Molecular cloning and differential expression of three GnRH mRNAs in discrete brain areas and lymphocytes in red drum

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Abstract

Three gonadotropin releasing hormones, seabream GnRH (GnRH-I), chicken GnRH-II (GnRH-II) and salmon GnRH (GnRH-III) cDNAs were isolated from the brain of the red drum, *Sciaenops ocellatus*. The GnRH cDNA sequences of red drum showed more similarity to those of Atlantic croaker, sea bass and sea bream. The real-time quantitative RT-PCR study revealed expression of GnRH-I and GnRH-III mRNAs in the olfactory bulb plus telencephalon (OB+TEL), and preoptic-anterior hypothalamic area (POAH), indicating an overlap of the GnRH-I and GnRH-III systems in these fore-brain regions. However, GnRH-II mRNA expression was detected only in the midbrain tegmentum (MT).

The GnRH-I mRNA expression in the POAH in fish undergoing gonadal recrudescence was significantly higher than that in gonadally immature individuals, suggesting involvement of the POAH GnRH-I in gonadal maturation. On the other hand, GnRH-III mRNA expression was significantly higher in the OB+TEL region compared with that in the POAH. Moreover, the demonstration of GnRH-I mRNA and peptide expression in red drum lymphocytes indicates that the GnRH-I is synthesized in these cells of the immune system similar to the situation in mammals.

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Introduction

Gonadotropin-releasing hormone (GnRH) plays an important role in the control of vertebrate reproduction. Similarly, GnRH or GnRH-like peptides occur in some protochordate and invertebrate species (Adams *et al.* 2003). To date, 24 isoforms of GnRH have been identified, 14 from vertebrates and 10 from invertebrates (Gorbman & Sower 2003). Traditionally, GnRH variants were named after the species in which they were first identified. The present study follows the GnRH classification described in Gorbman & Sower (2003) for the three GnRH variants identified in the red drum, *Sciaenops ocellatus*, as GnRH-I (sea bream GnRH), GnRH-II (chicken GnRH-II), and GnRH-III (salmon GnRH).

The existence of two or more forms of GnRHs in a single species is well established in all vertebrate groups. The species in which two molecular forms of GnRH have been identified include most salmonids, carps, African catfish, European eel, frog, chicken and mammals, including humans (Lethimonier *et al.* 2004). The species known to have three forms of GnRH include those belonging to Perciformes and Pleuronectiformes, and a primitive salmonid (Powell *et al.* 1994, Amano *et al.* 2002, Vickers *et al.* 2004). Previous studies in species expressing three forms of GnRH have reported that GnRH-I is expressed

in the caudal telencephalon-preoptic area (TEL-POA), GnRH-II in the midbrain tegmentum (MT) and GnRH-III in the olfactory bulb (OB) and TEL (White *et al.* 1995, Andersson *et al.* 2001, Amano *et al.* 2002). However, in two other perciform species, European sea bass (González-Martínez *et al.* 2001) and Atlantic croaker (Mohamed *et al.* 2005), GnRH-I and GnRH-III systems have been shown to overlap in the OB, TEL and POA. These studies are based mainly on the results of *in situ* hybridization or immunohistochemistry. However, these methods are limited in their ability to detect very low levels of expression in cells or tissues. A conventional reverse transcription-polymerase chain reaction (RT-PCR) method, which allows detection of only the final amplified product, has been employed to study expression patterns of GnRH mRNAs in discrete brain areas of rainbow trout (Ferriere *et al.* 2001) and Atlantic croaker (Mohamed *et al.* 2005). However, information on the absolute quantification of GnRH mRNAs from their sites of expression in fish brains using a real-time RT-PCR method is currently lacking.

In addition to the brain GnRH systems, sympathetic ganglia and spinal cord express GnRH-I in vertebrates (Dolan *et al.* 2003). GnRH-I expression has been reported also in a variety of mammalian non-neural tissues such as bone marrow, gastric smooth muscle cells, gonads, heart,

incisors, kidney, liver, pancreas, placenta, prostate, and skeletal muscle (Dolan *et al.* 2003, Chen *et al.* 2004, Tiong *et al.* 2004). In addition, GnRH-I expression and GnRH binding sites have been demonstrated in different tissues of the immune system of mammals (Chen *et al.* 1999). Similarly in teleosts, GnRH-I expression has been shown in gill, gonad, heart, kidney, liver, muscle, pituitary, and spleen (Ferriere *et al.* 2001, Mohamed *et al.* 2005). However, GnRH expression in tissues of the immune system, such as lymphocytes, has not been reported in any non-mammalian vertebrate.

The red drum is an economically important aquaculture species belonging to the family Sciaenidae and order Perciformes, the largest vertebrate order. Changes in the expression of three GnRH variants in relation to the reproductive stages have been reported previously in only the female phase of two perciform species, gilthead seabream and red seabream, which are protandrous and protogynous hermaphrodites, respectively (Gothilf *et al.* 1997, Senthilkumaran *et al.* 1999, Okuzawa *et al.* 2003). Thus, it is worthwhile to investigate the expression of three GnRH mRNAs in relation to reproductive stages in both males and females of a gonochoristic species, such as red drum.

