Journal of Biomedical Science (2005) 12:629–639
DOI 10.1007/s11373-005-7457-z

Chromosomal organization, evolutionary relationship, and expression of zebrafish GnRH family members

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Received 6 April 2005; accepted 17 May 2005

Key words: GnRH, LHRH, sGnRH, cGnRH-II, zebrafish, olfactory

Summary

Multiple forms of gonadotropin-releasing hormone (GnRH) are found in different vertebrates. In this study, we have cloned cDNA encoding the full-length *gnrh3* and *gnrh2* from zebrafish brain and characterized their structure and expression patterns. We performed phylogenetic analysis and compared conserved syntenies in the region surrounding the *GnRH* genes from human, chicken, pufferfish, and zebrafish genores. The *gnrh3* and *gnrh2* genes were mapped to LG17 and LG21, respectively. The zebrafish genome appears to lack an ortholog to human *GNRH1*, and the human genome appears to lack an ortholog of *gnrh3*. Expression of *gnrh3* began in the olfactory pit at 24–26 h postfertilization and expanded to the olfactory bulb during early larval stage. Expression of *gnrh2* is always in the midbrain. In addition, GnRH is also expressed in boundary cells surrounding seminiferous cysts of the testis. Thus, this detailed phylogenetic, chromosomal comparison, and expression study defines the identity and the evolutionary relationship of two zebrafish *gnrh* genes. We propose a model describing the evolution of *gnrh* genes involving ancestral duplication of the genes followed by selective loss of one gene in some teleosts.

Introduction

Gonadotropin-releasing hormone (GnRH, previously called luteinizing hormone-releasing hormone, LHRH) is a decapeptide that controls reproduction through the hypothalamus–pituitary–gonad axis in vertebrates [1]. GnRH is secreted from hypothalamus of the brain and it stimulates secretion of gonadotropins from the pituitary. Gonadotropins then circulate through the bloodstream to activate sexual maturation and reproductive functions of gonads.

To date, 14 structurally distinct GnRH forms are known in vertebrates [2–5]. Most GnRH isoforms are composed of 10 highly conserved amino acids which are spliced from longer precursor prehormone peptides. Different GnRH isoforms have been isolated from different species, and were originally named according to their species of origin [6–8], but this nomenclature is confusing, since most vertebrate species have two or three different forms of GnRH.

White and Fernald (1998) later classified GnRH subtypes into three classes according to the tissue of origin: hypothalamic form (GnRH-I), mesencephalic form (GnRH-II) and telencephalic form (GnRH-III) [9]. But the discovery of additional GnRH forms from salmon and catfish reveals that this nomenclature system does not account for all forms of GnRH. Parhar [3] described a different nomenclature system, based on expression pattern and ontogenic origins. Cells of olfactory origin gives rise to GnRH-I; the highly conserved mesencephalic GnRH as GnRH-II; sGnRH (salmon GnRH) of advanced fishes as GnRH-III;

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mdGnRH (medaka GnRH) and sbGnRH (seabream GnRH) in advanced fishes as GnRH-IV [3]. This nomenclature is again confusing, and few people are using it. A recent nomenclature system classified GnRH into three classes [1].

Zebrafish has become increasingly more important in the study of development and genetics. Many zebrafish genes have been isolated recently, which enables evolutionary studies [10, 11]. Two zebrafish *gnrh* genes have been cloned [12, 13]; they are expressed in the olfactory bulb-terminal nerve region and midbrain, respectively [12–15]. In this report, we investigated the evolutionary relationship of these two genes through analyses of phylogeny, chromosomal conserved syntenies, and expression patterns. Our analysis supports the classification of *GnRH* genes into three families. We also found that zebrafish lacks the *GnRH1* gene just like other primitive fish, while tetrapods do not have *GnRH3*. In addition, we detected testicular GnRH expression in the boundary cells surrounding seminiferous cysts. This provides a basis for further studies *of GnRH* genes.

Materials and methods

Animals

Zebrafish of AB and TL strains were maintained, bred, and reared at 28.5 °C following standard methods [16]. Developmental times refer to hours (hpf) or days (dpf) postfertilization.

gnrh3 and gnrh2 cDNA cloning

Total RNA was extracted from zebrafish testis using Tri-reagent (Sigma, St. Louis, MO). To obtain the *gnrh3* and *gnrh2* complete cDNA, rapid amplification of 5′- and 3′-cDNA ends (5′-RACE and 3′-RACE) was performed with brain total RNA using the SMART cDNA amplification kit (CLONTECH laboratories, Inc, Palo Alto, CA). The RACE products from this cDNA were subcloned into pGEM-T easy vectors (Promega, Madison, Wisconsin, USA) and sequenced.

Phylogenetic analysis

Nucleotide and predicted peptide sequences were compared with known *GnRH* genes in GenBank (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments were generated using sequences for unprocessed full-length proteins, and phylogenetic trees were constructed with the neighbor-joining method in a commercial sequence analysis program CLUSTAL W and NJplot (http://www.ebi.ac.uk/clustalw/ and http://pbil.univ-lyonl.fr/software/njplot.html). The genomic information for human, chicken, pufferfish (fugu and tetraodon), and zebrafish was compiled after searching the database (http://www.sanger.ac.uk/DataSearch/databases.shtml/and http://vega.sanger.ac.uk/Danio_rerio/).

