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Expression, Structure, Function, and Evolution of Gonadotropin-Releasing Hormone (GnRH) Receptors GnRH-R1^{SHS} and GnRH-R2^{PEY} in the Teleost, *Astatotilapia burtoni*

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Multiple GnRH receptors are known to exist in nonmammalian species, but it is uncertain which receptor type regulates reproduction via the hypothalamic-pituitary-gonadal axis. The teleost fish, *Astatotilapia burtoni*, is useful for identifying the GnRH receptor responsible for reproduction, because only territorial males reproduce. We have cloned a second GnRH receptor in *A. burtoni*, GnRH-R1^{SHS} (SHS is a peptide motif in extracellular loop 3), which is up-regulated in pituitaries of territorial males. We have shown that GnRH-R1^{SHS} is expressed in many tissues and specifically colocalizes with LH in the pituitary. In *A. burtoni* brain, mRNA levels of both GnRH-R1^{SHS} and a previously identified receptor, GnRH-R2^{PEY}, are highly correlated with mRNA levels of all three GnRH ligands. Despite its likely role in reproduction, we found that GnRH-R1^{SHS} has the highest affinity for GnRH2 *in vitro* and low responsivity to GnRH1. Our phylogenetic analysis shows that GnRH-R1^{SHS} is less closely related to mammalian reproductive GnRH receptors than GnRH-R2^{PEY}. We correlated vertebrate GnRH receptor amino acid sequences with receptor function and tissue distribution in many species and found that GnRH receptor sequences predict ligand responsiveness but not colocalization with pituitary gonadotropes. Based on sequence analysis, tissue localization, and physiological response we propose that the GnRH-R1^{SHS} receptor controls reproduction in teleosts, including *A. burtoni*. We propose a GnRH receptor classification based on gene sequence that correlates with ligand selectivity but not with reproductive control. Our results suggest that different duplicated GnRH receptor genes have been selected to regulate reproduction in different vertebrate lineages. (*Endocrinology* 148: 5060–5071, 2007)

IN VERTEBRATES, REPRODUCTION is regulated by photoperiod, physical environment, and social status, which act via a final common pathway: the hypothalamic-pituitary-gonadal (HPG) axis. The hypothalamus delivers GnRH (GnRH; see *Materials and Methods* for peptide nomenclature) to the pituitary causing release of gonadotropins (LH and FSH) that travel to the gonads to regulate gonadal growth and release of sex steroids. The effects of this signaling cascade depend critically on the response of the pituitary GnRH1 receptor. GnRH1 has also been associated with pituitary control of GH (1, 2) and prolactin secretion (3), although the details of these pathways are not as well understood.

Mammalian GnRH (mGnRH1) was first identified in the mammalian hypothalamus, and additional GnRH forms were discovered in nonmammalian vertebrates including teleost fish, which express three GnRH forms: GnRH1, GnRH2, and GnRH3 (4, 5). All species studied to date, including mammals, have two or three forms of GnRH produced by distinct but phylogenetically related genes (5–9). The amino acid sequences of GnRH2 and GnRH3 are conserved, but the structure of GnRH1 varies considerably across vertebrate species (5, 6, 8). The presence of two or three GnRH peptides within a single organism raises the possibility that GnRH receptors (GnRH-R) may have coevolved with their ligands.

Multiple GnRH-R types have been reported in mammals, birds, fish, and amphibians (8, 10–12). GnRH-Rs belong to the G protein-coupled receptor family, characterized by seven hydrophobic transmembrane domains linked by hydrophilic extra- and intracellular loops (8, 13–15). The first GnRH-R sequences were cloned from mammalian pituitary mRNA (8, 13, 14) and designated type I GnRH-Rs because they regulate reproductive function (16, 17). These receptors exhibit high affinity for mGnRH1 and low affinity for other naturally occurring forms of GnRH. A conserved amino acid motif (Ser-Asp/Glu-Pro, SDP), in extracellular loop 3 (EC3) of mammalian pituitary type I GnRH-Rs, determines their

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Abbreviations: EC3, Extracellular loop 3; GnRH-R, GnRH receptor; HPG, hypothalamic-pituitary-gonadal; ICA, independent component analysis; IP, inositol phosphate; mGnRH1, mammalian GnRH1; NT, nonterritorial; T, territorial.

