Four functional GnRH receptors in zebrafish: analysis of structure, signaling, synteny and phylogeny

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Synopsis Reproduction in all vertebrates requires the brain hormone gonadotropin-releasing hormone (GnRH) to activate a cascade of events leading to gametogenesis. All vertebrates studied to date have one to three forms of GnRH in specific but different neurons in the brain. In addition, at least one type of GnRH receptor is present in each vertebrate for activation of specific physiological events within a target cell. Humans possess two types of GnRH (GnRH1 and GnRH2) but only one functional GnRH receptor. Zebrafish, *Danio rerio*, also have two types of GnRH (GnRH2 and GnRH3), although in contrast to humans, zebrafish appear to have four different GnRH receptors in their genome. To characterize the biological significance of multiple GnRH receptors within a single species, we cloned four GnRH receptor cDNAs from zebrafish and compared their structures, expression, and cell physiology. The zebrafish receptors are 7-transmembrane G-protein coupled receptors with amino-acid sequence identities ranging from 45 to 71% among the four receptors. High sequence similarity was observed among the seven helices of zebrafish GnRHRs compared with the human GnRHR, the green monkey type II GnRHR, and the two goldfish GnRHRs. Also, key amino acids for putative ligand binding, disulfide bond formation, N-glycosylation, and G-protein coupling were present in the extracellular and intracellular domains. The four zebrafish receptors were expressed in a variety of tissues including the brain, eye, and gonads. In an inositol phosphate assay, each receptor was functional as shown by its response to physiological doses of native GnRH peptides; two receptors showed selectivity between GnRH2 and GnRH3. Each of the four receptor genes was mapped to distinct chromosomes. Our phylogenetic and syntenic analysis segregated the four zebrafish GnRH receptors into two distinct phylogenetic groups that are separate gene lineages conserved throughout vertebrate evolution. We suggest the maintenance of four functional GnRH receptors in zebrafish compared with only one in humans may depend either on subfunctionalization or neofunctionalization in fish compared with mammalian GnRH receptors. The differences in structure, location, and response to GnRH forms strongly suggests that the four zebrafish GnRH receptors have novel functions in addition to the conventional activation of the pituitary gland in the reproductive axis.

Introduction

Gonadotropin-releasing hormone (GnRH) has been tightly linked to the control of reproduction in vertebrates through the hypothalamic-pituitary-gonadal axis (Schally et al. 1971). However, there are additional forms of GnRH found in most chordate species that are expressed in many different brain and peripheral locations. Accumulating evidence suggests that GnRH has functions outside of pituitary regulation. The key to understanding novel GnRH functions depends on locating other GnRH target tissues.

Although researchers have now identified 14 distinct GnRH family members in vertebrates, the members can be classified into four GnRH clades: GnRH1, GnRH2, GnRH3 (Lethimonier et al. 2004; Vickers et al. 2004), and GnRH4 (Silver et al. 2004). Fish each possess either two or three GnRH forms with zebrafish falling into the group with two forms (GnRH2 and GnRH3) (Sherwood and Adams 2005).

In zebrafish, neurons containing GnRH3 (i.e., salmon GnRH) are present mainly in the olfactory bulb, area of the terminal nerve and ventral telencephalon; these GnRH neurons are thought to be recruited for control of pituitary function (Torgersen et al. 2002; Steven et al. 2003). This arrangement is closer to that in mammals, where GnRH1 (i.e., mammalian GnRH) is in both the preoptico-hypothalamic and terminal nerve areas (Schwanzel-Fukuda 1999).

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In addition, zebrafish, along with most jawed vertebrates, have a highly conserved form, GnRH2 (i.e., chicken GnRH-II), which has been localized in the midbrain (Kuo et al. 2005), although the exact role of GnRH2 is unclear.

The obvious clues to understanding novel GnRH functions are the location, signaling pathways and effectors of GnRH reception. The effects of GnRH on target tissues depend on specific GnRH receptors (GnRHRs), which are members of the G-protein coupled receptor (GPCR) family A. Vertebrate GnRHRs share a common overall structure in that they have an N-terminal extracellular region with seven hydrophobic transmembrane domains (TMD) connected by alternating hydrophilic intracellular and extracellular loops (ICL and ECL) terminating in a cytoplasmic C-terminal tail, although the tail is missing in the type I mammalian GnRHRs (Millar et al. 2004). GnRHRs are widely conserved in evolution, as demonstrated recently by the cloning of a functional GnRHR from the octopus (Kanda et al. 2006) and three functional receptors in the invertebrate tunicate (Kusakabe et al. 2003; Tello et al. 2005). Since the cloning of the first GnRHR from the mouse αT3 gonadotrope cell line (Reinhart et al. 1992; Tsutsumi et al. 1992) and the first teleost GnRHR from the African catfish (Tensen et al. 1997), many teleost GnRHRs have been identified. Some species have been shown to encode up to five GnRHRs in their genome, as is the case for each of two pufferfish (*Fugu rupripes* and *Tetraodon nigroviridis*), the cherry salmon (*Oncorhynchus masou*), and the European seabass (*Dicentrachus labrax*), although the functional status of only a few fish GnRH receptors is known (Jodo et al. 2003; Ikemoto and Park 2005; Moncaut et al. 2005).

The zebrafish, *Danio rerio*, is an excellent vertebrate model to elucidate novel functions of the ligand–receptor partnership both during development and in the adult. We have shown previously that both GnRH2 and GnRH3 contribute to early brain development in zebrafish in regard to the anterior and posterior midbrain boundaries as well as to the development of the eye cup and stalk (Wu et al. 2006). Recent reports have elucidated the ontogeny of both GnRH2 and GnRH3 in the developing zebrafish brain and placed salmon GnRH (GnRH3) in context with the well-accepted view of GnRH migration from the region of the nasal placode/anterior neural plate into the hypothalamus (Schwanzel-Fukuda 1999; Wray 2002; Whitlock 2005; Whitlock et al. 2006; Palevitch et al. 2007). In addition to the conventional midbrain GnRH2 population, a promoter–reporter study identified novel forebrain and hindbrain GnRH2 populations (Palevitch et al. 2007). Despite these recent findings, there has been a paucity of investigation into the receptors that mediate GnRH signaling in the zebrafish. The complete characterization of the zebrafish GnRH receptors, including their locations is a key part of understanding the functional significance of two GnRH populations during vertebrate development as well as in the adult. With sequencing of the genome nearly complete, three GnRHRs and one partial GnRHR have been annotated in the genome, but not cloned. In the present study, we characterize the zebrafish GnRH receptor system at the molecular level by cloning four complete zebrafish GnRHRs. Zebrafish GnRH receptor functions are assessed by measuring ligand-induced intracellular accumulation of inositol phosphate (IP) and cAMP in transfected COS7 cells. Each zebrafish GnRHR sequence is mapped to its chromosomal location and placed into its evolutionary context using both synteny mapping and phylogenetic methods.

Materials and methods

Animals

All experiments were approved by the Animal Care Committee at the University of Victoria. Adult zebrafish (*D. rerio*) were obtained from a local dealer (Safari Pets, Victoria, BC). Zebrafish were anesthetized with tricaine methanesulfonate (MS222; Argent Chemical Laboratories, Inc., Redmond, WA) and tissues were dissected under a microscope. Tissues used for RNA extraction were immediately frozen in liquid nitrogen.

Gene organization

To identify the complete GnRHR gene complement in the zebrafish, genomic sequences from The Sanger Institute's Zebrafish *Danio rerio* Genome Sequencing Project (http://www.sanger.ac.uk/Projects/D_rerio/) were screened. The entire open reading frame (ORF) nucleotide sequences of all reported fish GnRHRs were used with default parameters. Each search generated closely matched fragments. The DNA regions encoding four putative GnRHRs were compiled, examined for exon/intron boundaries, and analyzed for an ORF. Primers were designed and the complete receptor ORFs were amplified from brain tissue using PCR and sequenced. Exon/intron boundaries were established by comparing the cDNA to the genome-sequencing project or to genomic DNA directly. To complete any missing regions in the coding sequences, 5′- and 3′-rapid amplification of cDNA ends (RACE) were implemented.

Isolation of zebrafish mRNA for cDNA synthesis and RACE

The mRNA was isolated from brain tissue using a Micro Poly (A) Pure mRNA isolation kit according to the manufacturer's protocol (Ambion Inc., Austin, TX). Complementary DNA was synthesized in a 50 µl reaction that contained mRNA, 2 µM oligo dT, 2 mM deoxynucleoside triphosphates, 1 × first strand reaction buffer, 0.01 M dithiothreitol, 40 U RNase inhibitor, and 100 U Superscript-II reverse transcriptase (Invitrogen, Burlington, ON). The reaction was incubated at 42°C for 90 min, and the enzyme was heat-inactivated at 70°C for 15 min.

For RACE–PCR, 250 ng of mRNA were used to prepare RACE-ready cDNA using the RLM-RACE kit (Ambion) according to the manufacturer's instructions, except that the DNA was redissolved in DNase/RNase-free distilled water.

PCR and sequencing of cDNA

Oligonucleotides were designed to regions encoding candidate GnRHRs based on the compiled sequences for zebrafish GnRHR genes 1, 2, 3, and 4. The cDNA encoding each GnRHR ORF was altered to include a strong context Kozak sequence (GCCACCATGG) with ATG as the start codon (Kozak 1996). Each 50 µl reaction contained 2.5 U Platinum *Taq* polymerase High Fidelity (Invitrogen), 1 × High Fidelity PCR buffer, 2.5 mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphates (Invitrogen), and 0.4 µM of each Koz forward (F) and Stop reverse (R) primer (Table 1). PCRs were performed under the following conditions: 94°C for 2 min; 35 cycles at 94°C for 30 s; 56°C for 30 s; 72°C for 2 min; and a 5-min final extension. Amplicons generated from this PCR and the RACE–PCR were separated by electrophoresis on a 1.3% (w/v) agarose gel and visualized with ethidium bromide staining using an Eagle-Eye-II still video system (Stratagene, La Jolla, CA). Bands were selected, isolated (QIAGEN, Valencia, CA), and cloned or directly cloned as amplicons into pGEM Vector-T (Promega Corp., Madison, WI) and sequenced. The SequiTherm EXCEL II DNA sequencing kit was used by the University of Victoria Sequencing Centre.