Isolation of zebrafish BAC clones containing GnRH genes

Genomic clones containing the *gnrh* genes were isolated from a zebrafish BAC genomic DNA library (Genome Systems Inc, St. Louis, Missouri, USA) using *gnrh3* cDNA fragments as probes. Four clones (81d01, 131o09, 135e16 and 148c10) were further analyzed. The 135e16 and 148c10 BAC clones consisted of the complete *gnrh3* gene and a large fragment *of gnrh3* promoter.

RT-PCR

Reverse transcription was performed using the Superscript preamplification system (Gibco BRL) with 0.5 μg oligo$(dT)_{12–18}$ and 3 μg of each total RNA in a 20 μl reaction as described [17]. The cDNA product (1 or 2μl) was used in PCR with *gnrh3, gnrh2* and actin primers for 30 cycles at 95 °C for 60 s, 55 °C for 60 s and 72 °C for 60 s.The *gnrh3* primers (CACAGCAGTTTTAGCATGGAGTG and ACACTCTTCCCCGTCTGTCGG) amplified cDNA from nt.13–310. The *gnrh2* primers (ATGGTGCTGGTCTGCAGGCTG and GTAGGAACTGCTGCAAATGGGT) amplified cDNA from nt.167–635. The actin primers [18] (TCACACCTTCTACAACGAGCTGCG and GAAGCTGTAGCCTCTCTCGGTCAG) generated a 340-bp fragment. PCR products were analyzed on 1.5% agarose gels.

In situ *hybridization*

Whole-mount *in situ* hybridization was performed using digoxigenin-labeled antisense RNA probe and anti-digoxigenin alkaline phosphatase-conjugated

antibody as described previously [19]. The *gnrh3* probe was generated from the *pGEM-T-gnrh3*/*Ncol*-linearized template using SP6 RNA polymerase, and the *gnrh2* probe was from the *pGEM-T-gnrh2*/*SpeI*-linearized template using T7 RNA polymerase. These *gnrh3* and *gnrh2* probes were 298 and 469 bp in length containing all coding region and some untranslated region, respectively. After staining and mounting, images were captured with a digital camera.

Immunohistochemistry

For immunocytochemical analysis, adult testes were fixed in 4% paraformaldehyde/PBS solution for 16 h, following our own established method [10] and VECTASTAIN ABC kit (Vector Laboratories, Inc, Burlingame, CA, USA). The anti-Gnrh3 antibody (gift from Dr. Chen-Chih Kao, Institute of Marine Biology, National Sun Yat-Sen University, Kaohsiung, Taiwan) and anti-3β-HSD antibody were diluted at 1:1000 and 1:5000, respectively. The anti-3β-HSD antibody was produced in our own lab (Chung et al., unpublished result). After staining and mounting, images were captured with a digital camera.

Results

Cloning and characterization of Zebrafish GnRH genes

We isolated cDNA clones encoding *gnrh3* and *gnrh2* from zebrafish brain RNA using the method of 5′-and 3′-Rapid Amplification of cDNA Ends. The *gnrh2* cDNA is 655 bp in length and contains a predicted open reading frame of 86 amino acids. Comparing this sequences with those from the database (GenBank accession number: AF511531 and NM_181439), there is a single nucleotide change in *gnrh2* changing Codon 11 from ATG (Met) to GTG (Val).

The *gnrh3* cDNA is 430 bp in length and contains a predicted open reading frame of 94 amino acids. Genomic clones containing the *gnrh3* gene were isolated from a zebrafish BAC genomic DNA library using *gnrh3* cDNA fragments as probes. The 135e16 and 148c10 BAC plasmids contained the complete *gnrh3* gene and a large 5′-flanking region (Figure 1). Both the cDNA and the genomic clones have been sequenced. Two other sequences were also present in the database. Comparing all four sequences, there were three