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specificity for mGnRH1 (18, 19). Receptors that lack the SDP motif in EC3 are not specific for mGnRH1 (8, 10, 20–22) (see GnRH-R amino acid alignment, supplemental Fig. A, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). GnRH-Rs with a PEY motif in EC3 (GnRH-R1^{PEY}) were initially classified as a nonmammalian type I GnRH-R, and they were predicted to regulate reproduction in response to GnRH1 (10). GnRH-Rs with a PPS motif were originally proposed to be the receptors for GnRH2 (10), and indeed, mammalian type II GnRH-Rs, which are specific for GnRH2, have a PPS motif (8, 23, 24).

Unfortunately, GnRH-Rs have been named numerically in the order of their discovery, without consideration of functional or evolutionary relationships (11, 25–27). Several GnRH-R nomenclature systems have been proposed that, although based on phylogenetic relationships, contradict one another (supplemental Table A, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). For example, the *Astatotilapia burtoni* GnRH-R1^{SHS} described here was initially named GnRH-R2, following Troskie *et al.* (10) but was renamed as a type I GnRH-R to be consistent with Lethimonier *et al.* (12). Classification of receptors based on function has been hampered by lack of physiological evidence about regulation of the HPG axis in nonmammalian vertebrates.

We used an African cichlid fish species, *A. burtoni*, in which reproduction is socially regulated, to identify which GnRH-R regulates reproductive function and to clarify the evolutionary relationship among known receptors. *A. burtoni* males exhibit one of two distinct social phenotypes: dominant (territorial, T) animals, which are larger, brightly colored, and reproductively capable or subordinate (nonterritorial, NT) males, which are smaller and camouflaged, have regressed gonads, and school with females (28, 29). Shifts in social status produce a corresponding change in the gonads via the HPG axis (29, 30). We have previously shown that [Ser^8] GnRH1 is the only form of GnRH found in the pituitary of *A. burtoni*, and it is up-regulated in T males, implying that it must regulate gonadotropin release (31).

We previously cloned a GnRH-R2^{PEY} from *A. burtoni* and showed that it has a relatively poor response to [Ser^8] GnRH1 *in vitro* (32) and is expressed in the dorsal-anterior and posterior pituitary (33). We also identified a partial sequence corresponding to EC3 of a second GnRH-R, now designated GnRH-R1^{SHS} (32), which is expressed in the ventral anterior and posterior pituitary and up-regulated in T males compared with NT males (33, 34). We now report the complete cloning and tissue distribution of GnRH-R1^{SHS} and compare the functional activities and regulation of GnRH-R2^{PEY} and GnRH-R1^{SHS} to determine whether expression and structure-function relationships support the identification of GnRH-R1^{SHS} as the receptor regulating reproduction in *A. burtoni*.

Materials and Methods

Nomenclature of GnRH ligands and receptors

GnRH1 denotes the peptide that regulates reproduction in vertebrates, and we use mGnRH1 to distinguish the mammalian form. For other GnRH peptide sequences, we show amino acid changes relative to the mGnRH1 sequence (35). GnRH peptides used in this study were mGnRH1 (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) and the three forms of GnRH found in *A. burtoni*, [Ser^8] GnRH1 (also called seabream GnRH), GnRH2 ([His^5,Trp^7,Tyr^8] GnRH) (36), and GnRH3 ([Trp^7,Leu^8] GnRH, also called salmon GnRH) (35, 37). GnRH-Rs are designated as in GenBank with the addition of a three-letter superscript, which is the conserved motif in EC3 (SHS, PEY, PPS, or SDP) to indicate their GnRH-R type classification. Hence, the receptor identified by our experiments becomes GnRH-R1^{SHS}, because it was designated GnRH-R1 in GenBank and contains the SHS sequence in EC3. Correspondingly, the other *A. burtoni* GnRH-R is named GnRH-R2^{PEY}. In receptors where the EC3 motif is not identical to the conserved motif, the motif in the receptor is used (*e.g.* GnRH-R1^{PDY} for one of the catfish receptors, which has PDY instead of PEY in EC3).

Animals

A. (Haplochromis) burtoni derived from a wild-caught population were raised in aquaria under conditions similar to their native equatorial habitat in Lake Tanganyika in Africa (pH 8, 28 C) (28). Both male and female fish (3–4 cm length) were used in these experiments. To collect brain tissue, fish were killed via rapid cervical transection. All work was performed in compliance with Stanford University (APLAC) guidelines.