The aims of the present study were to: (i) isolate the cDNAs encoding three different GnRH variants in red drum; (ii) study expression of the three GnRH mRNAs in discrete brain areas; (iii) estimate the GnRH mRNAs by real-time quantitative RT-PCR at their site(s) of expression in both male and female fish with immature and recrudescing gonads in order to understand their potential role during gonadal recrudescence and (iv) localize and quantify the expression of GnRH-I in peripheral lymphocytes by *in situ* hybridization, immunocytochemistry, and real-time RT-PCR.

Materials and Methods

Animals and tissue preparation

Red drums were captured in the vicinity of Port Aransas and were reared in the laboratory for various periods in a recirculating seawater system under simulated natural photoperiod and temperature conditions. Male and female fish with immature and recrudescing gonads were used for this study. Fish were sacrificed under deep anesthesia following experimental procedures approved by the Animal Care and Use Committee of the University of Texas at Austin. Whole brains were quickly removed, immediately frozen in liquid nitrogen, and maintained at -80°C until used for total RNA isolation or micro-dissection. Body and gonad weights were recorded to calculate gonadosomatic index ($\text{GSI} = \text{gonad weight} \times 100 / \text{body weight without gonads}$), and gonads were processed for routine histology to determine the reproductive stage of each fish sampled. Total blood was

collected from seven individual fish of both sexes from the caudal vein using sterile, heparinized syringes and processed immediately for the separation of the lymphocytes.

Isolation of lymphocytes

The lymphocytes were isolated from fresh heparinized blood using lymphocyte separation medium (Cellgro, Herndon, VA, USA) according to standard protocols used previously for fish (Kollner *et al.* 2004). Briefly, the blood was diluted 1:1 with PBS, 4 ml diluted blood was carefully layered onto a 3 ml lymphocyte separation medium (Density = $1.077\text{--}1.080\text{ g/ml}$) in a 15 ml nuclease-free centrifuge tube and centrifuged at 400 g for 45 min at room temperature. The interface containing lymphocytes was carefully aspirated and washed with PBS by centrifugation at 260 g for 10 min at 4°C . The cell pellet was re-suspended in PBS and immediately used for total RNA isolation, *in situ* hybridization and immunocytochemistry.

Total RNA isolation

Total cellular RNA was isolated from brains and lymphocytes using TRIzol reagent (Invitrogen) as per manufacturer's directions and stored at -80°C until used for the first strand cDNA synthesis or one-step RT-PCR assays. The frozen brains used for micro-dissection were thawed $>16\text{ h}$ in RNAlater-ICE Frozen Tissue Transition Solution (Ambion, Austin, TX, USA) at -20°C . Each brain was dissected into six parts: olfactory bulb (OB), telencephalon (TEL), preoptic-anterior hypothalamic area (POAH), MT, cerebellum plus optic tectum (CE+OT) and medulla oblongata (MO) as described previously (Mohamed *et al.* 2005). Total cellular RNA was isolated from the micro-dissected brain parts using an Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA, USA) and stored at -80°C until used for one-step RT-PCR assays.

Rapid amplification of cDNA ends (RACE)

First-strand cDNA synthesis was carried out using the RNA ligase mediated-RACE system (GeneRacer kit, Invitrogen) according to the manufacturer's protocol. Approximately $5\text{ }\mu\text{g}$ of total RNA was used to synthesize cDNA and the reaction was incubated at 42°C for 50 min followed by 37°C for 20 min with 2 units of RNase H and stored at -20°C until used for RACE. The primers used in all PCRs are shown in Table 1. Two rounds each of PCRs were performed for 3' RACE, 5' RACE and full length cDNA synthesis (Fig. 1). For 3' RACE, six degenerate primers, GnRH-I primer 1 (GnRH-I DP1) and GnRH-I primer 2 (GnRH-I DP2), GnRH-II primer 1 (GnRH-II DP1), GnRH-II primer 2 (GnRH-II DP2), GnRH-III primer 1 (GnRH-III DP1), GnRH-III primer 2 (GnRH-III DP2), were designed to be sense to