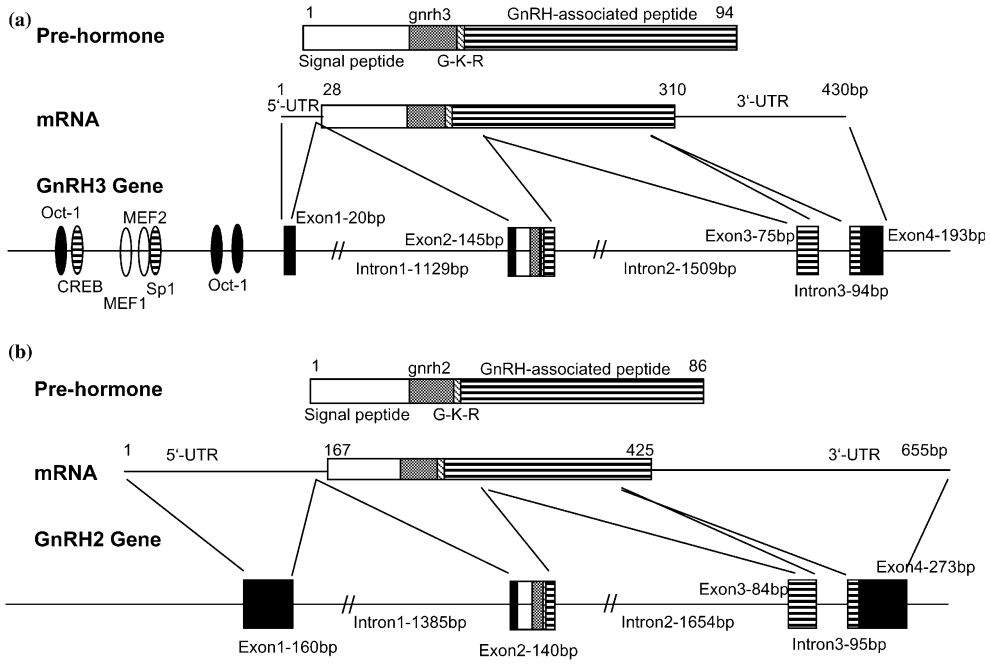


Figure 1. Structure of the *gnrh3* and *gnrh2* prepropeptide, mRNA and gene. The Gnrh3 preprohormone and cDNA are 94 a.a. and 430 bp long, respectively. The *gnrh3* gene consists of 4 exons (20, 145, 75 and 193 bp each) and three introns (1129, 1509 and 94 bp each). The Gnrh2 preprohormone and cDNA are 86 a.a. and 655 bp long, respectively. The *gnrh2* gene consists of four exons (160, 140, 84 and 273 bp each) and three introns (1385, 1654 and 95 bp each). G-K-R, protease cleavage site.

polymorphic changes without affecting codon usage, which were Leu8 (CTG or TTG), Pro50 (CCT or CCA) and one nucleotide in the 3′-untranslated region. Computer analysis (http://www.cbil.upenn.edu/tess/) predicted the presence of binding sites for transcription factors like Oct-1, MEF-1, MEF-2, Otx-2, Sp1, and CREB in this 5′-flanking region (Figure 1). Both Oct-1 and MEF transcription factors have been shown to regulate *GnRH* transcription [20–23].

The structure of *gnrh2* gene is similar to that of *gnrh3*, with four exons and three introns. It encodes a polypeptide composed of the signal peptide, the GnRH peptide, and GnRH-associated peptide. Computer analysis (http://www.cbil.upenn.edu/tess/) predicted the presence of binding sites for transcription factors like Oct-1, MEF-2, AP-1, Sp 1, GR, AR and CREB in this 5′-flanking region (Figure 1). As yet, the transcriptional regulation of *gnrh2* is still not clear.