RNA isolation, amplification, sequencing, and analysis of A. burtoni GnRH-Rs

Partial sequences corresponding to the cichlid GnRH-R1^{SHS} were originally amplified using degenerate primers designed to conserved regions of transmembrane TM6 and TM7 (10, 38). We subsequently designed a pair of degenerate primers to conserved amino acid sequences identified by an alignment with amberjack (CAB65407), striped bass (AAF28464) (32), medaka (BAB7056), and European seabass (DLA419594) GnRH-Rs. Sense primer S2 [5′-gtgggcigcica(t/c)(a/t)(g/c)iga(c/t)ggiaa-3′] was designed to the conserved motif WAAHSDGK, and the antisense primer AS1 [5′-gtticc(c/t)tcia(a/g)(g/a)tc(g/a)t-cigg(g/a)aa-3′] was designed to the conserved motif FPDDLEGK. These primers were used to amplify a 750-nucleotide product from *A. burtoni* brain cDNA that was identified as a partial sequence for GnRH-R1^{SHS}. Sequence information from this clone was used to design gene-specific primers for 5′ RACE and 3′ RACE to clone the full-length GnRH-R1^{SHS} cDNA.

5′ RACE and 3′ RACE cDNA were prepared according to the manufacturer's instructions (SMART RACE cDNA Amplification Kit; BD Biosciences, Franklin Lakes, NJ), from total RNA extracted from *A. burtoni* brain. Antisense primers CH3gsp (5′-tgcaggcaaagtctccggcaagc-3′) and CH3ngsp (5′-aatcagcaccctcacgtgggattttcg-3′) were used in combination with the 5′ universal (UPM) and nested universal (NUP) primers to amplify the 5′ end of GnRH-R1 cDNA from the 5′ RACE *A. burtoni* brain cDNA. The 3′ end of the cichlid GnRH-R1^{SHS} receptor was amplified using the sense primers 3′ gsp1 (5′-gcccgagagcccggatgagaactctg-3′) and 3′ ngsp1 (5′-gtgattgttctgtctttcatcatctg-3′) in combination with the universal (UPM) and nested universal (NUP) from the 3′ RACE *A. burtoni* brain cDNA. Commercial kits (BD Advantage 2 PCR Enzyme System; BD Biosciences) were used according to the manufacturer's instructions for both 5′ and 3′ PCR amplification reactions.

Sequence information from the 5′ RACE and 3′ RACE cDNA clones was used to design a sense and antisense primer that, respectively, included the start and stop codons of the GnRH-R1^{SHS} mRNA transcript, and which also included either an additional *Bam*HI or *Xho*I site (italic): FLs 5′-gcgc*ggatcc*accatggtggatgggcac-3′ and FLas 5′-ggccgg*ctcgag*tcataa-gatgctctcag-3′. The full-length GnRH-R1^{SHS} coding region was amplified from *A. burtoni* brain cDNA, using this pair of primers and subcloned into a *Bam*HI/*Xho*I-digested pcDNA3.1(+) expression vector (Invitrogen). Expression plasmids were purified using the PureYield Plasmid Miniprep System according to the manufacturer's alternative protocol (Promega) and sequenced using T7 and BGH reverse primer (Invitrogen, Carlsbad, CA). The nucleotide sequence of the full-length coding region is in GenBank [AY705931 (GnRH-R1, GnRH-R1^{SHS})].

Phylogenetic analysis of GnRH-R

We used phylogenetic analysis to situate *A. burtoni* GnRH-Rs with respect to previously cloned GnRH-Rs. All publicly available (Feb-

ruary 14, 2007) vertebrate full-length GnRH-Rs were obtained from GenBank. Multiple GnRH-R sequences were aligned using the predicted sequences of GnRH-R polypeptides (ClustalW program in MEGA3.1) (39). This multiple sequence alignment was used to generate a neighbor-joining tree with bootstrap values using the Jones-Taylor-Thornton matrix. Lamprey GnRH-R was used to root the phylogenetic tree. Full species names and GenBank accession numbers for the receptor cDNAs are listed in the supplemental material (published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Functional assay of GnRH-R1^{SHS}

For functional tests, the *A. burtoni* GnRH-R1^{SHS} cDNA expression plasmid was transfected into COS-1 cells using diethylaminoethyl dextran, as previously described (40). The ligand structure-function relationships of the putative GnRH-R1^{SHS} were directly compared with *A. burtoni* GnRH-R2^{PEY} (32) by measuring ligand-stimulated inositol phosphate (IP) production, as described previously (40), except that IP was extracted with formic acid (10 mM). Ligand concentrations ranged from 10^{-10} to 10^{-5} M. Data points were determined in duplicate, and EC_{50} values are the means of three separate experiments.