Isolation of total RNA for receptor tissue expression (TE)

Total RNA was isolated from male and female tissues separately using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Total RNA samples were run on the Eukaryote Total RNA Nano chip to assess RNA quality using the Agilent 2100 Bioanalyzer with all RIN values ranging between 5.6 and 9.2 (Agilent Technologies Canada Inc., Mississauga, ON). Total RNA (1.5 µg) from each tissue was DNased with the DNA-free kit according to the manufacturer's directions (Ambion). DNased total RNA (800 µg) from male and female tissues were pooled to create an equal mix of male and female RNA for each tissue. The RNA was reverse transcribed in a 20 µl reaction that contained 2.5 µM oligo dT, 0.5 mM deoxynucleoside triphosphates, 1 × first strand reaction buffer, 5 mM dithiothreitol, 40 U RNaseOUT, and 200 U Superscript-III reverse transcriptase (Invitrogen). The reaction was incubated at 50°C for 60 min, and the enzyme was heat inactivated at 70°C for 15 min.

PCR for tissue distribution

Primer pairs used in each tissue–expression PCR reaction are listed in Table 1. Each 50 µl reaction contained 2 U Platinum Taq polymerase High Fidelity (Invitrogen), 1 × High Fidelity PCR buffer, 2 mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphates, and 0.2 µM of each TE primer pair. PCRs were performed under the following conditions: 94°C for 5 min; 35 cycles at 94°C for 30 s; 55°C for 30 s; 72°C for 30 s; and a 10-min final extension. The PCR amplicons were separated by electrophoresis on a 3% (w/v) agarose gel, stained with ethidium bromide and visualized using a UV transilluminator. A no template control was included as a negative control for each primer pair and TATA-box binding protein (TBP) was used as a housekeeping gene for each tissue.

IP accumulation assay

The GnRHR ORFs each containing the Kozak sequence mentioned previously were cloned into pcDNA3.1(-) (Invitrogen). COS7 cells (Invitrogen) were seeded and grown into monolayer cultures in T-175 cm^2 flasks in growth medium consisting of Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 4 mM L-glutamine, 0.1 mM non-essential amino acids (Invitrogen) and 10% fetal bovine serum (Invitrogen) at 37°C in 5% CO_2. After 3 days, the monolayers were trypsinized with TrypLE (Invitrogen) and seeded in 24-well tissue culture treated plates (Corning-Costar Corp., Cambridge, MA) at a density of 55,000 cells per well and grown overnight in growth medium. At 85–95% confluence, ~24 h post seeding, the cells were washed and incubated with serum-free medium (VP-SFM, Invitrogen), then transfected with 0.8 µg/well of receptor encoded plasmid DNA using Lipofectamine as per the manufacturer's protocol (Invitrogen). After another 24 h, the cells were washed with labeling medium: Medium

Table 1 Primer names and sequences used to amplify target cDNA for GnRH, GnRHR, and housekeeping genes in the zebrafish, *D. rerio*

Primer	Sequence (5′–3′)	Target	Target size (bp)
zfR1Koz F	TCTAGAGCCACCATGGCAGGTAACGTGT	*zfGnRHR1*	1165
zfR1Stop R	GCGGCCGCGGATTGACCACCTATGCTGTTTG		
zfR2Koz F	TCTAGAGCCACCATGGACACAACTCAATTGATCGAGGAT	*zfGnRHR2*	1266
zfR2Stop R	GCGGCCGCTGGCTATTCATTCCACTGTGGCGA		
zfR3Koz F	ATGCTAGCGCCACCATGGCTGGTAACTGGTCTCA	*zfGnRHR3*	1052
zfR3Stop R	ATCTCGAGCTCCAGTGTTCCTCCTTTGT		
zfR4Koz F	TCTAGAGCCACCATGGATGACAGCTCTC	*zfGnRHR4*	1256
zfR4Stop R	GCGGCCGCTGCTTTCTTTCCCCACTATTCT		
zfR1 TE F	GCGATGCCATGTGTAAACTTCTCTG	*zfGnRHR1*	582
zfR1 TE R	GGGCGTATACCGGCGTGACCT		
zfR2 TE F	TGATTAGTCTGGATCGACAGG	*zfGnRHR2*	765
zfR2 TE R	GCACAAACTCAGCATCCCATTCTAGC		
zfR3 TE F	CGCCGGGGACGTGGTGTGTA	*zfGnRHR3*	421
zfR3 TE R	GCGCCTCAGAGAATCACCTTTGTTG		
zfR4 TE F	TGGCCTGGGCGATGAGTGTTGTTCT	*zfGnRHR4*	298
zfR4 TE R	CGGGCCTTGGGGATGTTGCTGT		
GnRH2 TE F	ATTAGACTGAAGTGATGGTG	*zfGnRH2*	493
GnRH2 TE R	AGCCTTTATTGTAGGAACTG		
GnRH3 TE F	AAGGTTGTTGGTCCAGTTGTTGCT	*zfGnRH3*	226
GnRH3 TE R	CAAACCTTCAGCATCCACCTCATTCA		
TBP TE F	ACACCACTTTATACCACACC	*zfTBP*	100
TBP TE R	ACAATATTCTGTAACTGCGGG		

Koz, Kozak; F, forward; R, reverse; TE, Tissue Expression; TBP, TATA-box binding protein.

Table 2 Amino acid sequences for the peptides tested in the receptor activation assay

Peptide	Sequence
GnRH1	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
GnRH2	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2
GnRH3	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2

GnRH1 (mammalian GnRH), GnRH2 (chicken GnRH-II), and GnRH3 (salmon GnRH).

199 (Invitrogen) containing 0.3% (w/v) bovine albumin (Sigma-Aldrich, St. Louis, MO) and subsequently labeled with 0.9 µCi/well of *myo*-[2-^{3}H]-Inositol (Amersham, Piscataway, NJ) in labeling medium for 24 h. The cells were washed and pre-incubated for 30 min at 37°C in labeling medium containing 10 mM LiCl. The cells were stimulated with various concentrations of GnRH (Table 2) for 1 h at 37°C with gentle agitation, and then lysed using 200 µl of 0.1 M formic acid after the medium in each well was removed. Measurement of total IPs in cell extracts was performed by the multi-well filtration method described by Chengalvala et al. (1999).

cAMP accumulation assay

COS7 cells were grown, seeded, and transfected in 24-well plates as described above. The cells were allowed to grow in VP-SFM for 24 h post-transfection; then the medium was replaced with Medium 199 (Invitrogen) containing 0.3% (w/v) bovine albumin (Sigma-Aldrich). After 24 h these cells were washed with Hank's Buffered Salt Solution (HBSS) (Invitrogen) supplemented with 20 mM HEPES and 300 µM IBMX (Sigma), pH 7.4 and pre-incubated for 15 min at 37°C. These cells were stimulated with various concentrations of ligands for 1 h at 37°C with gentle agitation. Intracellular cAMP concentrations were measured using a cAMP Direct Enzyme Immunoassay following the manufacturer's protocol (Amersham).

Analysis of data

All IP and cAMP samples were measured at least in duplicate within each assay. All assays were repeated on three independent occasions. Data were analyzed using nonlinear regression; GnRH concentrations

inducing half-maximal stimulation (EC_{50}) were calculated using PRISM5 software (GraphPAD Software, Inc., San Diego CA). The Log EC_{50} values were generated from the mean ± SEM of three independent experiments and differences between Log EC_{50} values were analyzed by the one-way analysis of variance (ANOVA) statistical procedure followed by the Tukey's post-test, where $P<0.05$ was considered statistically significant.

Phylogenetic analysis

The deduced amino-acid sequences for the four zebrafish GnRHRs and all full-length teleost GnRHRs that have been published or reported in GenBank were aligned using the ClustalW program (a component of the Mega software suite, Version 3.1). Other select GnRHRs were included in the alignment to help distinguish each GnRHR group: mammalian type I, non-mammalian type I, type II, and type III; the human oxytocin and arginine vasopressin type 1A receptors were added as outgroups. Receptor names are as reported and shown in Table 3. The phylogenetic tree was constructed using the maximum likelihood method with 500 bootstrap resamplings using default parameters (available online at http://atgc.lirmm.fr/phyml/).

Chromosomal synteny

The chromosomal locations and organization of neighboring gene clusters surrounding each GnRHR loci in select model genomes were determined using the following genome assemblies available in ensemble at http://www.ensembl.org/index.html (zebrafish assembly Version 6, Zv7; *Xenopus tropicalis* assembly version 4.1; chicken genome assembly Version 2.1; cow preliminary genome assembly 3.1; and human genome assembly NCBI 36).

Results

Zebrafish encode four full-length GnRH receptors

We cloned each zebrafish GnRHR cDNA to functionally characterize individual receptors. The four zebrafish GnRHRs were amplified by RT–PCR from reverse-transcribed poly$(A)^+$ mRNA extracted from zebrafish brain tissue. An *in silico* study performed previously by Lethimonier et al. (2004) identified the putative amino-acid sequence of three full-length zebrafish GnRHRs and one partial receptor sequence from the draft zebrafish genome assembly. To avoid unnecessary confusion, we have maintained the same naming convention. Our complete ORFs were deposited in GenBank under the following accession numbers: zfgnrhr1, EF571592; zfgnrhr2, EF571593; zfgnrhr3, EF571594; zfgnrhr4, EF571595.

The four zebrafish GnRHR cDNAs encode proteins with the profile characteristics of family A GPCRs. The isolated *zfgnrhr1* cDNA clone encoded a putative GnRHR similar to the zfGnRHR1 previously predicted (Lethimonier *et al.* 2004) with the exception of eight amino-acid differences. The 1134 bp ORF (including the stop codon) spans ~12.6 kb on chromosome 19 and encodes a putative protein of 377 amino acids. The ORF contains three exons of 519 bp, 208 bp, and 407 bp, separated by two introns of 9312 bp and 2150 bp. The ORF encoding zfGnRHR2 is 1239 bp, spans ~4.1 kb on chromosome 7, and encodes a putative protein of 412 amino acids. The ORF has three exons of 531 bp, 205 bp, and 503 bp interrupted by two introns of 2451 bp and 406 bp. The translated protein matches the previously identified zfGnRHR2, except for two amino-acid differences. After performing 5′-RACE, we isolated a complete 1011 bp ORF for *zfgnrhr3* encoding a putative protein of 336 amino acids. The ORF spans ~6.2 kb when compared to isolated genomic DNA. The zfGnRHR3 ORF is comprised of three exons of 474 bp, 205 bp, and 332 bp separated by a first intron of ~3.7 kb and a second intron of 1518 bp. When the zebrafish genome sequencing project was searched with this cDNA sequence, only a partial match resulted without significant hits for the first exon. Inspection of exons 2 and 3 revealed that this receptor maps to a region of chromosome 16 embedded in ambiguous sequence, probably the result of problems in assembling the genome. Finally, the ORF encoding zfGnRHR4 was shown to be 1221 bp, which spans ~9.8 kb on chromosome 18 and encodes a putative protein of 406 amino acids. The ORF contains three exons of 543 bp, 205 bp, and 473 bp with two large introns (4559 bp and 4052 bp). Interestingly, our translated zfGnRHR4 had one amino-acid substitution and included an additional five amino acids encoded at the junction of exons 2 and 3 when compared to the previously annotated sequence.