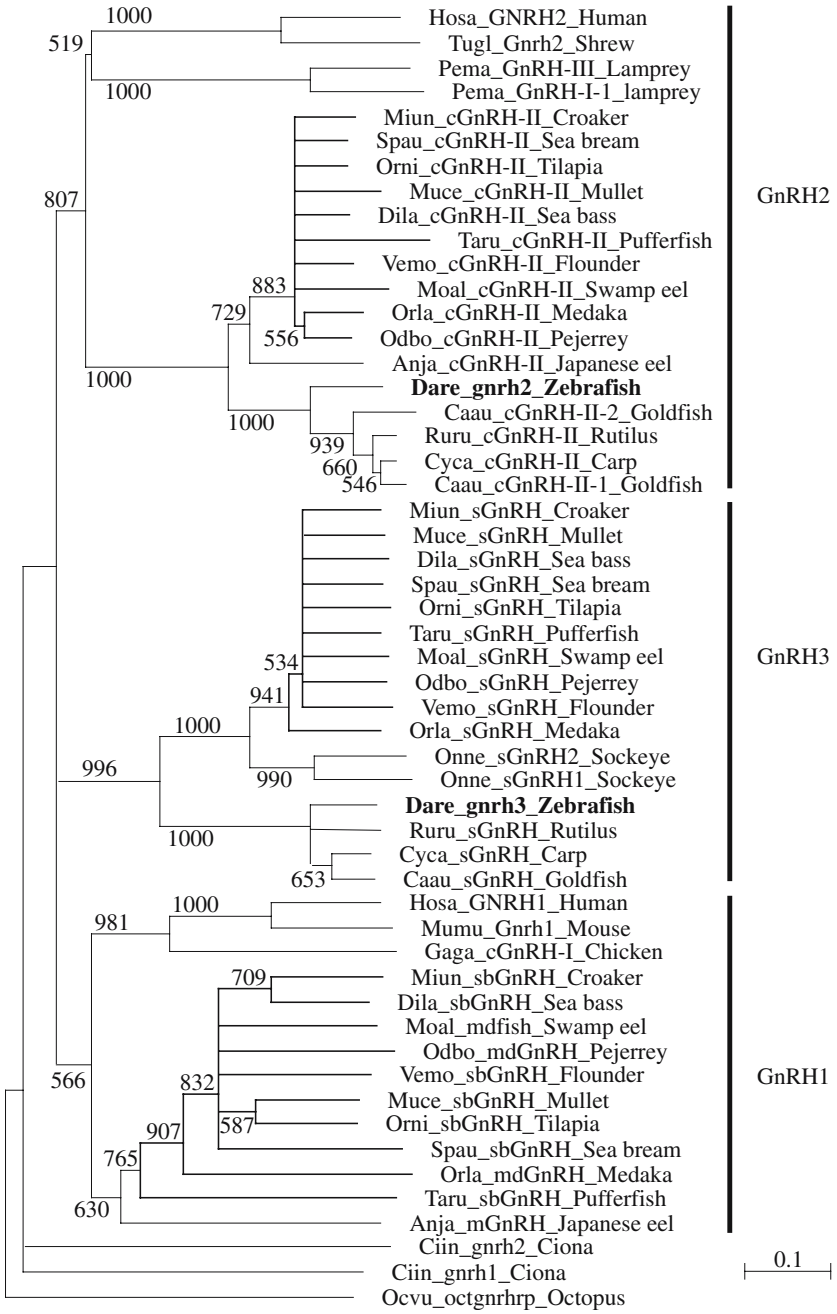
Phylogenetic analysis of GnRH genes

The classification of GnRH isoforms is still in flux because new isoforms continue to be discovered and researchers have not yet exhaustively examined all GnRH isoforms from very many species. To understand how the two zebrafish GnRH isoforms fit into the evolutionary scheme, we performed phylogenetic analysis of a large list of GnRH preprotein isoforms in genome databases. The results showed that *GnRH* genes were already present in stem chordates, and probably much earlier as well, given the presence of a *GnRH-like* gene in octopus. Independent duplication of a *GnRH2-like* gene appears to have occurred in the lamprey lineage. Other vertebrate *GnRH* genes usually branched as expected from the known evolutionary relationships of the species; and GnRH isoforms fell into three natural clades, GnRH1, GnRH2, and GnRH3, which were formerly called sea bream, chicken, and salmon GnRH, respectively (Figure 2). The zebrafish *gnrh2* gene clustered with other *GnRH2* family members, while *gnrh3* clustered on the *GnRH3* branch, and neither was close to the *GnRH2* branch. Each of the GnRH3 and GnRH2 peptides from zebrafish, goldfish and rutilus clustered together as expected from species phylogenies. Two *Gnrh2* genes in goldfish clustered as expected for an independent genome duplication [24], as did two *Gnrh3* genes in sockeye salmon, whose lineage also experienced a recent genome duplication event [25]. Many fish of the Percomorpha Clade had a copy of each of the three *GnRH* genes.

Figure 2. Phylogenetic tree connecting the known GnRH peptides. The common names of the organism, followed by GnRH form. Zebrafish gnrh3 and gnrh2 are closed to the gnrh3 and gnrh2 of goldfish and rutilus, respectively, as expected from organismal relationships. The Percornorpha blade is shown by heavier lines. The accession number for the sequences are as follows: Orni_sbGnRH_Tilapia (AB104861); Vemo_sbGnRH Flounder (AB066360); Dila_sbGnRH_Sea bass (AF224279); Spau_sbGnRH_Sea bream (U30320); Orla_mdGnRH_Medaka (AB041336); Anja_mGnRH_Japanese eel (AB026991); Mumu _mGnRH_Mouse (M14872); Hosa_mGnRH_human (NM_ 000825); Gaga_cGnRH-I_Chicken (P37042); Miun-sbGn RH_Croaker (AY324668); Moal_mdGnRH_swamp eel (AY8 58056); Odbo_mdGnRH_Pejerrey (AY744689); Muce_ sbGnRH_Mullet (AY373450); Taru_sbGnRH_Pufferfish (scaffold 155:1:291812); Ruru_sGnRH_Rutilus (U60667); Dare_ gnrh3_Zebrafish(AJ304429); Caau_sGnRH_Goldfish (U30 301); Orla_sGnRH_Medaka (ABO41335); Orni_SGnRH_Tilapia (AB104863); Spau_sGnRH_Sea bream (U30311); Vemo_ sGnRH_Flounder (AB066358); (X79712); Onne_sGnRH1_ Sockeye (AF232212); Onne-sGnRH2_Sockeye (AF232213); Miun-sGnRH_Croaker (AY324670); Moal_sGnRH_swamp eel (AY858055); Odbo_sGnRH_Pejerrey (AY744688); Muce_SGnRH_Mullet (AY373449); Dila_sGnRH_Sea bass (AF224280); Taru_sGnRH_ Pufferfish (SINFRUP0000013 1193); Cyca_sGnRH_carp (AY189960); Hosa-GNRH2_Human (NM_001501); Tugl_ Gnrh2_Shrew (U63327); Orni_cGnRH-II_Tilapia (AB104862); Spau_cGnRH-II_Sea bream (U30325); Vemo_cGnRH-1I_Flounder(AB066359); Orla_cGnRH-II_ Medaka (AB041334); Anja_cGnRH-II_Japanses eel (AB026990); Caau_ cGnRH-II-1_Goldfish (U30386); Ruru_cGnRH-II_Rutilus (U60668), Caau_cGnRH-II-2_Goldfish (U40567); Dare-_gnrh2_Zebrafish (AF511531); Miun-cGnRH-II_Croaker (AY324669); Moal_cGnRH-Il_swamp eel (AY786183); Odbo_cGnRH-II_Pejerrey (AY744687); Muce_cGnRH-II_Mullet (AY373451); Dila_cGnRH-II_Sea bass (AF224281); Taru_cGnRH-II Pufferfish (SINFRUP0000 0150964); Cyca_cGnRH-II_carp (AY189961) Pema_1GnRH-I-1_lainpfey (AF144481); Pema_lGnRH-III_ Lamprey (AY05 2628); Ciin_gnrhl_Ciona (AAP06794); Ciin_gnrh2_Ciona (AAP06796); Ocva_octgnrhrp_Octopus (BAB86782).