For competition binding assays, [His^5,D-Tyr^6] GnRH1 was radioiodinated as previously described (41). Transfected cells were washed twice with cold PBS and incubated (5 h at 4 C) with ^{125}I-labeled [His^5,D-Tyr^6] GnRH1 (10^5 cpm) in the absence (B0) or presence of various concentrations (10^{-9} to 10^{-5} M) of unlabeled peptides in 0.5 ml HEPES-buffered DMEM with BSA (0.1%). Unbound ^{125}I-labeled [His^5,D-Tyr^6]-GnRH was removed by washing twice with PBS, and cell-associated radioactivity was solubilized in 1 ml 1 M NaOH and counted. Nonspecific binding was determined in the presence of excess GnRH2 (10^{-5} M). Data points were determined in duplicate, and the IC_{50} values are the means of three separate experiments.

Localization of GnRH-R1^{SHS} mRNA transcripts

GnRH-R1^{SHS} expression was localized using PCR on tissue samples from adult *A. burtoni* (brain, pituitary gland, retina, gill, heart, intestine, kidney, liver, muscle, ovary, retina, spleen, and testes) and *in situ* hybridization using antisense receptor-specific probes in the brain and pituitary.

In situ hybridization

In situ hybridization methods are described in detail elsewhere (33), so only essential details are given here. Reproductively active males (n = 3) were killed by rapid cervical transection, and brains were removed and placed in sterile PBS. Tissue was frozen in mounting medium (OCT; Tissue-Tek, Torrance, CA) on dry ice, sectioned coronally, and stored at −80 C until use. Probes for GnRH-R1^{SHS} and GnRH-R2^{PEY} were synthesized, based on GenBank sequences (GnRH-R1^{SHS}, GenBank AY705931; GnRH-R2^{PEY}, AY028476), and plasmids were linearized to generate both sense and antisense templates for each receptor. GnRH-R1^{SHS} and GnRH-R2^{PEY} probes were from 151-1035 bp and from 55–1129 bp, respectively, and were generated by transcribing with SP6 or T7 polymerase in the presence of [^{35}S]UTP (Maxiscript kit; Ambion, Austin, TX). Slides with brain tissue were treated as described previously (33). Photomicrographs were captured digitally (SPOT camera system; Diagnostic Instruments, Sterling Heights, MI) and optimized for clarity (PhotoShop; Adobe, San Jose, CA).

PCR on tissue samples

Tissue from T males (~6 cm; ovaries from sexually mature female ~6 cm standard length) was homogenized and RNA was extracted (RNeasy Micro Kit; QIAGEN Inc., Valencia, CA). cDNA was synthesized from total RNA from each tissue sample (3′ RACE, SMART kit; Clontech Laboratories Inc., Palo Alto, CA). Sense (5′-attgttctgtctttcatcatctgctgga-3′) and antisense (5′-tgctctcagcacgactctcgt-3′) primers specific to *A. burtoni* GnRH-R1^{SHS} generated a 420-bp PCR product, whereas sense (5′-ggctgctcagttccgagtt-3′) and antisense (5′-gtgaggaccctctctggtggacatt-3′) primers specific to the *A. burtoni* GnRH-R2^{PEY} generated a 961-bp PCR product from the cDNA template. The GnRH-R2^{PEY} primer pair spans two introns predicted from the conserved GnRH-R genomic structure in fish (12). Positive controls for primers were performed using brain cDNA because *in situ* hybridization assays have shown both GnRH-R1^{SHS} and GnRH-R2^{PEY} expression in the brain (33). Negative controls were performed using the same procedure as for the experimental group without adding cDNA from any tissue. PCR was performed with a 68–60 C touchdown protocol as follows: 3 min denaturation at 95 C, followed by 16 cycles of 30 sec denaturation at 95 C, 30 sec annealing (68–60 C), and 15 min extension at 72 C. These reactions yielded a single product, as revealed by gel electrophoresis.

Immunocytochemical staining

To localize the somatotropes and gonadotropes, reproductively active fish (T males; n = 2) were killed by rapid cervical transection, and their brains removed and placed in 4% paraformaldehyde in PBS (pH 7.4) overnight and then transferred into a 30% sucrose solution overnight. Fixed tissue was frozen in OCT on dry ice and stored at −80 C. Three series of sections were cut on a cryostat (Microm; Zeiss, Oberkochen, Germany) in coronal or sagittal planes at 14 μm.