Table 1 Nucleotide sequence of primers

GnRH-I		GnRH-II		GnRH-III	
Primers ^a					
DP1	5'-CARCAYTGGTCTTAYGGICTIAG-3'	5'-CARCAYTGGTCTTAYGGICTTCTCTG-3'	5'-CARCAYTGGTCTTAYGGITGGYT-3'	5'-TAYGGITGGTCTTAYGGITGGYT-3'	5'-TAYGGITGGTCTTAYGGITGGYT-3'
DP2	5'-TAYGGICTIAGYCCIGGIGG-3'	5'-TAYGGICTIAGYCCIGGIGG-3'	5'-TAYGGICTIAGYCCIGGIGG-3'	5'-TAYGGICTIAGYCCIGGIGG-3'	5'-TAYGGICTIAGYCCIGGIGG-3'
GSP1	5'-CACGTGTGGAACCTGTCACATATGG-3'	5'-CACGTGTGGAACCTGTCACATATGG-3'	5'-CACGTGTGGAACCTGTCACATATGG-3'	5'-CACGTGTGGAACCTGTCACATATGG-3'	5'-CACGTGTGGAACCTGTCACATATGG-3'
GSP2	5'-CGTGTGGAACCTGTCACATATGG-3'	5'-CGTGTGGAACCTGTCACATATGG-3'	5'-CGTGTGGAACCTGTCACATATGG-3'	5'-CGTGTGGAACCTGTCACATATGG-3'	5'-CGTGTGGAACCTGTCACATATGG-3'
GSP3	5'-AGTATTCACAGACGCGGAG-3'	5'-AGTATTCACAGACGCGGAG-3'	5'-AGTATTCACAGACGCGGAG-3'	5'-AGTATTCACAGACGCGGAG-3'	5'-AGTATTCACAGACGCGGAG-3'
GSP4	5'-ATTTCACAGACGCGGAG-3'	5'-ATTTCACAGACGCGGAG-3'	5'-ATTTCACAGACGCGGAG-3'	5'-ATTTCACAGACGCGGAG-3'	5'-ATTTCACAGACGCGGAG-3'
qRT-PCR F	5'-GCCTCTCCGCTCTGTGACTCA-3'	5'-GCCTCTCCGCTCTGTGACTCA-3'	5'-GCCTCTCCGCTCTGTGACTCA-3'	5'-GCCTCTCCGCTCTGTGACTCA-3'	5'-GCCTCTCCGCTCTGTGACTCA-3'
qRT-PCR R	5'-GAGCGTGGTACTCTCTTCTAC-3'	5'-GAGCGTGGTACTCTCTTCTAC-3'	5'-GAGCGTGGTACTCTCTTCTAC-3'	5'-GAGCGTGGTACTCTCTTCTAC-3'	5'-GAGCGTGGTACTCTCTTCTAC-3'
Actin F	5'-CAGGACTCCATACCGAGGA-3'	5'-CAGGACTCCATACCGAGGA-3'	5'-CAGGACTCCATACCGAGGA-3'	5'-CAGGACTCCATACCGAGGA-3'	5'-CAGGACTCCATACCGAGGA-3'
Actin R					

^aDP, Degenerate primer; GSP, Gene specific primer; qRT-PCR, quantitative RT-PCR; F, Forward; R, Reverse; R=A or G; Y=C or T; I=A, T, C or G.

Figure 1 Schematic representation of the red drum prepro GnRH-I (A), GnRH-II (B), GnRH-III (C) cDNAs showing the 5' untranslated region (5' UTR), the signal peptide (SP), the gonadotropin-releasing hormone decapeptide (GnRH-DP), the proteolytic cleavage site (GKR), the GnRH associated peptide (GAP), the 3' untranslated region (3'UTR), and positions of degenerate primers (DP), gene specific primers (GSP), GeneRacer primers (GRP) and nested primers (NP) used for RT-PCR.

nucleotides encoding GnRH-I, GnRH-II and GnRH-III amino acids 1–8 (DP1), and 5–10 plus the first amino acid (Gly) of the processing site (DP2) (Table 1). Gene specific primers (GSPs) for 5' RACE and almost full length cDNAs were synthesized based on the sequence information of the 3' RACE and 5' RACE products, respectively.

All PCRs were performed in a final volume of 50 µl solution containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 200 nM each primer, 200 µM dNTPs, 2.5 units Nova Taq DNA polymerase (Novagen, Madison, WI, USA) and 1 µl template DNA. The PCR was carried out 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 second round PCR products were separated on 1.2% agarose gel by electrophoresis and the band of expected size was purified using MinElute Gel Extraction Kit as per manufacturer's protocol (Qiagen). The purified PCR products were cloned into pGEM-T Easy vector plasmids and transformed into *E. coli* JM 109 competent cells (Promega). The plasmid DNAs were purified using QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions. Both strands of the plasmid DNA containing candidate GnRH cDNA inserts were sequenced by T7 and SP6 universal primers. The full length sequences of the three GnRH cDNAs were submitted to the GenBank (Accession numbers: GnRH-I, AY677172; GnRH-II, AY677171; GnRH-III, AY677170). The entire open reading frame (ORF) of the deduced amino acid sequences of red drum GnRH cDNAs and other closely related representatives of teleosts (obtained from Genbank) were aligned by CLUSTAL W (<http://www.ebi.ac.uk/clustalw>) alignment program with default settings.