Chromosomal analysis of GnRH genes

The lack of a GnRH1 isoform from zebrafish and a GnRH3 from human could be due to problems in zebrafish *GnRH1* cDNA cloning and gene identification, or due to the true absence of these genes from the genomes of these taxa. To differentiate between these two possibilities, we compared the genomic context of *GnRH* genes from various vertebrates. We mapped *gnrh3* and *gnrh2*



genes to LG17 and LG21, respectively (Figure 3). Searching genome database (http://www.sanger.ac.uk/DataSearch/databases.shtml/ and http://vega.sanger.ac.uk/Danio_rerio/), we also obtained genomic information surrounding *GnRH* genes from human, chicken, pufferfish (fugu and tetraodon), and zebrafish (Figure 3).

Comparing these sequences, we detected strong conservation in chromosomal synteny in the region surrounding the *GnRH2* gene among these species. The nearest neighbor of *GnRH2* is *ptpra* in zebrafish (LG21, 5.3–5.4 Mb), tetraodon (LG11, 880–890 kb), chicken (chromosome 4, 88.6–88.7 Mb), and human (chromosome 20p13, 2.8–

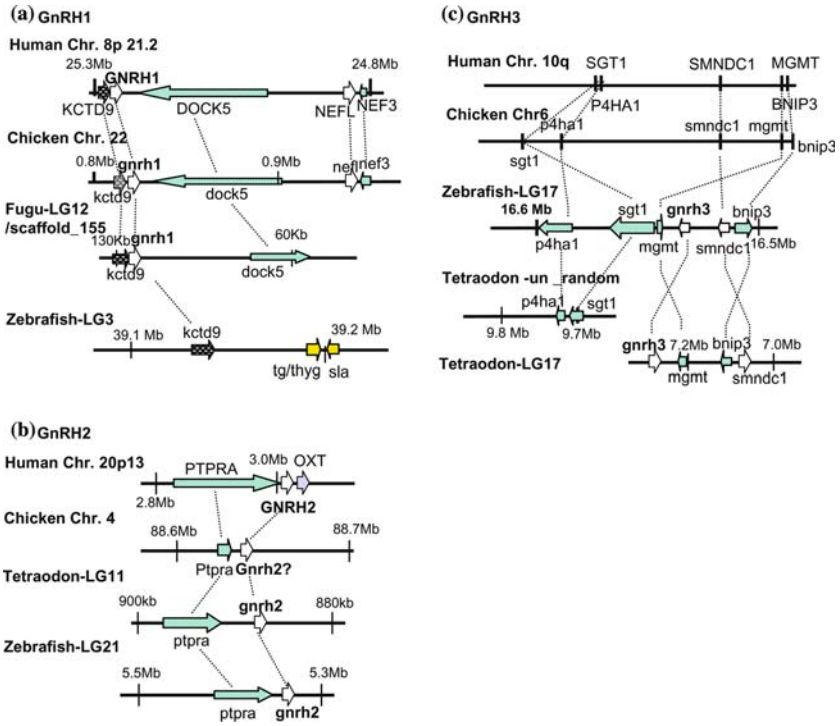


Figure 3. Chromosome maps of human, chicken, pufferfish, and zebrafish genes surrounding the locus of *GNRH*. (a) *GNRH1* is located between *DOCK5* and *KCTD9* in chicken and human chromosomes. A *gnrh1* ortholog is not found downstream from *dock5* and *kctd9* in pufferfish and zebrafish. (b) *GNRH2* is conserved and located near *PTPRA* from zebrafish to human. (c) The *gnrh3* gene is located near *mgmt* in pufferfish and zebrafish. *GNRH3* is not found in human and chicken genomes.

3.1 Mb) (Figure 3b). Our genomic analysis shows that this chromosome region has remained intact in ray-fin (zebrafish and pufferfish) and lobe-fin (human and chicken) evolution.