Sections were rehydrated (PBS) and incubated in blocking solution (0.3% Triton X-100, 0.2% BSA, 10% normal goat serum diluted in PBS). Slides were incubated separately in three primary antisera [rabbit anti-GH antibody (lot 8502), which labels somatotropes, and rabbit anti-LH antibody (lot 8506) and rabbit anti-FSH antibody (lot 8707), which label gonadotropes in *A. burtoni*, all generously provided by Dr. A. Takahashi] diluted 1:1000 in blocking solution at 4 C overnight (42). The other set of slides was incubated in blocking solution instead of primary antiserum for the control group. All of the sections were washed in PBS, and the signal was amplified (ABC kit; Vector Laboratories, Burlingame, CA). Sections were incubated in 3′,3′-diaminobenzidine (Sigma-Aldrich, St. Louis, MO) to visualize the bound antibody and counterstained with cresyl violet to visualize cell nuclei. The slides were dehydrated in an ethanol/xylene series and coverslipped with Permount medium for microscope viewing.

Regulation of GnRH-R mRNA abundance

Tissue preparation. To discover whether expression of GnRH-R mRNA was regulated, either as a function of diurnal time or with respect to GnRH ligand expression patterns, animals (n = 24) were housed in separate aquaria with three animals of mixed sex including one T male in each of eight tanks to provide minimal disturbance of other animals in the room when the animals were killed. Tissue was collected from animals in a single tank at 3-h intervals over a 24-h period beginning at 1000 h (fish were maintained on a 12-h light, 12-h dark cycle with dark-to-light transitions at 0900 h). At night, the aquarium room was accessed through a light-tight door, and all tissue-processing steps were performed in complete darkness using minimal infrared illumination. Fish were killed by rapid cervical transection, and their brains and pituitaries were rapidly removed. Brain and pituitary samples were stored at −80 C until use.

Tissue taken at each time point was separately homogenized in chilled TRIzol (Invitrogen) with a Tissue Tearor (Biospec Products, Bartlesville, OK) and kept frozen in TRIzol reagent at −80 C until RNA extraction.

RNA extraction and PCR sample preparation. Total RNA was extracted from samples following a standard protocol (TRIzol; Invitrogen). RNA was DNase treated to remove genomic DNA contamination (TURBO DNA-free; Ambion), and 1.0 μg total RNA from each tissue sample was reverse transcribed to cDNA (SuperScript II RNase H reverse transcriptase; Invitrogen).

Quantitative real-time PCR of A. burtoni GnRH ligands and receptors. Primers for quantitative real-time PCR for [Ser^8] GnRH1 were 5′-cagacacactgggcaatatg-3′ and 5′-ggccacactcgcaaga-3′, generating a 128-bp product; for GnRH2, primers were 5′-tggactcctttggcacatcagaga-3′ and 5′-ctctggctaaggcatccagaagaa-3′, generating a 126-bp product; for GnRH3, 5′-atggatggctaccaggtggaaaga-3′ and 5′-tggatttgggcatttgcctcatcg-3′, generating a 11- bp product; for GnRH-R1^{SHS}, 5′-tcagtacagcggcgaaag-3′ and 5′-gcatctacgggcatcacgat-3′, generating a 187-bp product; and for GnRH-R2^{PEY}, 5′-ggctgctcagttccgagtt-3′ and 5′-cgcatcaccaccataccact-3′, generat-

ing a 220-bp product. Three housekeeping genes, actin, 18S rRNA, and tubulin, cloned in *A. burtoni* were used to control for sample differences in total cDNA content. PCR were performed (iCycler; Bio-Rad, Hercules, CA), and reaction progress in 30-μl reaction volumes was monitored by fluorescence detection at 490 nm during each annealing step. Reactions contained 2× IQ SYBR Green SuperMix (Bio-Rad), 10 μM of each primer, and 1 ng cDNA (RNA equivalent). Reaction conditions were 1 min at 95 C and then 35 cycles of 30 sec at 95 C, 30 sec at 60 C, and 30 sec at 72 C, followed by a melting curve analysis over the temperature range from 95 C to 4 C. All reactions were run in duplicate.