After querying the National Center for Biotechnology Information's (NCBI) non-redundant protein database with the four translated zebrafish GnRHRs, two zebrafish receptors displayed the highest sequence similarity to the two goldfish GnRH receptors; zfGnRHR1 had 93% similarity to the goldfish type B and zfGnRHR3 had 89% similarity to goldfish type A. ZfGnRHR2 generated the closest match to the spotted green pufferfish GnRHR type1/III-3 (65% identity and 76% similarity), whereas zfGnRHR4 had a closest match to the European sea bass (*Dicentrarchus labrax*) GnRHR-2B (76% identity and 84% similarity).

Table 3 GnRH receptor amino acid sequences used to generate phylogram

Common name	Genus species	Receptor name	GenBank accession number
GnRH receptors			
human	*Homo sapiens*	GnRHR1	NP_000397
green monkey	*Cercopithecus aethiops*	GnRHR2	AAK52746
rhesus monkey	*Macaca mulatta*	GnRHR2	NP_001028014
cow	*Bos taurus*	GnRHR1	NP_803480
house mouse	*Mus musculus*	GnRHR1	AAA37716
chicken	*Gallus gallus*	GnRHR1	NP_989984
chicken	*Gallus gallus*	GnRHR2	NP_001012627
bullfrog	*Rana catesbeiana*	GnRHR1	AAG42575
bullfrog	*Rana catesbeiana*	GnRHR2	AAG42949
bullfrog	*Rana catesbeiana*	GnRHR3	AAG42574
pipid frog	*Xenopus tropicalis*	GnRHR1	From scaffold 22 & ENSXETP00000038462
pipid frog	*Xenopus tropicalis*	GnRHR2	From scaffold 3972 & ENSXETP00000036235
spotted green pufferfish	*Tetraodon nigroviridis*	GnRHR type 1/III-1	BAE45694
spotted green pufferfish	*Tetraodon nigroviridis*	GnRHR type 1/III-2	BAE45697
spotted green pufferfish	*Tetraodon nigroviridis*	GnRHR type 1/III-3	BAE45699
spotted green pufferfish	*Tetraodon nigroviridis*	GnRHR type 2/nmI-1	BAE45701,
spotted green pufferfish	*Tetraodon nigroviridis*	GnRHR 2/nmI-2	BAE45702
Japanese flounder	*Paralichthys olivaceus*	GnRHR	AAY28982
orange-spotted grouper	*Epinephelus coioides*	GnRHR1	ABF93210
European sea bass	*Dicentrarchus labrax*	GnRHR1A	CAE54804
European sea bass	*Dicentrarchus labrax*	GnRHR1B	CAE54806
European sea bass	*Dicentrarchus labrax*	GnRHR2A	CAD11992
European sea bass	*Dicentrarchus labrax*	GnRHR2B	CAE54807
European sea bass	*Dicentrarchus labrax*	GnRHR2C	Moncaut et al. 2005
striped seabass	*Morone saxatilis*	GnRHR	AAF28464
Nile tilapia	*Oreochromis niloticus*	GnRHR type 1	BAC77240
Nile tilapia	*Oreochromis niloticus*	GnRHR type 2	BAC77241
tilapia hybrid	*O. aureus* x *O. niloticus*	GnRHR type 1	AAQ88391
tilapia hybrid	*O. aureus* x *O. niloticus*	GnRHR type 3	AAQ88392
African cichlid	*Astatotilapia burtoni*	GnRHR1	AAU89433
African cichlid	*Astatotilapia burtoni*	GnRHR2	AAK29745
black sea bream	*Acanthopagrus schlegelii*	GnRHR1	AAV71128
amberjack	*Seriola dumerili*	GnRHR	CAB65407
dwarf gourami	*Colisa lalia*	GnRHR1-1	BAE87048
dwarf gourami	*Colisa lalia*	GnRHR1-2	BAE87049
dwarf gourami	*Colisa lalia*	GnRHR2-1	BAE87050
Atlantic croaker	*Micropogonias undulatus*	GnRHR2A	ABB97085
pejerrey	*Odontesthes bonariensis*	GnRHR2A	AB175336
Japanese medaka	*Oryzias latipes*	GnRHR1	BAB70506
Japanese medaka	*Oryzias latipes*	GnRHR2	BAB70505
Japanese medaka	*Oryzias latipes*	GnRHR3	BAC97833
African catfish	*Clarias gariepinus*	GnRH-II-R	O42329
African catfish	*Clarias gariepinus*	GnRHR2	AAM95605
zebrafish	*Danio rerio*	GnRHR1	EF571592
zebrafish	*Danio rerio*	GnRHR2	EF571593

(continued)

Table 3 Continued

Common name	Genus species	Receptor name	GenBank accession number
zebrafish	*Danio rerio*	GnRHR3	EF571594
zebrafish	*Danio rerio*	GnRHR4	EF571595
goldfish	*Carassius auratus*	GnRHR type A	AAD20001
goldfish	*Carassius auratus*	GnRHR type B	AAD20002
rainbow trout	*Oncorhynchus mykiss*	GnRHR	CAB93351
Japanese eel	*Anguilla japonica*	GnRHR	BAB11961
lamprey	*Petromyzon marinus*	GnRHR	AAQ04564
sea squirt	*Ciona intestinalis*	GnRHR1	NP_001028997
sea squirt	*Ciona intestinalis*	GnRHR2	NP_001028996
sea squirt	*Ciona intestinalis*	GnRHR3	NP_001028995
sea squirt	*Ciona intestinalis*	GnRHR4	NP_001028994
octopus	*Octopus vulgaris*	GnRHR	BAE66648
Outgroups			
human	*Homo sapiens*	Oxytocin - OXTR	NP_000907
human	*Homo sapiens*	Arginine Vasopressin - AVPR1A	NP_000697

Table 4 Amino-acid identity among zebrafish (zf) GnRHRs

1	*2*	*3*	*4*		
100	47	71	45	1	zfGnRHR1
	100	49	58	2	zfGnRHR2
		100	47	3	zfGnRHR3
			100	4	zfGnRHR4

Amino-acid identity was generated using AlignX, a component of the Vector NTI Advance 10 program suite (Invitrogen).

Among the four zebrafish GnRHR protein sequences, zfGnRHR1 displayed the highest identity in sequences to zfGnRHR3 (71%), whereas zfGnRHR2 showed the highest identity to zfGnRHR4 (58%) (Table 4).

GnRHRs have conserved domains

The four zebrafish receptor proteins show high conservation with other vertebrate GnRHRs in the seven TMDs and some conservation in ICL and ECL. All zfGnRHRs possess potential N-glycosylation sites in their N-terminal domains (at positions 4 and 15 for zfGnRHR1; 2 and 13 for zfGnRHR2; 4 and 9 for zfGnRHR3; 2, 22, and 32 for zfGnRHR4). These N-terminal extracellular regions showed little conservation with other vertebrate GnRHRs. However, at the other end of the protein, zfGnRHR1 and R4 have intracellular C-terminal tails that are highly similar to those of other teleost GnRHRs, but zfGnRHR2 and R3 have tails that show little conservation. All the zebrafish GnRHRs share conserved residues (Fig. 1) and motifs with other vertebrate GnRHRs including human; the conserved residues and their putative functions for zfGnRHR3 are highlighted in Fig. 2. Several residues previously characterized to be involved in ligand binding or formation of binding pockets are conserved in the four zebrafish GnRHRs: $Asp^{2.61(98)}$ (D), $Trp^{2.64(101)}$ (W), and $Asn^{2.65(102)}$ (N) in TMD2; $Lys^{3.32(121)}$ (K) in TMD3; $Trp^{6.48(280)}$ (W), $Tyr^{6.51(283)}$ (Y), and $Tyr^{6.52(284)}$ (Y) in TMD6; and $Trp^{6.59(291)}$ (W) in ECL3. Conserved microdomains that are involved in receptor activation are also present, with $Asn^{1.50}$ (N) in TMD1, $Asp^{2.50}$ (D) in TMD2, the DRxxxI/V motif within TMD3, and N/DPxxY in TMD7 (DPxxY in all zfGnRHRs) (Millar et al. 2004).

GnRH receptors show distinct distributions in tissues in adults

Expression of each GnRH and GnRHR gene was analyzed by RT–PCR using pooled total RNA isolated from four male and four female adult zebrafish. Tissues examined included skeletal muscle, gill, eye, brain, ovary, testes, heart, and intestine. Representative data regarding the GnRH2, GnRH3, zfGnRHR1, zfGnRHR2, zfGnRHR3, zfGnRHR4, and TBP mRNAs from each tissue are shown in Fig. 3. As to the hormone, the GnRH2 primer pair generated PCR amplicons of the expected size (493 bp) predominantly in brain, eye, testis, ovary, and skeletal muscle and produced a faint band in the gill. The GnRH3 target sequence (226 bp) was prominent in brain, ovary, and testis and showed a faint band in the rest of the tissues examined. In the receptor set, the zfGnRHR1 primer