The *GNRH1* gene is located between *DOCK5* and *KCTD9* in human (chromosome 8p21.2, 25.0–25.3 Mb), chicken (chromosome 22, 0.8–0.9 Mb) and fugu (LG12, 60–130 kb). Analysis of similar regions from the zebrafish genome did not reveal a *gnrh* gene near the *kctd9* locus (Figure 3a). Likewise, *DOCK5*, the nearest neighbor on the other side of *GNRH1*, is on zebrafish genome segment Zv4_NA11200, which has no trace of a *gnrh* gene. In addition, the *NEF* gene was found to reside next to the *DOCKS5* gene in the human, chicken, and tetraodon genomes, and *nef3* and *nefl* are adjacent on zebrafish genome segment BX005111.4.1-169747 without a *gnrh* gene nearby, therefore this region of the chromosome has been preserved except for zebrafish genome, which is greatly rearranged in this region. The lack of *gnrh1* gene from this region of the zebrafish chromosomes probably indicates that zebrafish does not have an ortholog of *GNRH1*. This finding is consistent with the report that only two forms of GnRH peptides were isolated from zebrafish [26]. We hypothesize that the chromosome rearrangement in the zebrafish lineage that separated *kctd9* from *dock5* resulted in the deletion of *gnrh1* from the genome.

Reciprocally, the *GnRH3* gene appears to be missing in human and chicken genomes (Figure 3c). The *GnRH3* gene was located near *sgtl, p4hal, mgmt smndc1*, and *bnip3* genes in tetraodon (LG17, 7.2–7.3 Mb) and zebrafish (LG17, 16.5–16.6 Mb). These five genes were found linked in the chicken and human, therefore this gene synteny is conserved in the fish, human and chicken genomes. But the *gnrh3* gene is clearly missing from the human and chicken genomes. Again, the location predicted for *GNRH3* in tetrapod genomes is at the breakpoint of an inversion, an event which may have precipitated the loss of an ancient *GNRH3* gene. The best model to account for these data is that the last common ancestor of zebrafish and human had a *gnrh3* gene between *SMNDC1* and *SGT1*.

Model of GnRH gene evolution

What can we say about the origin of 3 GnRH genes? GnRH genes have been isolated from octopus and urochordates [4, 27, 28], showing that the gene is ancient among bilaterians and possessed by the original chordates. Although the urochordate *Ciona* and the lamprey, a basally diverging vertebrate, have multiple forms of *GnRH* genes, these likely arose from independent duplication events in their respective lineages (Figure 2).

The human chromosome segment containing *GNRH1* on Hsa8p21-p11.2 and the predicted location of *GNRH3* in Hsa10q23 is the third largest paralagon (duplicated chromosome segment) in the human genome [29] (Human Paralagon Website at http://wolfe.gen.tcd.ie/dup/human5.28/). In addition, the chromosome region containing *GNRH1* on Hsa8p21.1 and *GNRH2* on Hsa20p13 are also paralogous [29]. These paralogy relationships suggest that the last common ancestor of human and zebrafish possibly had all three *GNRH* genes. After the divergence of ray-fin and lobe-fin fish, the *GNRH3* gene was lost sometime in the human lineage, and the *GNRH1* gene was lost at least in the zebrafish lineage, and perhaps independently in the salmonid lineage. Gene loss in the zebrafish lineage must have occurred after it diverged from the lineage giving rise to the Percomorpha Clade of teleosts because many of these species have been shown to have a copy of each of the three *GNRH* genes (see Figure 2).

In another model (Figure 4), the ancestral *gnrh* gene was first duplicated to become ancestors for *gnrh1* and *gnrh2* before the separation of vertebrates and invertebrates. After the separation of ray-fin and lobe-fin fishes, the ancestor of *gnrh1* was duplicated to give rise to ancestors for *gnrh1* and *gnrh3*. These genes were lost in some teleost lineages to yield present day clades with *gnrh1* and *gnrh3*, some clades with *gnrh2* and *gnrh3*, and others with all three forms. We prefer this model because the divergence of the *gnrhl* and *gnrh3* genes was calculated from the ratio of synonymous vs. non-synonymous substitutions to be about 200–400 million years ago (Kuo and Chung, unpublished data), at around the time of teleost speciation. This model can account for all *gnrh* genes so far, but its validity will be further tested only when more *gnrh* genes are isolated.

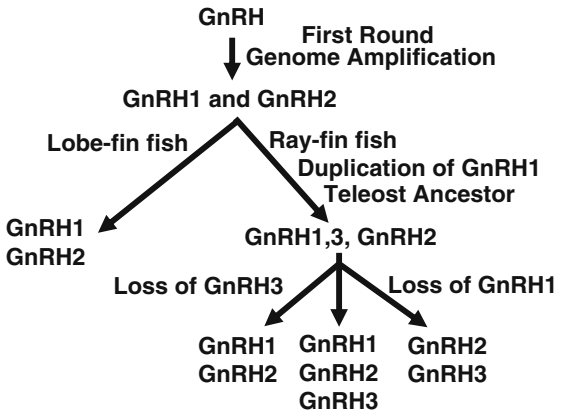


Figure 4. A model for the evolutionary relationship of *gnrh* genes. Two *gnrh* ancestral genes were present and retained in tetrapods. In teleosts, *gnrh1* was further duplicated to give rise to *gnrh3*. These three genes are present in some fishes, but are randomly lost in other fishes lineages. This results in the presence of *gnrh1* and *gnrh3* in some fishes, while other fishes have *gnrh2* and *gnrh3*.