Synthesis of recombinant GnRH RNAs for generation of standard curves

Plasmids containing the full-length cDNAs of the three GnRHs were used to synthesize sense cRNAs using Riboprobe *In Vitro* Transcription Systems (Promega) as described previously (Mohamed *et al.* 2005). Following the transcription reaction the plasmid DNA template was removed by digestion with TURBO DNase (Ambion) for 30 min at 37 °C. The riboprobes were purified by MEGA clear kit (Ambion) and stored at -80 °C until used for real-time RT-PCR. The cRNA concentrations were estimated on a spectrophotometer (SPECTRAMax 190, Molecular Devices, Sunnyvale, CA, USA) at 260 nm and confirmed on 1.4% denatured formaldehyde agarose gel with a known RNA weight marker. The weight of a single molecule of GnRH cRNA was calculated by the following formula: $W = MW / 6.022 \times 10^{23}$, where MW = molecular weight and 6.022×10^{23} is the Avogadro number. Five 10-fold serial dilutions of GnRH-I cRNA (10^3 to 10^7 molecules per 5 µl cRNA), six 10-fold serial dilutions of GnRH-II (10^3 to 10^8 molecules per 5 µl cRNA) and GnRH-III cRNAs (10^2 to 10^7 molecules per 5 µl cRNA) were prepared to generate the standard curves.

Qualitative and real-time quantitative RT-PCR

The qualitative RT-PCR reaction was performed by one step RT-PCR kit (Qiagen) as shown previously

(Mohamed *et al.* 2005). One step SYBR Green real-time quantitative RT-PCR amplifications were performed using the Mx3000P quantitative PCR system (Stratagene). After optimization of each of the primer pairs (Table 1) for both cRNA and sample RNA, reactions were assayed by Brilliant SYBR Green QRT-PCR Master Mix Kit (Stratagene) in a 25-µl reaction mixture containing 12.5 µl 2x SYBR-QRT-PCR master mix, 50 nM each primer, 0.375 µl diluted reference dye (ROX, 1:500), 0.0625 µl StrataScript RT/RNase block enzyme mixture and 100 ng of total RNA or 5 µl of 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, 57 °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. 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 standards. Standards were run in every plate and sample RNAs were normalized with β -actin.

Riboprobe synthesis and in situ hybridization

The full length coding regions of the red drum GnRHs were used to synthesize riboprobes. Alexa flour 488-chroma Tide UTP (Molecular Probes Inc., Eugene, OR, USA) labeled single-stranded sense and anti-sense RNA probes were synthesized for fluorescence *in situ* hybridization as described previously (Mohamed *et al.* 2005). Similarly, a β -actin anti-sense RNA probe was synthesized and used as a positive control. The isolated lymphocytes were smeared on nuclease-free silane coated slides (VWR, West Chester, PA, USA) and air dried at room temperature. The slides were fixed in 4% paraformaldehyde for 30 min, washed in PBS, dehydrated and air dried. The hybridization and secondary signal amplification were performed as described previously (Mohamed *et al.* 2005). TO-PRO-3 (1:1000 dilution; Molecular Probes) was applied on the slides for 10 min to stain the nucleus of lymphocytes, washed in PBS and mounted with Prolong Gold anti-fade reagent (Molecular Probes). The GnRH-I mRNA expressing and total lymphocytes were counted in 15 separate viewing areas to calculate percent of cells expressing the mRNA.

Immunocytochemistry

The isolated lymphocytes were smeared on silane coated slides and air dried at room temperature. The slides were fixed in 4% paraformaldehyde for 30 min, washed in PBS and hydrated. Sections were blocked in 10% normal goat serum for 1 h at room temperature and incubated in GnRH-I primary antibody (1:5000 dilution) raised in rabbit (AS-691; Senthilkumaran *et al.* 1999) or 10% normal goat serum (for control) overnight at 4 °C in a moist chamber. Sections were washed in PBS (3 × 10 min)