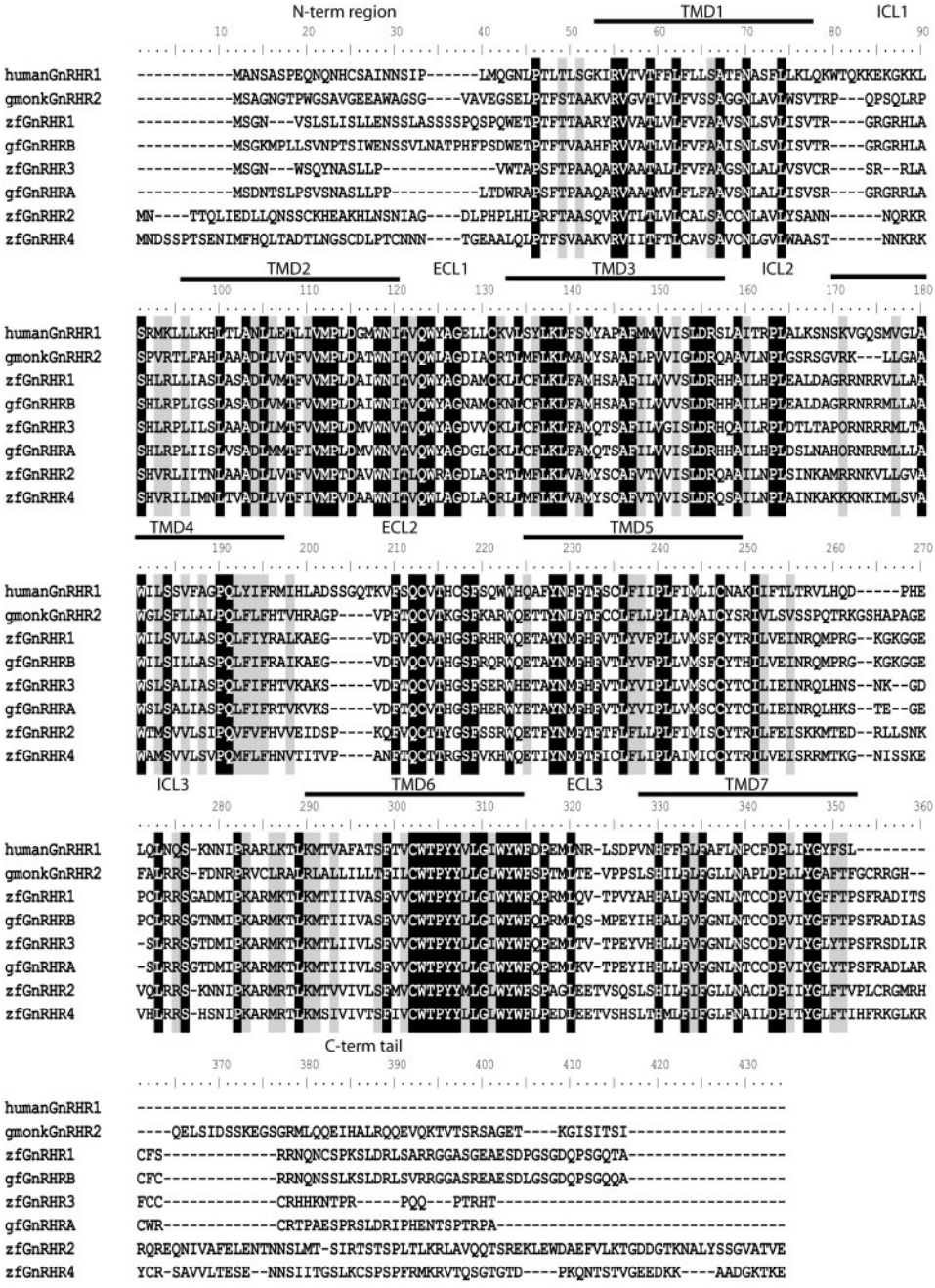

Fig. 1 Sequence alignment of cloned zebrafish GnRHRs with representative vertebrate GnRHRs. Deduced amino acid sequences were aligned using ClustalW. Dashes indicate gaps introduced in the sequence to optimize the alignment. The putative TMDs are indicated by horizontal bars. Intracellular loops (ICL) and extracellular loops (ECL) are noted above the alignment. Sequences are shaded according to the Blosum62 matrix.

pair amplified the expected target sequence (582 bp) in the brain, eye, gill, ovary, and testis and a faint band was seen in both the heart and the intestine. The zfGnRHR2 PCR product (765 bp) was visualized in the eye, brain, ovary, and testis. The zfGnRHR3 target sequence (421 bp) was observed in all tissues except for the heart, whereas the zfGnRHR4 target sequence (298 bp) was visualized in all tissues examined.

The four GnRH receptors signal through the IP pathway

To confirm the functional identity of the four zfGnRHR cDNAs, each receptor cDNA was expressed in COS7 cells and exposed to graded concentrations of GnRH1, GnRH2, and GnRH3. Each native ligand (GnRH2 and 3) stimulated IP accumulation in zfGnRHR-expressing cells (Fig. 4), indicating that all four receptors have the ability to couple to G_q/G_{11}.

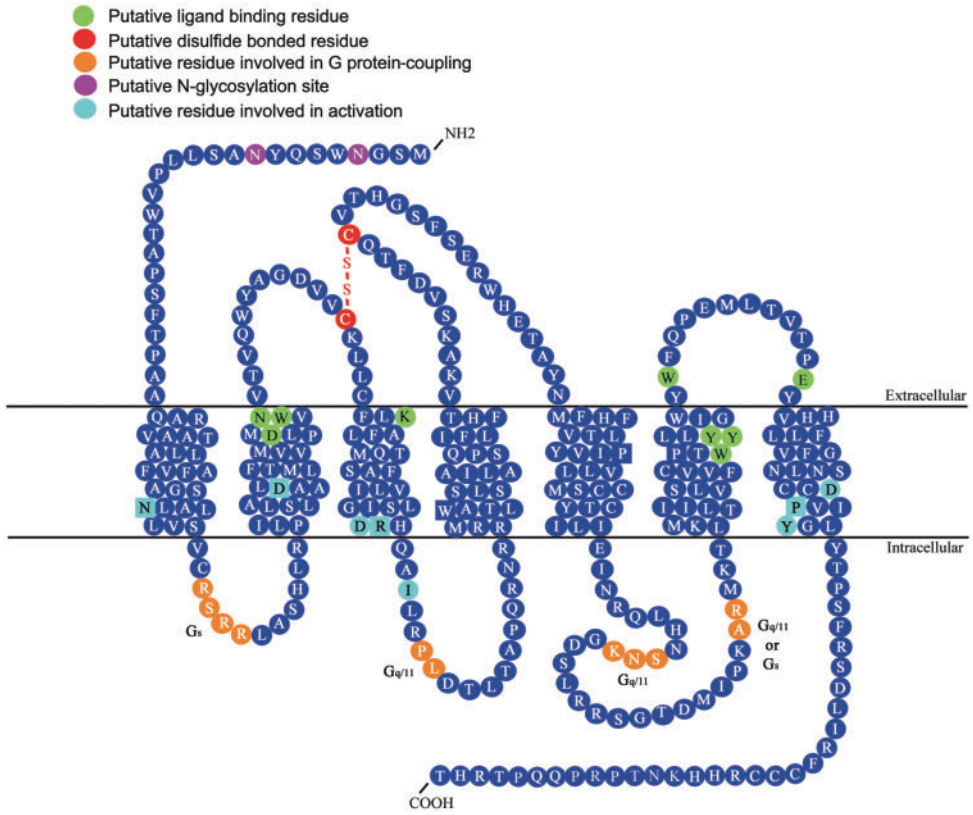

Fig. 2 Helical net representation of the zebrafish GnRHR3. Key functional residues that are conserved with other GnRHRs are highlighted (see key). The reference residue in each helix is indicated by a square. For the GnRHR numbering convention see reference (Sealfon et al. 1997).

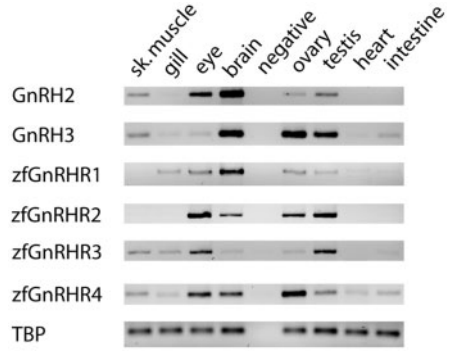

Fig. 3 PCR amplicons displaying tissue expression of zebrafish GnRHR cDNAs. Two control reactions were run; one negative (no template DNA) for each primer set and one positive control TBP for each tissue examined. A representative amplicon from each primer pair was cloned and sequenced to confirm that each primer pair was specific.

Both zfGnRHR1 and R3 showed a greater preference for GnRH2 compared with GnRH3, whereas zfGnRHR2 and R4 showed equal potency with both native ligands (Table 5). GnRH2 was the most potent with zfGnRHR3, inducing a dose-dependent stimulation with an EC_{50} value of 0.1 nM. The least potent endogenous ligand–receptor pair was GnRH3 with zfGnRHR1, stimulating intracellular IP accumulation dose dependently with EC_{50} values of 308 nM. Another distinct characteristic of the receptors is that zfGnRHR2 and R4 showed a distinct response to the non-native GnRH1 (Log EC_{50} -7.75 and -7.97), whereas zfGnRHR1 and R3 did not respond to the peptide in the physiological range (Table 5). As a control, IP levels were measured in COS7 cells transfected with empty vector and stimulated with either GnRH2 or GnRH3; IP did not rise above basal levels (data not shown).

Only one zebrafish GnRHR activates the intracellular cAMP signaling pathway

The ability of GnRH2 and GnRH3 to induce cAMP accumulation in zfGnRHR transfected COS7 cells was analyzed. Only treatment with increasing concentrations of GnRH2 elicited a rise in cAMP accumulation in zfGnRHR3-expressing cells. The maximal response was at least two-fold over basal levels with an EC_{50} of 56 nM (Fig. 5). We were unable to detect any significant responses with the

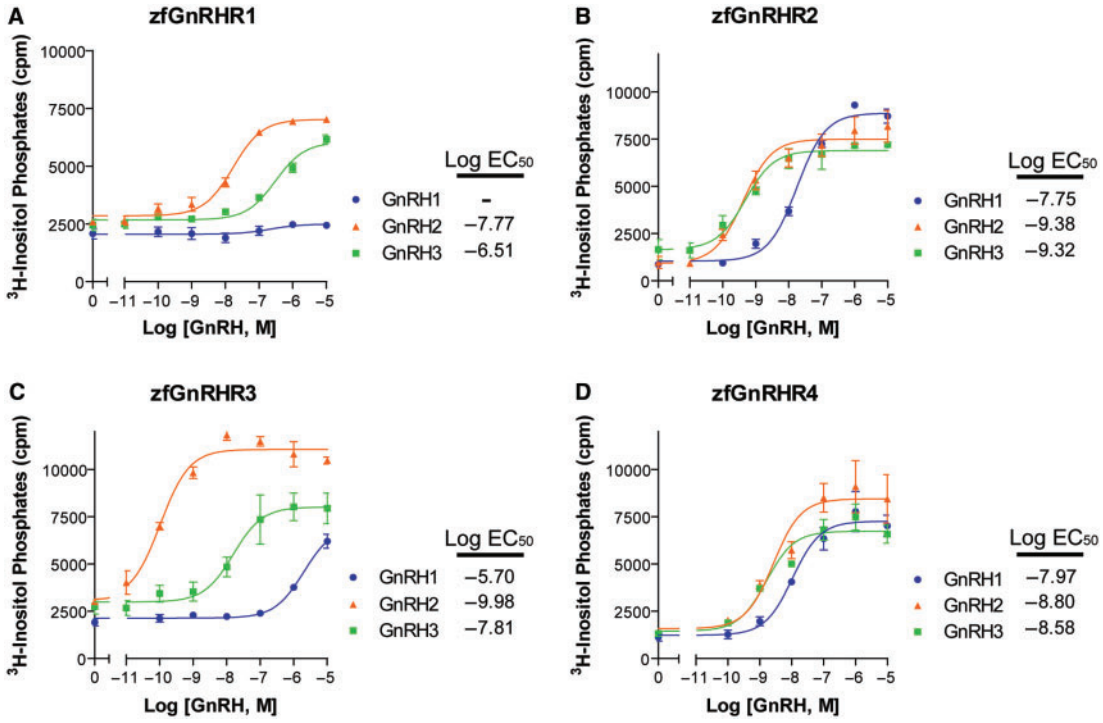

Fig. 4 *myo*-[2-^{3}H]IP accumulation in COS7 cells expressing either zfGnRHR1 (A), zfGnRHR2 (B), zfGnRHR3 (C), or zfGnRHR4 (D) induced with graded concentrations of indicated peptide for 1h. Cells were transfected with 0.8 μg of vector containing GnRHR cDNA. At 24 h after transfection, labeled inositol was added and 48 h post-transfection the cells were washed and then stimulated. The intracellular ^{3}H-IP concentrations are shown as scintillation counts per minute (cpm) and *error bars* represent mean ± SEM of a minimum of three independent experiments performed in triplicate.