Expression of gnrh3 and gnrh2 genes during development

One way to classify gene family is by way of their expression patterns. Both zebrafish *gnrh2* and *gnrh3* genes are expressed in adult brain (Figure 5). In addition, there are other minor sites of gene expression like liver and ovary for *gnrh3*, and ovary, gill, muscle, testis, and heart for *gnrh2*. Expression patterns of *gnrh3* and *gnrh2* genes were also examined during embryogenesis using RT-PCR. Expression of *gnrh3* and *gnrh2* first appeared at about the pharyngula stage (Figure 5), which is about 24 h postfertilization (hpf). These transcripts then persisted throughout life.

It has been show that *gnrh2* and *gnrh3* are expressed in the midbrain and olfactory nerve of zebrafish embryos, respectively [14]. Adult *Gnrh1* cells are located in the preoptic area [12]. To examine the migratory process of these *gnrh*-expressing cells, we performed whole-mount *in situ* hybridization to track *gnrh* cells in older larvae (Figure 6). The *gnrh3* transcripts first started to appear at 26–30 hpf in two small cell cluster in the ventromedial forebrain. Double *in situ* hybridization with the *atoh2b (ndr1b)* gene, which is expressed in the olfactory bulb [30], reveals that *gnrh3* expression is at the level of olfactory placode (Figure 6a, b). The number of *gnrh3-expressing*

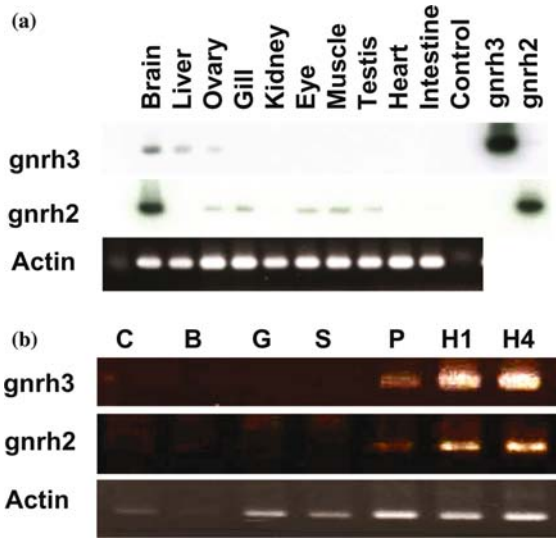


Figure 5. Expression pattern of *gnrh3* and *gnrh2* genes during development and in adult tissues are detected by RT-PCR. RNA was isolated from different stage, and primers specific for different *gnrh3* and *gnrh2* genes were used for RT-PCR. (a) *gnrh3* and *gnrh2* genes were mainly expressed in brain. *gnrh3* is also expressed in liver and ovary. *gnrh2* is expressed at ovary, eye, and testis. (b) Expressions of *gnrh3* and *gnrh2* genes start in the pharyngula stage. The developmental stages are as follows: C, cleavage; B, blastula; G, gastrula; S, segmentation; P, pharyngula; H1, 1 day after hatching; H4, 4 days after hatching.

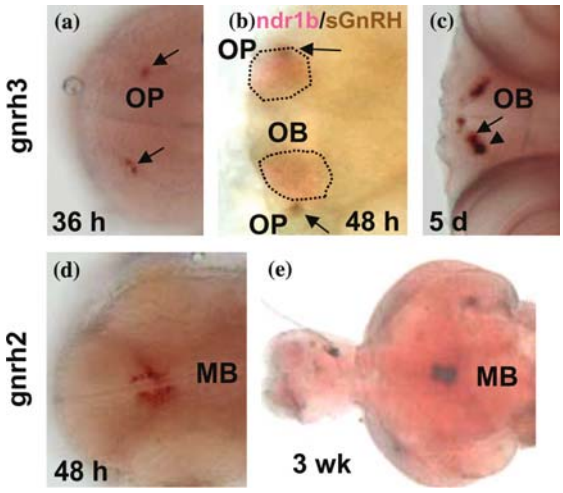


Figure 6. Expression of *gnrh3* and *gnrh2* in zebrafish embryonic and larval brains as detected by *in situ* hybridization. (a) *gnrh3* is expressed in the olfactory placode (OP) at 36 hpf. (b) Double *in situ* hybridization with *gnrh3* (brown) and *ndr1b* (pink) probes at 48 hpf; ***atoh2b*** is the marker for olfactory bulb (OB). Expression of *gnrh3* is near the OB. (c) At 5 days (d) postfertilization, *gnrh3* already occupied a larger region of OB. (d, e) Expression of *gnrh2* at midbrain (MB) both at 48 hpf and 3 weeks (wk), dorsal view.

cells gradually increased to cover a wider region of the olfactory bulb but this expression domain had not migrated out even by 5 dpf (Figure 6c and data not shown). Thus the migration of *gnrh3* cells appears to be a late event; they have not reached the preoptic area at 5 dpf.

The expression of *gnrh2* started at the midbrain before 48 hpf and continued to be detected in the same place even at 3 weeks of age (Figure 6d, e). Thus, *gnrh3* and *gnrh2* are expressed in different parts of the brain, where they may carry out different functions.