Quantitative real-time PCR data analysis. Fluorescence readings for each sample were baseline subtracted, and suitable fluorescence thresholds were automatically determined by the MyiQ software. To determine the number of cycles needed to reach threshold, the original fluorescence reading data were analyzed using a special PCR algorithm (43). To calculate the relative mRNA amount, target gene levels were normalized using the geometric mean of housekeeping gene mRNA values.

Statistical analyses

To test the hypothesis that GnRH ligands and/or receptors were expressed with a diurnal rhythm, we analyzed the mRNA samples taken around the clock for rhythmic variation using generalized additive models (44, 45). This technique identifies nonlinear effects that might produce rhythmicity by analyzing the residuals of a scatterplot smoother fit to the data with a back-fitting algorithm. For the generalized additive models and the linear regressions, we took $P < 0.05$ as the initial significance level and applied a Bonferroni step-down correction to account for family-wise error due to multiple testing. These analyses were computed using R (R:Statistical computing environment; R Foundation, Vienna, Austria).

To identify potential linear relationships between ligands/receptors in a larger data set, we used robust regression (Siegel repeated means) (46) that allowed for the observed nonconstant variance (heteroskedasticity) in the data without requiring data transformation. As a control, we similarly compared the relationships between the ligands/receptors and the Clock 1a gene (GenBank DQ923857). To look for independent sources underlying the data distribution, we used independent component analysis (ICA), which is a recently developed technique (see survey in Ref. 44) related to latent variable and factor analyses. Unlike more traditional approaches, ICA yields unique (except for the sign) solutions to the problem of finding underlying sources of variation by maximizing the information contained in each independent source of interaction (44). The resulting sources may thus be interpreted as latent variables containing information about independent sources of variation in the data. We examined the relationships of each of the ligands/receptors with these latent variables by regressing each of the ligands/receptors on each of the independent sources, again using robust regression. We used a consensus source matrix from 100 solutions arrived at using the maximum-likelihood ICA package (computed using R). It is important to note that because the ICA signs are arbitrary, we can discover whether ligands/receptors relate to sources in the same or opposite directions or not at all (*i.e.* the signs can be reversed, but not the relative relationships between variables).

Results

Cloning of full-length GnRH-R1^{SHS} from A. burtoni

Analysis of the full-length putative GnRH-R cDNA sequence from *A. burtoni* tissue (supplemental Fig. A) shows it to be a second GnRH-R, with conserved Asp residues in transmembrane segments 2 and 7 and the carboxyl-terminal tail that are characteristic of GnRH-Rs that are not mammalian SDP-type GnRH-Rs (47, 48). There are conserved motifs SCAFVT in transmembrane segment 3 and VSHS in EC3 characteristic of teleost receptors (supplemental Fig. A) as identified by Lethimonier *et al.* (12). This sequence is most similar to other SHS-type GnRH-Rs especially from teleost fish (*e.g.* GnRH-R1^{SHS} *O. niloticus*, GenBank AY705931). Consistent with our proposed nomenclature, we named this receptor GnRH-R1^{SHS} (12, 33, 34). The sequence also has residues conserved among all GnRH-Rs (supplemental Fig. A), Asp^{107}, Trp^{110}, Asn^{111}, Lys^{130}, Asn^{217}, and Tyr^{298}, which are equivalent to residues Asp^{98}, Trp^{101}, Asn^{102}, Lys^{121}, Asn^{212}, and Tyr^{290} of the human GnRH-R1^{SDP}, known to be important in ligand binding (8).

Phylogenetic analysis

Phylogenetic analysis of vertebrate GnRH-Rs identified two families of GnRH-Rs (a and b), each containing two subfamilies, which are distinguished by conserved motifs in EC3 (Fig. 1 and supplemental Fig. A). The a1 family includes GnRH-Rs from all nonmammalian vertebrate taxa, including fish, reptiles, birds, and amphibians that are characterized by a conserved T*PEY*VH motif in EC3. All mammalian GnRH-Rs, known to regulate reproduction, form a distinct subfamily a2, characterized by a consensus V*SDP*VN motif in EC3 and the absence of a C-terminal tail (supplemental Fig. A). With less bootstrap support, family b GnRH-Rs can be split into two subfamilies that are also characterized by different but related motifs in EC3. Teleost and amphibian receptors in subfamily b2 have a V*SHS*LT motif, whereas GnRH-Rs from amphibians and some mammals belong to subfamily b1, characterized by the V*PPS*LS motif. It should be noted that although the GnRH-R2^{PPS} from chicken appears to be an exception, its phylogenetic inclusion in subfamily b2 has a very low bootstrap value. Phylogenetic analysis with alternative algorithms (*e.g.* minimal evolution) yielded very similar topology (data not shown). These and other data (8, 10, 12) showing that GnRH-R families can be distinguished by conserved amino acid motifs in EC3 allow an unambiguous classification system for vertebrate GnRH-Rs. The phylogenetic tree shows that the two types of GnRH-Rs found in teleosts, GnRH-R^{SHS} and GnRH-R^{PEY}, diverged early in vertebrate evolution and are neither recent duplications nor limited to teleosts.