	Signal peptide	GnRH-I DP	GnRH-associated peptide	
Red drum	---MAPQTSAML---LLLVGSVLWC---HGCCQHWSYGLSPGGKRDLDGRSDT---IVDSFPHVDSPCSVLSQVED---FPKIYRMKGFLDGVTDRENGRRTFKK	90		
Atlantic croaker	---PQTSAML---VGSVLWC---HG---D---GR.D---MVDS..HVDS..S.LSCVEE---FPKI..M..FLDGVTDREN.R.TFKK	90 (97)		
European seabass	---AQTFALR---VGTLLGTLGQS---E..GL.E.LGNQIVGS..HVAT..R.LGCAEESPFPKI..M..FLDAVTDREN.N.TYKK	99 (75)		
Striped seabass	---PQTFALW---VGTLLG---QG---E..GL.E.LGNQIVGS..HVET..R.LGCAVESPFPKI..M..FLDAVTDREN.P.TYKK	95 (74)		
Red seabream	---PQTSNLW---VVMVMMS---QG---D..SL.D.LG-DIIER..HADS..S.LGCAEESPFPKM..M..FIGSGTDRDN.H.TYKK	95 (70)		
Gilthead seabream	---PQTSNLWIL---VVMVMMS---QG---D..SL.D.LG-NIIER..HVDS..S.LGCVPEHPVPM..M..FIGS---ERDI.H.MYKK	95 (60)		
Barfin flounder	MHRK..VKTLSSVW---VGTLPV---QH---Q..SL.Q.LG-NVVEE..RVDS..S.LGCAEESPFAGI..M..FLGSITDR---N.TQNI	96 (58)		
Cichlid	---AKTLALW---AGTVFP---QG---D..NF.D.LG-NMVEE..RVEA..S.FGCAEESPFAM..V..LLASVAEREN.H.TFKK	94 (57)		
	Signal peptide	GnRH-II DP	GnRH-associated peptide	
Red drum	MCVSRIVLLGLLLCVGAQLSNAQHWSHGWPGGKRELDSEFGTSEISEEIKLCEAGECSYLRPQRSILRNILLDALARELQKRK	85		
Atlantic croaker	..V..V..L...A..Q..NA.....R...GV..S..L.....	85 (98)		
European seabass	..V..V..F...V..Q..NA.....R...SV..N..I.....	85 (95)		
Gilthead seabream	..V..V..L...V..Q..NG.....T...SV..N..L.....	85 (95)		
Striped seabass	..V..V..F...V..Q..NA.....R...NV..N..I.....	85 (95)		
Cichlid	..V..A..L...V..Q..FA.....R...SI..N..L.....	85 (94)		
Barfin flounder	..A..V..L...V..H..SG.....R...NI..N..L.....	85 (91)		
	Signal peptide	GnRH-III DP	GnRH-associated peptide	
Red drum	MEVSSRVVMQVLLLALVQVTLAQHWSYGWLPGGKRSVGELEATIRMMGTGGVSLPEEASAQTERLPYVINDSSSHFDRKKRFPNN	90		
Atlantic croaker	..VS...MVQ.....A.....G...E...S...T.....V..N...H...KF..N	90 (98)		
Red-seabream	..AS...TVQ.....S.....G...E...S...T.....V..K...H...RF..K	90 (94)		
Gilthead seabream	..AS...TVQ.....S.....G...E...S...T.....V..K...P...RF..K	90 (93)		
European seabass	..AN...MVR.....S.....E...E...S...T.....V..N...H...RS..K	90 (92)		
Cichlid	..AG...IMQ.....S.....G...D...N...I.....I..N...H...RF..N	90 (90)		

Figure 2 Theoretical translation of the three red drum GnRH cDNAs aligned with amino acid sequences of respective GnRHs from closely related species. Numbers in parentheses indicate percent identities between the three GnRH precursors of red drum and other species. DP, decapeptide.

and incubated for 1 h at room temperature in horseradish peroxidase conjugated goat anti-rabbit IgG (Molecular Probes; 1:100 in 1% blocking reagent) and washed three times in PBS. The secondary signal amplification was performed as described previously (Mohamed *et al.* 2005). Sections were mounted with Prolong Gold anti-fade reagent (Molecular Probes). The negative controls included omission of the GnRH-I antibody and blocking of the antibody with GnRH-I peptide (Bachem, Torrance, CA, USA). The GnRH-I peptide expressing and total lymphocytes were counted in 15 separate viewing areas to calculate percent of cells expressing the peptide.

Monochromatic photomicrographs were taken using Nikon Eclipse E600 microscope (Nikon, Japan) with fluorescein isothiocyanate (FITC; green) filter. The images were captured by CoolSNAP RS Photometrics camera and MetaMorph software (Universal Imaging, Downingtown, PA, USA). Dichromatic photomicrographs were taken using Nikon Eclipse TE 2000-U confocal laser scanning microscope (Nikon Instruments, Melville, NY, USA) equipped with epifluorescence filters. An argon laser (Ar 488) with an excitation wavelength of 488 nm was used to activate the green fluorescence (Alexa fluor 488) and the helium/neon laser (HeNe 633) with an excitation wavelength of 633 was applied to activate the far-red fluorescence (TO-PRO-3). Image processing was carried out with Nikon computer software (EZ-C1, Gold Version 2.10). All text and symbols were inserted in Adobe Photoshop.

Statistical analysis

Student's *t*-test was performed for statistical analysis of GnRH mRNA expressions in two brain areas,

between males and females, and between immature and recrudescing fish.

Results

Molecular characterization of GnRH cDNAs

Nucleotide sequences of primers used in this study are shown in Table 1. In the 3' RACE, GnRH-I DP1 and GR3', and GnRH-I DP2 with GR3' nested primers, yielded 208 base pairs (bp) C-terminal region of red drum GnRH-I cDNA (Fig. 1). Similarly, GnRH-II DPs and GnRH-III DPs with GR3' primers yielded 359 and 180 bp C-terminal regions in GnRH-II and GnRH-III cDNAs, respectively. In the 5' RACE, gene specific primers (GSP) 1 and GSP 2 yielded 204 bp GnRH-I, 312 bp GnRH-II, and 167 bp GnRH-III cDNAs of the N-terminal regions. The overlapping of 5' and 3' RACE sequences provided 412 (GnRH-I), 606 (GnRH-II) and 313 (GnRH-III) bp long cDNAs (excluding poly A tail). These GnRH cDNAs have a 270 (GnRH-I), 255 (GnRH-II) and 270 (GnRH-III) bp ORF with 5' and 3' untranslated regions (UTR). The primers for full length cDNAs (GSPs-3 and 4) yielded almost full length GnRH cDNAs, which matched with their partial cDNAs obtained by 3'-RACE and 5'-RACE. The deduced amino acid sequences of GnRH-I and GnRH-III cDNAs consist of a 90 aa polypeptide comprising a 23 aa signal peptide (SP), GnRH decapeptide (GnRH-DP), 3 aa signal processing site (GKR) and 54 aa GnRH associated peptide (GAP) (Fig. 2). The deduced amino acid sequences of GnRH-II cDNA consist of an 85 aa polypeptide comprising a 23 aa SP, GnRH-DP, 3 aa GKR and 49 aa GAP (Fig. 2).