Table 5 I P accumulation after incubation of GnRH1, GnRH2, and GnRH3 ligands with each zebrafish GnRHR (zfGnRHR)*

	IP accumulation EC50 (log_{10} M)**			
Ligand	**zfGnRHR1**	**zfGnRHR2**	**zfGnRHR3**	**zfGnRHR4**
GnRH1	–	-7.75 ± 0.08^{d}	-5.70 ± 0.12^{f}	-7.97 ± 0.14^{d}
GnRH2	-7.77 ± 0.11^{d}	-9.38 ± 0.13^{b}	-9.98 ± 0.10^{a}	-8.80 ± 0.13^{c}
GnRH3	-6.51 ± 0.14^{e}	-9.32 ± 0.18^{b}	-7.81 ± 0.23^{d}	-8.58 ± 0.18^{c}

*Log EC_{50} ± SEM of IP measurements in COS7 cells, which had been transfected with receptor cDNA 48 h earlier. Also added to the cells was 0.9 μCi/well *myo*-[2-^{3}H]-inositol at 24 h and GnRH peptides at 1 h before measurements.

**Dose of peptide stimulating half-maximal IP response (EC_{50}). Data were derived from the means of triplicate samples from three or more independent experiments.

$^{a–f}$Log EC_{50} values that are significantly different are indicated by *different superscript letters*. GnRH concentrations that induce dose-dependent responses that are not significantly different from one another share the *same superscript letter* ($P < 0.05$)(–) denotes no response detected.

other combinations of GnRHs with the four zfGnRHRs.

Phylogeny and synteny of zebrafish GnRH receptors show conserved relationships

The locations of chromosomes and neighboring gene clusters surrounding GnRHR loci are shown for human, cow, chicken, pipid frog, and zebrafish (Fig. 6).

A molecular phylogenetic tree highlighting teleost GnRHRs was constructed for each putative amino-acid sequence using the maximum likelihood algorithm (available at http://atgc.lirmm.fr/phyml/). Figure 7 shows a maximum likelihood tree based on the degapped regions encoding transmembranes 1–7. Human oxytocin and vasopressin receptors were included as outgroups. The robustness of the internal branches was estimated by 500 bootstrap resamplings. Vertebrate GnRHRs separated into four groups described by Millar et al. (2004). The zebrafish GnRHRs cloned in the present study segregated into two types: type III and non-mammalian type I.

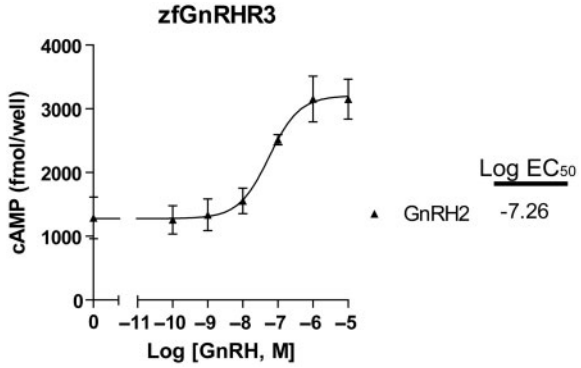

Fig. 5 cAMP accumulation in COS7 cells expressing zfGnRHR3, induced with graded concentrations of GnRH2 for 1 h. Cells were transiently transfected with 0.8 μg of vector containing zfGnRHR3 cDNA. At 48 h post-transfection, cells were washed and stimulated with GnRH2. The intracellular concentrations are shown as femtomoles per well and error bars represent mean ± SEM of a minimum of three independent experiments performed in duplicate. Other zebrafish GnRHRs did not activate the cAMP pathway.

Discussion

The present study reports the presence of four full-length zebrafish GnRHRs with conserved structural characteristics. Each receptor is physiologically active with two native GnRH forms in IP intracellular signaling assays. The spatial expression of each receptor is widespread, with high expression in brain, eye, and gonads. Finally, the mapping of each zebrafish GnRHR to its respective chromosome and the neighboring syntenic regions helps to elucidate each receptor's gene history in the context of vertebrates, including humans.

Structural motifs for folding, activation, signaling, and internalization are conserved

The cloning of four complete GnRH receptors from the zebrafish brain confirms that each transcript encodes a putative protein characteristic of non-mammalian GnRH receptors. Unlike the human type I GnRH receptor, which lacks an intracellular tail, the zebrafish receptors have tails ranging from 28 to 81 amino acids. The C-terminal tail has been implicated in controlling expression, receptor desensitization, membrane cycling, and activation of G-proteins (Sealfon et al. 1997; Blomenröhr et al. 2002; Millar et al. 2004). At the other end of the receptor molecule, zfGnRHR1, R2, and R3 have two consensus glycosylation sites (N-x-S/T) on their N-terminal extracellular extension, whereas zfGnRHR4 has three. Mutation of two N-glycosylation sites in the mouse GnRHR caused a decrease in membrane expression, but had no effect on ligand binding or on activation of intracellular IP accumulation. This suggests that glycosylation has a role in maintaining receptor expression levels, presumably by improving receptor trafficking or stability (Davidson et al. 1995).

Zebrafish GnRHRs contain key residues and motifs for activation, G-protein coupling, and internalization. All zebrafish GnRHRs contain the arginine-cage motif (DRxxxI/V) at the intracellular junction of TM3 and a DPxxY domain in TM7. The arginine in the cage motif has been shown to be necessary for the transition of the receptor into an active confirmation by coordinating interactions with the conserved N/DPxxY micro-domain in TM7 (Ballesteros et al. 1998; Oliveira et al. 1999). The N/DPxxY motif has been shown to be important for internalization, agonist–induced receptor activation and G-protein signal transduction (Arora et al. 1996). Like other GPCRs in family A, the zfGnRHRs possess conserved cysteine residues, two of which form a disulfide bond between the first two ECLs necessary for correct receptor folding (Karnik et al. 1988; Gether 2000). ZfGnRHR2 and R4 retain an additional cysteine residue in the N-terminus that may form a second disulfide bond, as in the human type I GnRH receptor (Davidson et al. 1997). Each receptor has at least one cysteine residue in its C-terminal tail in context with neighboring basic and/or hydrophobic residues that may facilitate tail palmitoylation. This may create an additional fourth ICL by anchoring the tail into the plasma membrane. Palmitoylation of other GPCRs in family A has been shown to contribute to the accessibility of regulatory phosphorylation sites located downstream of the palmitolyated cysteine (Moffett et al. 1996). Each zfGnRHR has one or more consensus phosphorylation motif (S/T) × (R/K) thought to be targets of protein kinase C (PKC) (Hanyaloglu et al. 2001). Only zfGnRHR3 has a putative Src homology 3 (SH3) binding motif (PxxP) in its intracellular tail which could facilitate the coupling to mitogen-activated protein kinases (MAPKs) (Millar et al. 2004).

The four GnRH receptors activate the IP pathway

Both GnRH2 and GnRH3 stimulated dose-dependent accumulation of IP after heterologous expression of the four zebrafish GnRH receptors in COS7 cells, indicating that each was functional and able to activate the IP signaling pathway. The sensitivity of the receptors showed that GnRH2 was more potent than GnRH3 with zfGnRHR3 and R1, but was equally as potent with zfGnRHR2 and R4. GnRH1 has been identified in bony fish that evolved before teleosts but was lost early in the teleost lineage (Sherwood et al. 1991). As shown in Fig. 4B and D, two zebrafish

