnRH-II and GnRH-III could be more important during larval development. The overall increasing trend of GnRH-II and GnRH-III mRNAs during the larval development in cobia is also consistent with the increasing number and size of the

GnRH immunoreactive cells reported during early development of other teleosts (Parhar *et al.* 1998, González-Martínez *et al.* 2002).

One of the most striking features of the expression patterns in this study was the detection of concomitant peaks of the GPR54 and three GnRH mRNAs in cobia at 26 DAH. Similar peaks of three GnRH mRNAs have been reported in gilthead seabream at 28 DAH (Wong *et al.* 2004). The GnRH mRNA peaks also correspond with those of the selected markers of pituitary-gonadal axis in seabream, such as the mRNAs of GnRH receptor, FSH β , FSH, and LH receptors, and Vasa (Wong *et al.* 2004). Based on their data in seabream, Wong *et al.* (2004) have suggested that the ontogeny and organization of the HPG axis may start early and reach a more stable development at 28 DAH. Although the peaks of GnRH mRNAs in cobia were observed slightly earlier than those in seabream possibly due to relatively faster growth and development in cobia when compared with seabream, the overall patterns were remarkably similar between the two species. These similar expression patterns in cobia further support the idea of early establishment of the HPG axis. In addition, the concomitant peaks of GPR54 and multiple GnRH mRNAs in cobia suggest that the GPR54 may also be an important component of the development and maturation of the HPG axis in fish.

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