Amino acid sequences of GnRH cDNAs in red drum and other closely related species are shown in Fig. 2. The red drum GnRH-I cDNA matched highly with the GnRH-I cDNA of Atlantic croaker (97%), followed by European sea bass (75%) and striped sea bass (74%). The highest similarity of red drum GnRH-II cDNA was noticed with the GnRH-II cDNAs of Atlantic croaker (98%), European sea bass, striped sea bass, and gilthead sea bream (95% each), and GnRH-III cDNA in red drum with those of Atlantic croaker (98%), red sea bream (94%) and gilthead sea bream (93%). The conservation of the GnRH cDNAs was consistent with the traditional phylogenetic models for these species at the level of families (Sciaenidae: croaker and red drum; Moronidae: sea bass and striped bass; and Sparidae: sea breams).

GnRH mRNAs in discrete brain areas and their alterations with gonadal maturation

The gonads of immature fish were small (GSIs: 0.02–0.04%) with no apparent sign of gametogenesis, whereas recrudescing fish had relatively large gonads (GSIs: 0.31–2.73%) showing advanced stages of sperm or late-vitellogenic oocytes, in males and females respectively (photomicrographs not included).

The diagrammatic representation of the dissected brain areas of red drum used for one-step RT-PCR is shown in Figure 3A. The nucleotide sequences of red drum GnRH and β -actin primers used for RT-PCRs are shown in Table 1. The integrity of total RNA purified from the dissected brain areas were confirmed with β -actin primers. Actin primers yielded 233 bp PCR product of red drum β -actin from the total RNA of OB, TEL, POAH, MT, CE+OT and MO (Fig. 3B). GnRH-I and GnRH-III primers yielded 178 and 155 bp PCR products, respectively of GnRH-I and GnRH-III from the total RNA of OB, TEL and POAH but not from the RNAs of other brain areas (Fig. 3C and E). On the other hand, the GnRH-II primers yielded a 121 bp PCR product of GnRH-II only from the total RNA of MT. (Fig. 3D). The DNA sequencing results of the RT-PCR products matched completely with the GnRH cDNA sequences of red drum, indicating the specificity of the primers.

The real-time RT-PCR analyses of red drum GnRH mRNAs were performed using standard curves of GnRH cRNAs synthesized by *in vitro* transcription reactions. A strong linear relationship with a correlation coefficient of 0.999 between the fractional cycle number and the starting copy number was demonstrated for the standard curves of three GnRH cRNAs (Fig. 4A). The amplification efficiencies of GnRH-I, GnRH-II and GnRH-III cRNAs were $93.5 \pm 2.4\%$, $95 \pm 1.3\%$ and $96 \pm 1.6\%$, respectively.

The GnRH mRNA levels in the discrete brain areas, OB together with TEL (OB+TEL), POAH and MT were quantified by real-time RT-PCR. The results are shown as the absolute copy number per 100 ng total RNA.

Figure 3 Schematic representation of GnRH mRNAs expression in different parts of red drum brain. (A) The straight lines within the brain diagram demarcate dissected areas used for expression studies. (B–E) Total RNA (500 ng) from each brain area was used for 35 cycles of RT-PCR with β -actin (B), GnRH-I (C), GnRH-II (D), and GnRH-III (E) qRT-PCR primers. RT-PCR products were subjected to electrophoresis on 2% ethidium bromide-stained agarose gel. Total RNA was replaced with nuclease-free water for negative controls (NC). OB, Olfactory bulb; TEL, Telencephalon; POAH, Preoptic-anterior hypothalamic area; MT, Midbrain tegmentum; CE+OT, Cerebellum plus optic tectum; MO, Medulla oblongata.

The GnRH-I mRNA level was significantly higher in the POAH than that in OB+TEL region of both males and females (Fig. 4B). In addition, GnRH-I mRNA level in the POAH, but not in OB+TEL, was significantly higher in fish with recrudescing gonads compared with that observed in gonadally immature individuals (Fig. 4B). Moreover, GnRH-I mRNA expression in the POAH of recrudescing females was significantly higher than that in males. The GnRH-II mRNA expression in the MT was significantly higher in recrudescing fish than in immature fish, but here was no difference between the sexes at either reproductive stage (Fig. 4C). In contrast to GnRH-I, the GnRH-III mRNA level was significantly higher in the OB+TEL than that in the POAH of recrudescing males and both immature and recrudescing females (Fig. 4D). In addition, the GnRH-III mRNA level in the OB+TEL, but not in POAH, was significantly higher in fish with recrudescing gonads compared with that observed in gonadally immature individuals (Fig. 4D). Furthermore, the GnRH-III mRNA expression in the OB+TEL was significantly higher in females than that in males at both reproductive stages.