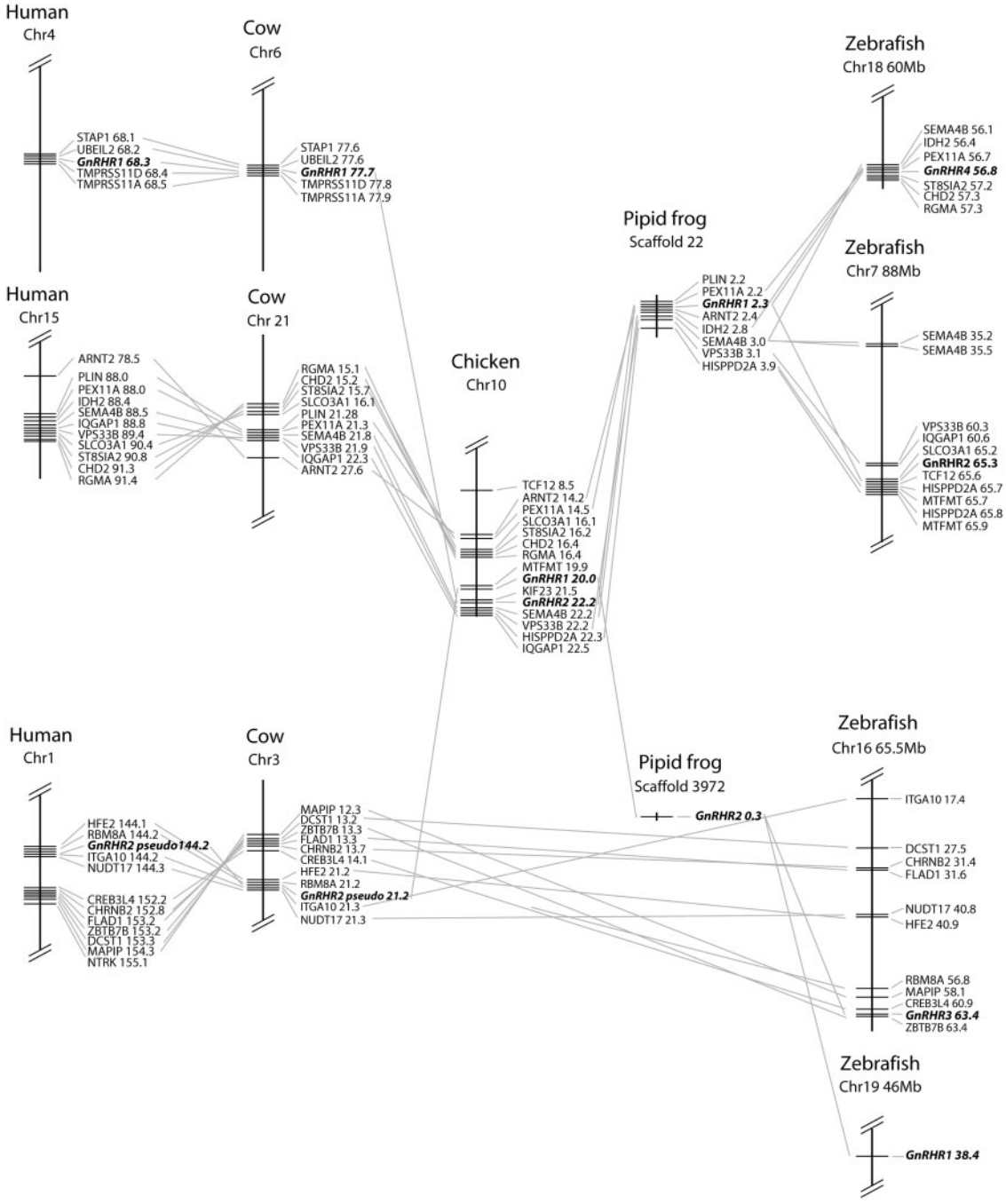

Fig. 6 ZfGnRHR gene synteny. Chromosomal arrangement of conserved neighboring genes surrounding each GnRHR gene loci in human, cow, chicken, pipid frog, and zebrafish (chromosome locations are shown in megabases). Gene abbreviations are: ARNT2, aryl-hydrocarbon receptor nuclear translocator 2; CHD2, chromodomain helicase DNA binding protein 2; CHRNB2, Neuronal acetylcholine receptor subunit beta-2 precursor; CREB3L4, cAMP responsive element binding protein 3-like 4; DCST1, DC-STAMP domain-containing protein 1; FLAD1, FAD1 flavin adenine dinucleotide synthetase homolog; GNRHR/GnRHR, gonadotropin-releasing hormone receptor; GNRHR pseudo, gonadotropin-releasing hormone receptor non-functional pseudogene; GPR89A, G protein-coupled receptor 89A; HISPPD2A, histidine acid phosphatase domain containing 2A; HFE2, hemochromatosis type 2; IDH2, isocitrate dehydrogenase 2; ITGA10, integrin, alpha 10; IQGAP1, Q motif containing GTPase activating protein 1; KIF23, kinesin family member 23; MAPIP, mitogen-activated protein-binding protein-interacting protein; MTFMT, mitochondrial methionyl-tRNA formyltransferase; NTRK1, neurotrophic tyrosine kinase, receptor, type 1; NUDT17, nudix (nucleoside diphosphate linked moiety X)-type motif 17; PEX11A, peroxisomal biogenesis factor 11A; PLIN, perilipin; RBM8A, RNA binding motif protein 8A; RGMA, RGM domain family, member A; SEMA4B, semaphorin 4B; SLCO3A1, solute carrier organic anion transporter family, member 3A1; STAP1, signaltransducing adaptor protein 1; ST8SIA2, ST8 alpha-N-acetyl-neuraminide alpha-2, 8-sialyltransferase 2; TCF12, transcription factor 12 (HTF4, helix-loop-helix transcription factors 4); TMPRSS11A, transmembrane protease, serine 11A; TMPRSS11D, transmembrane protease, serine 11D; UBE1L2, ubiquitin-activating enzyme E1-like 2; VPS33B, vacuolar protein sorting 33 homolog B; and ZBTB7B, zinc finger and BTB domain containing 7B.

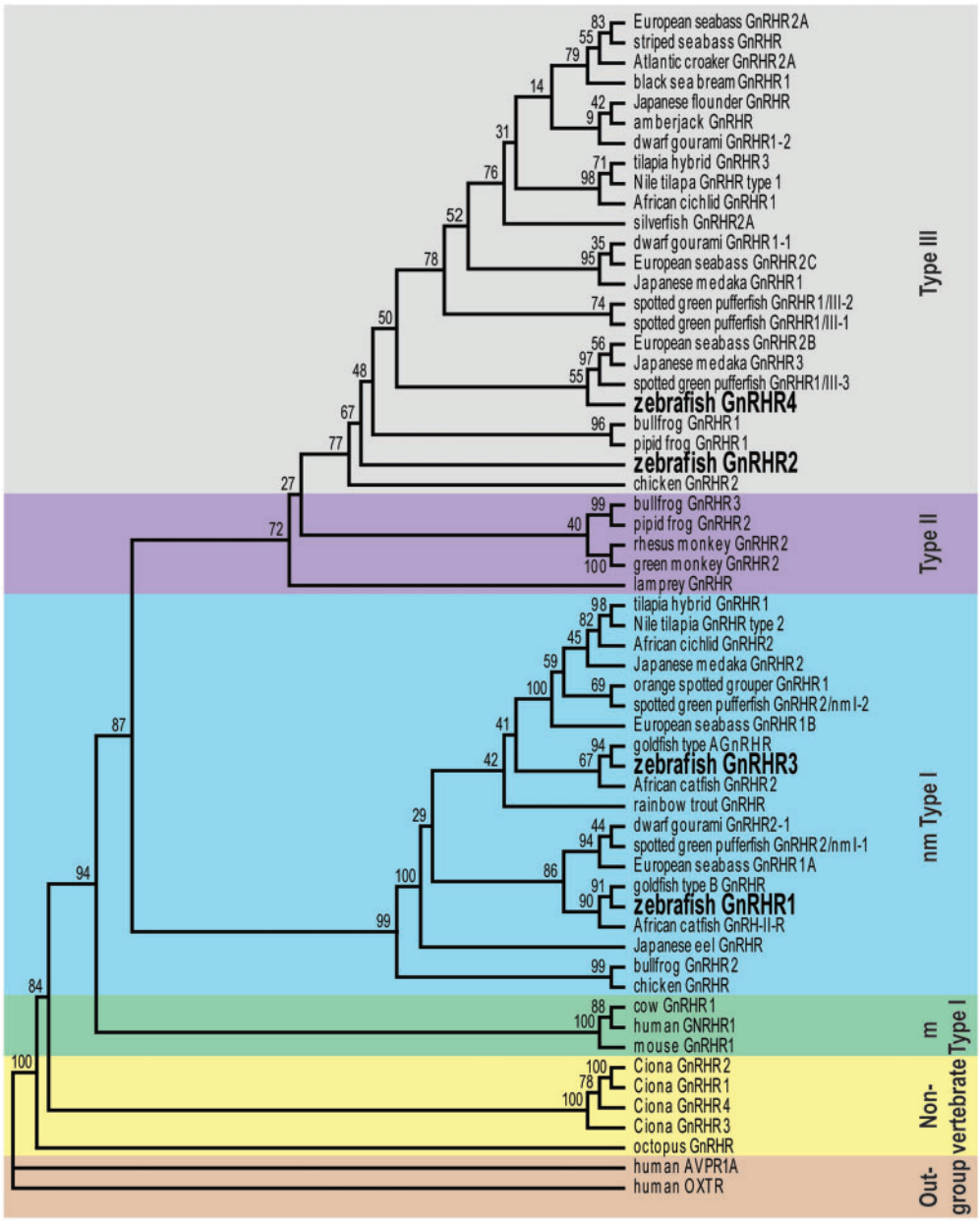

Fig. 7 Phylogenetic analysis of GnRH receptors. All complete teleost GnRHRs available in GenBank are used with other select vertebrates to resolve grouping. Human vasopressin and oxytocin receptors are used as outgroups. Receptors characterized in this report are bolded.

GnRHRs retained the ability to respond to GnRH1 at physiological concentrations.

ZfGnRHR1 had ~18-fold higher response to GnRH2 than to GnRH3 and zfGnRHR3 had ~149-fold higher potency to GnRH2 than to GnRH3. ZfGnRHR1 and R3 displayed a similar pattern of IP accumulation in response to GnRH2 and GnRH3 as the two goldfish GnRHRs, type B and A, respectively (Illing et al. 1999). There was a difference in absolute EC_{50} values between the two zebrafish and goldfish GnRHRs, with the goldfish receptors displaying ~four-fold higher sensitivity to each of the endogenous ligands tested. To date, equivalents to zfGnRHR2 and zfGnRHR4 have not been found in goldfish. Of the four zebrafish receptors, GnRHR1 was the least sensitive to physiological doses of GnRH. Two possibilites are that this receptor uses an intracellular signaling pathway other than the cAMP or IP path or that the *zfGnRHR1* gene has accumulated mutations that are associated with the transition to a pseudogene.

In addition to activation of the intracellular accumulation of IP, zfGnRHR3 elicited the intracellular accumulation of cAMP in response to graded

concentrations of GnRH2, presumably by coupling to an alternate G-protein ($G\alpha_s$) pathway. Previous studies have shown that some mammal, bullfrog, teleost, and protochordate GnRHRs show a ligand-induced cAMP turnover via activation of adenylyl cyclase (Levavi-Sivan and Yaron 1992; Bogerd et al. 2002; Oh et al. 2003; Tello et al. 2005). A more recent study demonstrated that only sustained stimulation of the GnRH receptor in LβT2 gonadotrope cells led to a concomitant increase in cAMP. These authors suggest that *in vivo* the cAMP-signaling pathway may be selectively recruited under intense GnRH release such as during the preovulatory surge (Larivière et al. 2007). The ability to activate this alternate pathway may indicate that zfGnRHR3 has additional functions *in vivo* unique from the other receptors.

ZfGnRHR3 was the most sensitive of the four receptors (EC_{50} 0.1 nM) and the most selective; the response to GnRH2 was ~149-fold higher than to GnRH3. GnRHR1 was similar but less sensitive and showed higher selectivity for GnRH2 over GnRH3. In contrast, both zfGnRHR2 and R4 were equally sensitive to GnRH2 and GnRH3, which was also demonstrated with the Japanese medaka GnRHR3 and the European seabass GnRHR2A (Okubo et al. 2003; Kah et al. 2007). In addition, zfGnRHR1 and R3 segregate to the same group (non-mammalian type I) in the phylogram, whereas zfGnRHR2 and R4 are type III, indicating that these receptors may also share a conserved function. The similar ligand preference of these receptors suggests that both GnRH2 and GnRH3 are endogenous ligands in this fish species.