Function of the A. burtoni GnRH-R1^{SHS}

To discover whether the *A. burtoni* GnRH-R1^{SHS} cDNA encoded a functional receptor, we analyzed its function using a recombinant system. Because fish cell lines were not available, we transfected COS-1 cells with GnRH-R1^{SHS} and showed specific binding of a radiolabeled GnRH analog, which was displaced by mGnRH1 and each of the three forms of GnRH expressed in *A. burtoni* (Fig. 2 and Table 1). We found that GnRH-R1^{SHS} had relatively low affinity for the three forms of GnRH that are expressed in *A. burtoni*. IC_{50} values ranged from 53.5 nM for GnRH2, which had highest affinity for GnRH-R1^{SHS}, to 20,300 nM for [Ser^8] GnRH1 (Table 1 and Fig. 2). Consistent with our previous report, GnRH-R2^{PEY} exhibited similar low affinities for all peptides, and peptides had the same rank order of IC_{50} values in cells expressing either GnRH-R1^{SHS} or GnRH-R2^{PEY} (GnRH2 < GnRH3 < mGnRH1 < [Ser^8] GnRH1; Table 1 and Fig. 2).

The four GnRH peptides stimulated IP production in cells transfected with *A. burtoni* GnRH-R1^{SHS} cDNA, confirming that the cDNA encodes a functional receptor that activates the Gq/11 family of G proteins in COS-1 cells and couples to