GnRH-I mRNA and peptide expressions in lymphocytes

The expression of GnRH-I mRNA in the peripheral lymphocytes was shown by RT-PCR methods (Fig. 5).

Figure 4 Standard curves of three GnRH cRNAs and their mRNA levels in different parts of the brain performed by real-time quantitative RT-PCR. (A) Standard curves of log starting copy numbers versus threshold cycles (C_t) to determine the initial template concentration. (B–D) GnRH mRNA levels in different areas of the brain in gonadally immature and recrudescent fish. Each bar represents mean $\pm$ S.E.M. of triplicate samples. Significant differences are indicated by different letters on the bar ($P < 0.05$). White and black bars represent male and female fish, respectively.

GnRH-I primers amplified a 178 bp PCR product from the RNA of lymphocytes. Subsequent cloning and DNA sequencing of the RT-PCR products confirmed that the quantitative RT-PCR (qRT-PCR) GnRH-I primers amplified a portion of DNA between the 72 bp and 249 bp in the ORF of the 309 bp prepro GnRH-I mRNA/cDNA. Neither GnRH-II nor GnRH-III primers amplified any PCR products from the RNA of lymphocytes (Fig. 5A). Actin primers amplified a 233 bp PCR product from the RNA of lymphocytes, demonstrating integrity of the RNAs. GnRH-I mRNA level in the red drum lymphocytes was 9794 ± 120 copies per 100 ng total RNA (Fig. 5B), approximately 15% of that in the POAH where it is highly expressed but comparable to that in the OB+TEL region of the brain.

GnRH-I mRNA and peptide expression was localized in the lymphocytes by *in situ* hybridization and immunocytochemistry, respectively (Fig. 6). A strong signal of GnRH-I mRNA expression was detected in the cytoplasm of the lymphocytes hybridized with GnRH-I

antisense probe (Fig. 6A and C). The nuclear stain (red) used in this study allowed visualization of the distinct morphology of lymphocytes as nucleated cells with a narrow cytoplasm showing GnRH-I mRNA signals (Fig. 6C). The β -actin mRNA expression in the cytoplasm of lymphocytes was slightly more widespread than the GnRH-I mRNA signals (photomicrographs not included). No signal was detected in the lymphocytes hybridized with the GnRH-I sense probe (Fig. 6B and D). The GnRH-I peptide expression was detected as bright green signals in the cytoplasm of lymphocytes (Fig. 6E). No GnRH-I expression was detected in the lymphocytes on negative control slides (Fig. 6F). Moderate to strong GnRH-I mRNA and peptide signals were observed in $55.6 \pm 2.9\%$ and $61.4 \pm 3.7\%$ of the lymphocytes, respectively.

Discussion

Three GnRH cDNAs have been isolated from red drum brain and expression of their mRNAs quantified by

Figure 5 Expression of GnRH-I mRNA in the lymphocytes. (A) Total RNA (500 ng) from lymphocytes was used for 35 cycles of RT-PCR with β -actin, GnRH-I, GnRH-II and GnRH-III qRT-PCR primers. RT-PCR products were subjected to electrophoresis on 2% ethidium bromide-stained agarose gel. Total RNA was replaced with nuclease-free water for negative controls (NC). (B) GnRH-I mRNA level in the lymphocytes was estimated by real-time quantitative RT-PCR. Each bar represents mean $\pm$ S.E.M. of 7 samples.

RT-PCR. Amino acid sequences of the three GnRH cDNAs between red drum and other closely related representatives of the order Perciformes, such as Atlantic croaker, sea bass and sea bream are very similar. Results of conventional RT-PCR show differential expression of the three GnRH mRNAs in distinct brain areas of red drum, however, the expression of GnRH-I and GnRH-III mRNAs overlaps in some forebrain regions, similar to the observations in Atlantic croaker (Mohamed *et al.* 2005). The real-time quantitative RT-PCR study shows more abundant expression of GnRH-I mRNA in the POAH, GnRH-II mRNA in the MT, and GnRH-III mRNA in the OB+TEL of fish undergoing gonadal recrudescence than that in the gonadally immature individuals. In addition, this study demonstrates for the first time the expression of GnRH-I mRNA and peptide in the lymphocytes of a non-mammalian vertebrate species.