Functional targets are widespread in peripheral organs

Although not reported here, all four GnRHRs are expressed in the pituitary (unpublished observation). Our studies here have focused on delineating possible autocrine/paracrine GnRH mechanisms within unconventional organs, mainly in gonadal tissues due to the presence of both GnRH and receptors (for reviews see Harrison et al. 2004 and Ramakrishnappa et al. 2005). We amplified transcripts of both forms of GnRH and all four receptors from the testes and ovaries of zebrafish, indicating that local networks may be present. In zebrafish, GnRH3 has been detected previously in the interstitial cells of the testis using immunoaffinity (Kuo et al. 2005). Studies in the testes of rats and humans showed that GnRH mRNA is expressed in Sertoli cells, whereas the receptor is expressed in Leydig cells (Bahk et al. 1995; Botté et al. 1998). In cells cultured from testes of rats, GnRH was shown to increase the expression of GnRH receptors and incubation with GnRH agonists blocked steroidogenesis (Dufau et al. 1984; Botté et al. 1999). GnRH-induced apoptosis occurs only during the late stage of spermatogenesis in the testes of mature goldfish (Andreu-Vieyra et al. 2005). Detailed studies with zfGnRHR antibodies will be essential for establishing specific testicular targets.

In the ovary, *in situ* studies of expression have shown GnRH1 mRNA in granulosa cells at many stages of follicular development, with the changes in ovarian receptor expression shown to correlate with the degree of development across the human estrous cycle (Kang et al. 2003). GnRH has been shown to have many effects in the ovary, such as inhibition of DNA synthesis, induction of apoptosis and activation of genes encoding factors important for follicular rupture and oocyte maturation. A recent study found that tissue-specific expression of the human GnRHR in granulosa-luteal cells is mediated by usage of alternative promoters in upstream regulatory elements not critical for expression in pituitary cell lines (Cheng et al. 2002). Other studies have suggested that the GnRH system is involved in the control of atresia in the ovary (Kang et al. 2003).

The presence of GnRH networks within the gonads of vertebrates may have been evolutionarily conserved. Our earlier studies using protochordate tunicates identified two genes expressing a total of six unique GnRH forms and showed that injection of these peptides near the gonads *in vivo* resulted in spawning (Powell et al. 1996; Adams et al. 2003; Tello et al. 2005). The absence of a developed pituitary in these animals suggests that GnRH acts directly on the gonads.

Expression of all four GnRH receptors and both native GnRHs was found in the zebrafish eye. Studies in the goldfish found GnRH axons from the terminal nerve projecting to the eye and a more recent study in the cichlid, *Astatotilapia burtoni*, has localized GnRHR1 receptor mRNA to the amacrine cell layer in the retina and GnRHR2 mRNA to the ganglion cells, which convey visual information to the brain (Demski and Northcutt 1983; Grens et al. 2005). Fernald's group suggested that GnRH from the terminal nerve could broadly influence sensory processing of retinal signals both in the lateral and vertical processing circuits via these two receptors, respectively (Grens et al. 2005). A more recent study found that application of both GnRH2 and GnRH3 increased excitatory postsynaptic currents from retinal fibers to the periventricular neurons in rainbow trout, which might have neuromodulatory effects on the brain to regulate both homing behavior in salmonids in addition to reproductive behaviors in other animals (Kinoshita et al. 2007). Also, we found expression of receptors in various other tissues, notably skeletal

muscle and gill. Although we did not sample blood for receptor expression, the heart tissue was negative for cDNAs of GnRH2 and GnRHR1, 2, and 3. A faint PCR product for GnRH3 and GnRHR4 in heart tissue suggests a minimal expression in blood can not be ruled out for this peptide and receptor.

Molecular evolution of the GnRH receptor loci in vertebrates

To delineate vertebrate GnRHR gene history, we analyzed the neighboring chromosomal regions surrounding each receptor locus from model genomes at important evolutionary positions. Often the diversification of vertebrates does not coincide with the phylogenetic relationships of a single gene family due to gene duplication, rapid gene losses after duplication, and the incomplete identification of the complete gene repertoire within a given species. To help resolve the gene history of each GnRHR, a combined synteny mapping and phylogentic approach was used in this study.

Our phylogram shows that the four zebrafish GnRHRs segregate into two distinct GnRHR types (type III and non-mammalian type I), with sister relationships shared between zfGnRHR1 and R3 and zfGnRHR2 and R4, respectively. The presence of four GnRH receptors in this teleost species relative to two in most tetrapods may be explained by genome duplication in the Actinopterygii lineage, in a common ancestor of teleost fish (Amores et al. 1998). The doubling of the genome (and the resultant GnRHR repertoire) is thought to have occurred before the divergence of zebrafish, medaka, and pufferfish. In support of this theory, two homologous genes can be found in zebrafish for each single copy found in the chicken, mouse, and human genomes. Many zebrafish paralogs are unlinked and are estimated to have been formed around the same time, beween 300 and 450 mya (Taylor et al. 2001). We found that neighboring genes clustered around zfGnRHR2 and R4 are present on a single locus in pipid frogs and chickens. Of interest in our two mammalian models is the fact that many of the conserved GnRHR neighbors are seen on chromosomes separate from the GnRHR gene loci. It appears that early in the mammalian lineage, the GnRHR gene was mobilized (to chromosome 4 in humans) possibly by a transposition event in the absence of its neighboring genes. The selective loss of the C-terminal tail in all mammalian GnRHRs supports the possibility of an interchromosomal rearrangement. Our phylogenetic analysis shows that the mammalian type I receptors diverge at the base of the vertebrates, suggesting that this group of receptors have undergone rapid evolution, possibly due to this loss of their tail.

Our analysis of synteny is less revealing regarding the history of the GnRHR2 locus. ZfGnRHR3 appears to be an ortholog of type II GnRHR gene, sharing many of the neighboring genes present in the GnRHR2 locus from the mammalian lineage. However, we did not find these shared genes surrounding the zfGnRHR1 locus. Phylogenetic comparisons and high amino acid similarity with zfGnRHR3 show that zfGnRHR1 is the paralog of zfGnRHR3. The incomplete-genome sequencing project of the pipid frog (*X. tropicalis*) is unable to clarify GnRHR2 relationships as the GnRHR2 gene is located on a very small scaffold without available data on neighboring genes. Adding to the complexity, the two chicken GnRHR gene loci are present on the same chromosome (chromosome 11) within three megabases of each other. Our phylogenetic analysis indicates that the receptors segregate into two distinct groups, implying that these genes were not the result of recent tandem duplication. This organization in the chicken may have been the result of an interchromosomal rearrangement, possibly by a chromosomal fusion present in the avian lineage. Surprisingly, the GnRHR2 gene locus in select mammals has been the target of mutational events acting to effectively silence this gene (Morgan et al. 2006). Our gene synteny and phylogeny indicate that mammalian type I and type III GnRHRs evolved from the same gene loci whereas type II and non-mammalian type I GnRHRs evolved from a separate GnRHR gene. Taken together, these analyses highlight vertebrate GnRHR evolution, suggesting that two GnRHR loci were present in a vertebrate ancestor over 450 mya

The maintenance of four functional receptors in the zebrafish after large-scale duplication indicates that each of the two duplicates may have partitioned functions that were previously covered by an ancestral ortholog (subfunctionalization). Alternatively, one of two gene paralogs may have undergone critical structural changes that impart a novel function (neofunctionalization) that allows them to be maintained within the genome by a method first proposed by Ohno (1970). Finally, the distribution of two distinct types of GnRH ligands, each segregated to specific areas in the brain with varied potencies at each receptor suggests that different physiological pathways may be served by each receptor in this model vertebrate.

Acknowledgments

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References

Adams BA, Tello JA, Erchegyi J, Warby C, Hong DJ, Akinsanya KO, Mackie GO, Vale W, Rivier JE, Sherwood NM. 2003. Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, *Ciona intestinalis*. Endocrinology 144: 1907–19.

Amores A, et al. 1998. Zebrafish hox clusters and vertebrate genome evolution. Science 282:1711–4.

Andreu-Vieyra CV, Buret AG, Habibi HR. 2005. Gonadotropin-releasing hormone induction of apoptosis in the testes of goldfish (*Carassius auratus*). Endocrinology 146:1588–96.

Arora KK, Cheng Z, Catt KJ. 1996. Dependence of agonist activation on an aromatic moiety in the DPLIY motif of the gonadotropin-releasing hormone receptor. Mol Endocrinol 10:979–86.

Bahk JY, Hyun JS, Chung SH, Lee H, Kim MO, Lee BH, Choi WS. 1995. Stage specific identification of the expression of GnRH mRNA and localization of the GnRH receptor in mature rat and adult human testis. J Urol 154:1958–61.

Ballesteros J, et al. 1998. Functional microdomains in G-protein-coupled receptors. The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor. J Biol Chem 273:10445–53.

Blomenröhr M, Bogerd J, Leurs R, Goos H. 2002. Differences in structure-function relations between nonmammalian and mammalian GnRH receptors: what we have learnt from the African catfish GnRH receptor. Prog Brain Res 141:87–93.

Bogerd J, Diepenbroek WB, Hund E, van Oosterhout F, Teves AC, Leurs R, Blomenröhr M. 2002. Two gonadotropin-releasing hormone receptors in the African catfish: no differences in ligand selectivity, but differences in tissue distribution. Endocrinology 143:4673–82.

Botté MC, Chamagne AM, Carre MC, Counis R, Kottler ML. 1998. Fetal expression of GnRH and GnRH receptor genes in rat testis and ovary. J Endocrinol 159:179–89.

Botté MC, Lerrant Y, Lozach A, Berault A, Counis R, Kottler ML. 1999. LH down-regulates gonadotropin-releasing hormone (GnRH) receptor, but not GnRH, mRNA levels in the rat testis. J Endocrinol 162:409–15.

Cheng CK, Yeung CM, Chow BK, Leung PC. 2002. Characterization of a new upstream GnRH receptor promoter in human ovarian granulosa-luteal cells. Mol Endocrinol 16:1552–64.

Chengalvala M, Kostek B, Frail DE. 1999. A multi-well filtration assay for quantitation of inositol phosphates in biological samples. J Biochem Biophys Methods 38:163–70.

Davidson JS, Assefa D, Pawson A, Davies P, Hapgood J, Becker I, Flanagan C, Roeske R, Millar R. 1997. Irreversible activation of the gonadotropin-releasing hormone receptor by photoaffinity cross-linking: localization of attachment site to Cys residue in N-terminal segment. Biochemistry 36:12881–9.