Expression of Gnrh in the testis

In addition to the main expression domain in the brain, *gnrh* is also expressed in other tissues with a lower abundance of transcript concentration as shown by our RT-PCR data (Figure 5b). To investigate this further, we used immunohistochemical methods to detect GnRH peptide in adult testis. GnRH peptide was detected in boundary cells surrounding testicular lobules, which are comprised of several seminiferous cysts (Figure 7a). The location of each cyst was detected by the antibody against 3ß-HSD, which is expressed in Leydig cells surrounding every cyst (Figure 7b). The lack of staining with negative control (without first antibody) indicates that this immunohistochemical staining is very specific (Figure 7c).

Discussion

In this paper, we described the cloning, structural analysis, expression pattern, and phylogenetic analysis of two zebrafish GnRH genes. Using methods of phylogenetic analysis, chromosomal comparison across many species, and comparison of expression patterns, we agree with the recent classification separating GnRH isoforms into three major classes [1]. GnRH1 refers to mostly mGnRH found in the hypothalamus. GnRH2 refers to cGnRH-II found in the midbrain. GnRH3 refers to the sGnRH found in the olfactory system. We provide evidence indicating that zebrafish does not have *gnrh1*, while human and chicken do not have *GNRH3*. We also present a model describing the evolutionary relationship of

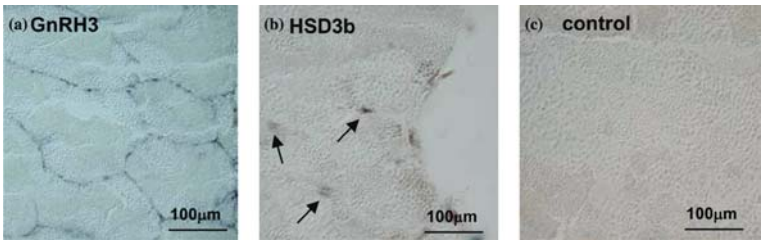


Figure 7. Expression of *gnrh3* and 3ß-HSD (Hsd3b) in zebrafish testis as detected by immunohistochemistry. (a) Gnrh peptide is expressed in the interstitial cells surrounding seminiferous cysts. (b) 3bHSD is expressed in Leydig-like cells. (c) negative control without first antibody.

these genes. In addition, we describe the expression patterns of *gnrh3* and *gnrh2* mRNA in zebrafish embryos.

Since zebrafish genome does not appear to have *gnrh1* one obvious question is which gene exerts the GnRH1 function. GnRH1 is usually secreted from the hypothalamus of mammals, yet human *GnRH1* promoter can drive gene expression in the olfactory bulb and telecephalon as demonstrated in a recent report [13]. The enhancer driving olfactory bulb and telecephalon expression is located between −976 and −929 of the zebrafish *gnrh3* promoter, and this sequence is conserved in *gnrh3* (African cichlid and Atlantic salmon) and *gnrh1* (*sbGnRH* from African cichlid) genes [9, 31, 32], suggesting that teleost *gnrh3* genes possess regulatory domains similar to mammalian *GnRHI* [13]. It is possible that zebrafish *gnrh3* has GnRHI function, as do other basally diverging teleosts (goldfish, eel and salmon) [33–35].

Clusters of GnRH-expressing cells were described as ganglia at the cribriform bone and the rostral olfactory bulb in salmon and goldfish [33, 35–37], but at the caudal-most part of the basal olfactory bulb in perciformes like tilapia [38]. In zebrafish, we observed major clusters of GnRH cells in the rostral olfactory bulb, suggesting that the zebrafish GnRH system is like the one in goldfish and salmon. Since both zebrafish and goldfish are cypriniformes, it is reasonable that their GnRH expression patterns are similar.

We found GnRH expression in the testis. Fish testes are bilateral elongated lobules consisting of seminiferous tubules [39]. We found that the seminiferous tubules of zebrafish are arranged in cyst-like structure, with each cyst containing sperm cells at the same developmental stage. We identified the Leydig-like cells by staining with 3β-HSD antibody, which is steroidogenic in nature. In addition, the Sertoli cells were identified by hybridization with *sox9a* probe (Tong and Chung, unpublished data), which was cloned by us [10]. The expression of GnRH does not overlap completely with Leydig cells. The exact nature of these GnRH-expressing cells and their function remain to be further examined. The function of GnRH in the testis is not clear, except that it could induce apoptosis important for spermatogenesis in mature goldfish testis [40]. GnRH-induced apoptosis occurs only during the late stage of spermatogenesis, mediated by increased levels of fas and fas ligand-like protein [41]. Thus GnRH may play an important role in the control of spermatogenesis in fish.

Acknowledgments

We thank Dr. Chen-Chih Kao (Institute of Marine Biology, National Sun Yat-Sen University, Kaohsiung, Taiwan) for gnrh3 antibody and Dr. Zhiyuan Gong (National University of Singapore) for *atoh2b* probe. This work was supported by grants NSC93-2321-B-001-018 from National Science Council and AS-94-TP-B04 from Academia Sinica, Republic of China, and R01RR10715 from NIH, USA.

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