The finding of GnRH-I mRNA and peptide expression in red drum lymphocytes appears to be consistent with GnRH-I mRNA expression in tissues of the mammalian immune system, such as lymphocytes, thymocytes, thymus, and spleen (Azad *et al.* 1991a,b 1993, Maier *et al.* 1992, Chen *et al.* 1999). In addition, both GnRH-I and GnRH-II are expressed in human T cells (Chen *et al.* 2002). However, neither GnRH-II nor GnRH-III mRNA expression was detected in red drum lymphocytes. Subsequent cloning and DNA sequencing of the RT-PCR products confirmed the identity of GnRH-I cDNA from lymphocytes being the same as that in the brain. Interestingly, the presence of GnRH-I mRNA and peptide in lymphocytes raises the question of possible contamination of other tissues with blood while assessing

Figure 6 Photomicrographs of *in situ* hybridization and immunocytochemistry showing GnRH-I mRNA and peptide expression in the lymphocytes. (A) Lymphocytes showing GnRH-I mRNA signals (arrow) in the cytoplasm hybridized with an anti-sense fluorescent probe (green). (B) Negative control corresponding with A using sense probe. (C) GnRH-I mRNA expression (arrows) in the cytoplasm of lymphocytes hybridized with an anti-sense fluorescent probe (green) and nuclear stain (red). (D) Negative control corresponding with C using sense probe. (E) Lymphocytes showing GnRH-I peptide signals (arrows) in the cytoplasm using GnRH-I antibody. (F) Negative control corresponding with E. Scale bar = 100 μ m. (This figure appears in colour at <http://joe.endocrinology-journals.org/content/vol188/issue3>).

GnRH expression in these tissues. Initial detection of GnRH-I mRNA expression in all the tissues examined in red drum without blood removal at the time of sampling prompted the investigation of its expression in blood cells of this species. Thus, data in literature on low level of GnRH expression in different tissues should be interpreted with caution, and care should be taken to remove excess blood from tissues in order to avoid false detection of GnRH peptide and/or mRNA expression.

To our knowledge, sex differences in GnRH-I and GnRH-III mRNA expressions in relation to the reproductive stages have not been reported for any fish species.

The increase in GnRH-I mRNA expression in the POAH of recrudescing versus immature fish was clearly more pronounced in females compared with that in males. In addition, the GnRH-III mRNA expression in the OB+TEL region was higher in both immature and recrudescing females. However, the functional significance of higher GnRH mRNA expressions in females is unknown.

The overall distribution pattern of GnRH-I and III mRNAs in the OB, TEL and POAH of red drum is similar to that in Atlantic croaker (Mohamed *et al.* 2005). In these two species, GnRH mRNAs have been absolutely quantified by conventional RT-PCR (croaker) and real-time RT-PCR (red drum) methods. The results clearly demonstrate that GnRH-I and GnRH-III mRNAs are more abundant, especially in the female fish in the POAH and OB+TEL, respectively than other brain areas in red drum. Other methods of localization such as *in situ* hybridization and immunohistochemistry show a similar pattern of expression. For example, in Atlantic croaker and European sea bass the number of GnRH-I expressing neurons in the POA and GnRH-III neurons in the OB and TEL are more than in other brain regions (González-Martínez *et al.* 2001, 2002, Mohamed *et al.* 2005). More abundant expression of GnRH-I mRNA in the POAH in red drum and other species supports its predominant role as a major hypophysiotropic regulator.

The observation of GnRH-II mRNA expression in the MT of red drum is consistent with most other species investigated to date (Collin *et al.* 1995, González-Martínez *et al.* 2001, Mohamed *et al.* 2005). GnRH-II expression has also been identified in the POA of goldfish (Lin & Peter 1997), roach (Penlington *et al.* 1997), frog (Collin *et al.* 1995), and macaque (Urbanski *et al.* 1999) and in frog spinal cord (Chartrel *et al.* 1998). However, RT-PCR methods could not detect GnRH-II mRNA expression in any part of red drum brain except in the MT, indicating GnRH-II mRNA is not expressed in other brain parts of this species. In this study, GnRH-II mRNA level in the MT was significantly higher in fish undergoing gonadal recrudescence than that in the gonadally immature individuals. Similarly in red seabream and gilthead seabream, GnRH-II mRNA levels increase around the spawning season (Gothilf *et al.* 1997, Okuzawa *et al.* 2003). However, it is unlikely that GnRH-II regulates gonadotropin secretion directly because neither GnRH-II peptide nor fibers have been detected in the pituitaries of these species (Senthilkumaran *et al.* 1999, Zmora *et al.* 2002).

The fact that GnRH-III mRNA expression is higher in the OB+TEL region of the red drum brain and that recrudescing fish express more GnRH-III mRNA in these regions compared with that in gonadally immature individuals suggests its potential involvement in some aspect of gonadal maturation in this species. On the other hand, the POAH GnRH-III mRNA expression was significantly lower in red drum with recrudescing gonads

compared with that observed in gonadally immature individuals. It is unclear at present whether GnRH-III synthesized in the OB and TEL regions can regulate gonadotropin secretion indirectly, or is involved in functions completely unrelated to a hypophysiotropic role.

The present study demonstrates differential expression of three GnRH mRNAs in the anterior to mid brain regions of red drum by RT-PCR methods. The higher expression of GnRH-I mRNA in the POAH of recrudescing red drum compared with that in immature fish agrees with the suggested role of POA GnRH in the control of reproductive maturation. However, little is known about the roles of GnRH-II and GnRH-III in the control of fish reproduction. Interestingly, the demonstration of GnRH-I mRNA and peptide expressions in red drum lymphocytes is consistent with similar observations in tissues of the immune system in mammals.

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