Davidson JS, Flanagan CA, Zhou W, Becker II, Elario R, Emeran W, Sealfon SC, Millar RP. 1995. Identification of N-glycosylation sites in the gonadotropin-releasing hormone receptor: role in receptor expression but not ligand binding. Mol Cell Endocrinol 107:241–5.

Demski LS, Northcutt RG. 1983. The terminal nerve: a new chemosensory system in vertebrates? Science 220:435–37.

Dufau ML, Warren DW, Knox GF, Loumaye E, Castellon ML, Luna S, Catt KJ. 1984. Receptors and inhibitory actions of gonadotropin-releasing hormone in the fetal Leydig cell. J Biol Chem 259:2896–9.

Gether U. 2000. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev 21:90–113.

Grens KE, Greenwood AK, Fernald RD. 2005. Two visual processing pathways are targeted by gonadotropin-releasing hormone in the retina. Brain Behav Evol 66:1–9.

Hanyaloglu AC, Vrecl M, Kroeger KM, Miles LE, Qian H, Thomas WG, Eidne KA. 2001. Casein kinase II sites in the intracellular C-terminal domain of the thyrotropin-releasing hormone receptor and chimeric gonadotropin-releasing hormone receptors contribute to beta-arrestin-dependent internalization. J Biol Chem 276:18066–74.

Harrison GS, Wierman ME, Nett TM, Glode LM. 2004. Gonadotropin-releasing hormone and its receptor in normal and malignant cells. Endocr Relat Cancer 11:725–48.

Ikemoto T, Park MK. 2005. Identification and molecular characterization of three GnRH ligands and five GnRH receptors in the spotted green pufferfish. Mol Cell Endocrinol 242:67–79.

Illing N, Troskie BE, Nahorniak CS, Hapgood JP, Peter RE, Millar RP. 1999. Two gonadotropin-releasing hormone receptor subtypes with distinct ligand selectivity and differential distribution in brain and pituitary in the goldfish (*Carassius auratus*). Proc Natl Acad Sci USA 96:2526–31.

Jodo A, Ando H, Urano A. 2003. Five different types of putative GnRH receptor gene are expressed in the brain of masu salmon (*Oncorhynchus masou*). Zoolog Sci 20:1117–25.

Kah O, Lethimonier C, Somoza G, Guilgur LG, Vaillant C, Lareyre JJ. 2007. GnRH and GnRH receptors in metazoa: a historical, comparative, and evolutive perspective. Gen Comp Endocrinol 153:346–64.

Kanda A, Takahashi T, Satake H, Minakata H. 2006. Molecular and functional characterization of a novel gonadotropin–releasing-hormone receptor isolated from the common octopus (*Octopus vulgaris*). Biochem J 395:125–35.

Kang SK, Choi KC, Yang HS, Leung PC. 2003. Potential role of gonadotrophin-releasing hormone (GnRH)-I and

GnRH-II in the ovary and ovarian cancer. Endocr Relat Cancer 10:169–77.

Karnik SS, Sakmar TP, Chen HB, Khorana HG. 1988. Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin. Proc Natl Acad Sci USA 85:8459–63.

Kinoshita M, Kobayashi S, Urano A, Ito E. 2007. Neuromodulatory effects of gonadotropin-releasing hormone on retinotectal synaptic transmission in the optic tectum of rainbow trout. Eur J Neurosci 25:480–84.

Kozak M. 1996. Interpreting cDNA sequences: some insights from studies on translation. Mamm Genome 7:563–74.

Kuo MW, Lou SW, Postlethwait J, Chung BC. 2005. Chromosomal organization, evolutionary relationship, and expression of zebrafish GnRH family members. J Biomed Sci 12:629–39.

Kusakabe T, Mishima S, Shimada I, Kitajima Y, Tsuda M. 2003. Structure, expression, and cluster organization of genes encoding gonadotropin-releasing hormone receptors found in the neural complex of the ascidian *Ciona intestinalis*. Gene 322:77–84.

Larivière S, Garrel G, Simon V, Soh JW, Laverrière JN, Counis R, Cohen-Tannoudji J. 2007. Gonadotropin-releasing hormone couples to 3′,5′-cyclic adenosine-5′-monophosphate pathway through novel protein kinase Cδ and -ε in LβT2 gonadotrope cells. Endocrinology 148:1099–107.

Lethimonier C, Madigou T, Munoz-Cueto JA, Lareyre JJ, Kah O. 2004. Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. Gen Comp Endocrinol 135:1–16.

Levavi-Sivan B, Yaron Z. 1992. Involvement of cyclic adenosine monophosphate in the stimulation of gonadotropin secretion from the pituitary of the teleost fish, tilapia. Mol Cell Endocrinol 85:175–82.

Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR. 2004. Gonadotropin-releasing hormone receptors. Endocrine Rev 25:235–75.

Moffett S, Adam L, Bonin H, Loisel TP, Bouvier M, Mouillac B. 1996. Palmitoylated cysteine 341 modulates phosphorylation of the β2-adrenergic receptor by the cAMP-dependent protein kinase. J Biol Chem 271:21490–97.

Moncaut N, Somoza G, Power DM, Canario AV. 2005. Five gonadotrophin-releasing hormone receptors in a teleost fish: isolation, tissue distribution and phylogenetic relationships. J Mol Endocrinol 34:767–79.

Morgan K, Sellar R, Pawson AJ, Lu ZL, Millar RP. 2006. Bovine and ovine gonadotropin-releasing hormone (GnRH)-II ligand precursors and type II GnRH receptor genes are functionally inactivated. Endocrinology 147:5041–51.

Oh DY, Wang L, Ahn RS, Park JY, Seong JY, Kwon HB. 2003. Differential G protein coupling preference of mammalian and nonmammalian gonadotropin-releasing hormone receptors. Mol Cell Endocrinol 205:89–98.

Ohno S. 1970. Evolution by gene duplication. Berlin: Springer-Verlag.

Okubo K, et al. 2003. A novel third gonadotropin-releasing hormone receptor in the medaka *Oryzias latipes*: evolutionary and functional implications. Gene 314:121–31.

Oliveira L, Paiva AC, Vriend G. 1999. A low resolution model for the interaction of G proteins with G protein-coupled receptors. Protein Eng 12:1087–95.

Palevitch O, Kight K, Abraham E, Wray S, Zohar Y, Gothilf Y. 2007. Ontogeny of the GnRH systems in zebrafish brain: *in situ* hybridization and promoter-reporter expression analyses in intact animals. Cell Tissue Res 327:313–22.

Powell JF, Reska-Skinner SM, Prakash MO, Fischer WH, Park M, Rivier JE, Craig AG, Mackie GO, Sherwood NM. 1996. Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. Proc Natl Acad Sci USA 93:10461–64.

Ramakrishnappa N, Rajamahendran R, Lin YM, Leung PC. 2005. GnRH in non-hypothalamic reproductive tissues. Anim Reprod Sci 88:95–113.

Reinhart J, Mertz LM, Catt KJ. 1992. Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor. J Biol Chem 267: 21281–84.

Schally AV, Arimura A, Kastin AJ, Matsuo H, Baba Y, Redding TW, Nair RM, Debeljuk L, White WF. 1971. Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones. Science 173:1036–38.

Schwanzel-Fukuda M. 1999. Origin and migration of luteinizing hormone-releasing hormone neurons in mammals. Microsc Res Tech 44:2–10.

Sealfon SC, Weinstein H, Millar RP. 1997. Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. Endocrine Rev 18:180–205.

Sherwood NM, Adams BA. 2005. Gonadotropin-releasing hormone in fish: evolution, expression and regulation of the GnRH gene. In: Melamed P, Sherwood NM, editors. Hormones and their receptors in fish reproduction, Vol. 4. Singapore: World Scientific Publishing Co. Pte. Ltd. p. 1–39.

Sherwood NM, Doroshov S, Lance V. 1991. Gonadotropin-releasing hormone (GnRH) in bony fish that are phylogenetically ancient: reedfish (*Calamoichthys calabaricus*), sturgeon (*Acipenser transmontanus*), and alligator gar (*Lepisosteus spatula*). Gen Comp Endocrinol 84:44–57.

Silver MR, Kawauchi H, Nozaki M, Sower SA. 2004. Cloning and analysis of the lamprey GnRH-III cDNA from eight species of lamprey representing the three families of *Petromyzoniformes*. Gen Comp Endocrinol 139:85–94.

Steven C, Lehnen N, Kight K, Ijiri S, Klenke U, Harris WA, Zohar Y. 2003. Molecular characterization of the GnRH system in zebrafish (*Danio rerio*): cloning of chicken GnRH-II, adult brain expression patterns and pituitary content of salmon GnRH and chicken GnRH-II. Gen Comp Endocrinol 133:27–37.

Taylor JS, Van de Peer Y, Braasch I, Meyer A. 2001. Comparative genomics provides evidence for an ancient genome duplication event in fish. Philos Trans R Soc Lond B Biol Sci 356:1661–79.

Tello JA, Rivier JE, Sherwood NM. 2005. Tunicate gonadotropin-releasing hormone (GnRH) peptides selectively activate *Ciona intestinalis* GnRH receptors and the green monkey type II GnRH receptor. Endocrinology 146:4061–73.

Tensen C, Okuzawa K, Blomenröhr M, Rebers F, Leurs R, Bogerd J, Schulz R, Goos H. 1997. Distinct efficacies for two endogenous ligands on a single cognate gonadoliberin receptor. Eur J Biochem 243:134–40.

Torgersen J, Nourizadeh-Lillabadi R, Husebye H, Alestrom P. 2002. *In silico* and *in situ* characterization of the zebrafish (*Danio rerio*) *gnrh3* (sGnRH) gene. BMC Genomics 3:25.

Tsutsumi M, Zhou W, Millar RP, Mellon PL, Roberts JL, Flanagan CA, Dong K, Gillo B, Sealfon SC. 1992. Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. Mol Endocrinol 6:1163–69.

Vickers ED, Laberge F, Adams BA, Hara TJ, Sherwood NM. 2004. Cloning and localization of three forms of gonadotropin-releasing hormone, including the novel whitefish form, in a salmonid, *Coregonus clupeaformis*. Biol Reprod 70:1136–46.

Whitlock KE. 2005. Origin and development of GnRH neurons. Trends Endocrinol Metab 16:145–51.

Whitlock KE, Illing N, Brideau NJ, Smith KM, Twomey S. 2006. Development of GnRH cells: setting the stage for puberty. Mol Cell Endocrinol 254–255:39–50.

Wray S. 2002. Development of gonadotropin-releasing hormone-1 neurons. Front Neuroendocrinol 23:292–316.

Wu S, Page L, Sherwood NM. 2006. A role for GnRH in early brain regionalization and eye development in zebrafish. Mol Cell Endocrinol 257–258:47–64.