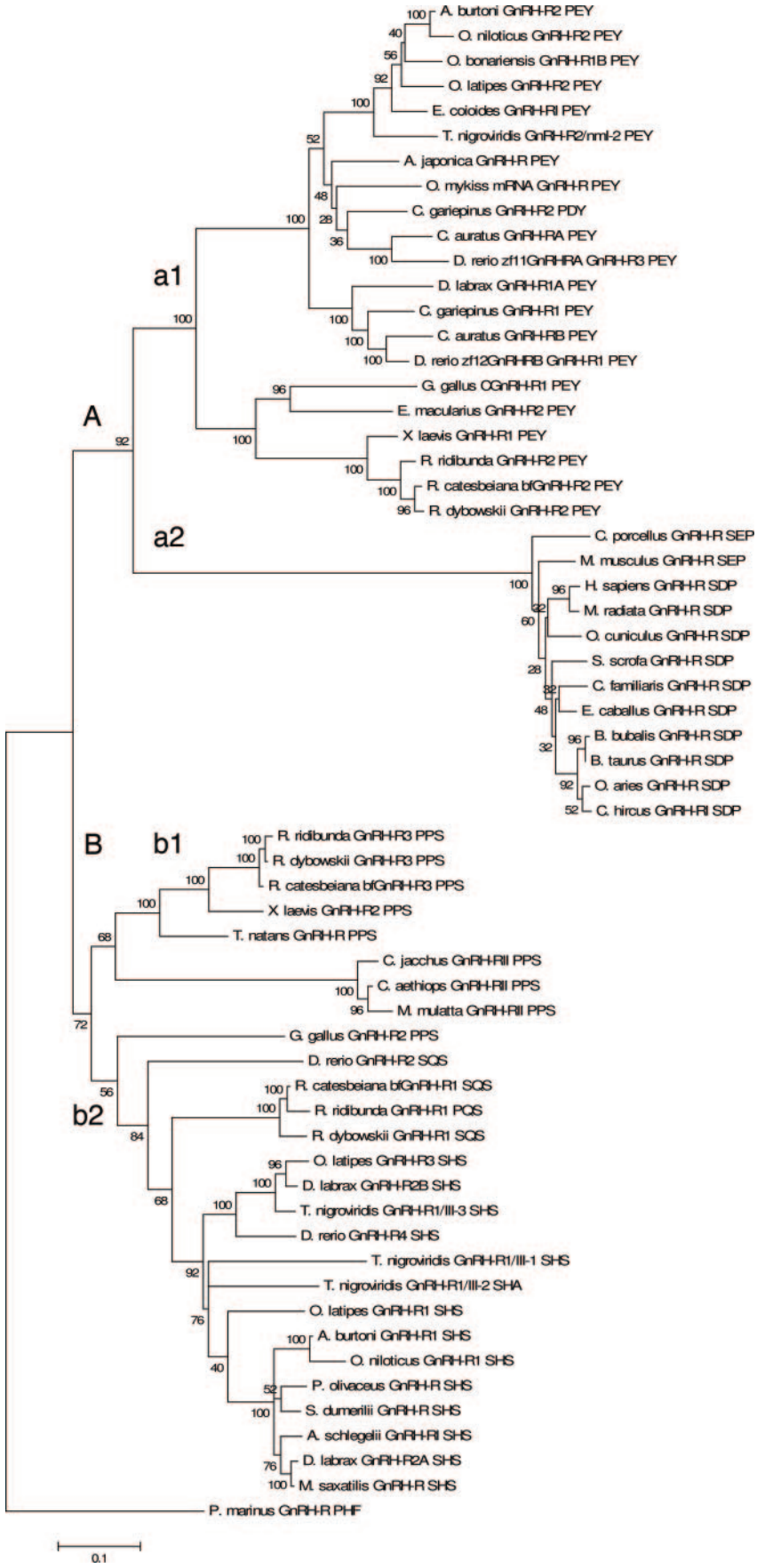

FIG. 1. Phylogenetic relationship among vertebrate GnRH-Rs. A neighbor-joining phylogenetic tree based on the multiple-sequence alignment of vertebrate GnRH receptor amino acid sequences (supplemental Fig. A). The tree was constructed using the Jones-Taylor-Thornton matrix, and bootstrap values are given at each node. The GenBank entry receptor name is given, followed by the EC3 motif. The GnRH-R sequence identified in the lamprey *M. marinus* was used as an out-group to root the tree. The two main GnRH-R families referred to in the text are labeled a and b.

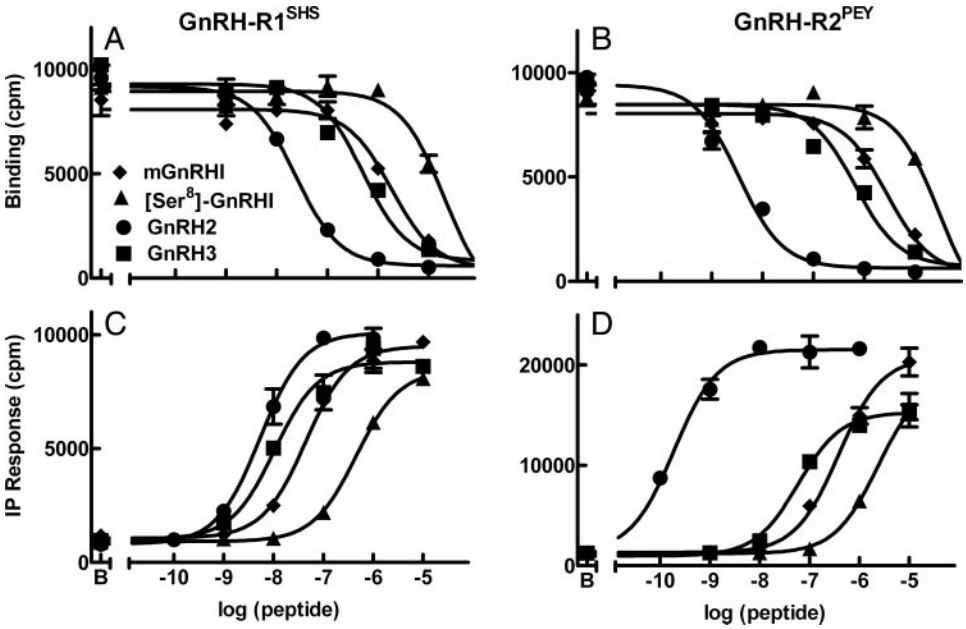

FIG. 2. Competition binding and IP production of GnRH-R1^{SHS} and GnRH-R2^{PEY} receptors. COS-1 cells transiently transfected with cichlid GnRH-R1^{SHS} (A and C) or cichlid GnRH-R2^{PEY} (B and D) were incubated with ^{125}I-labeled [His^5,D-Tyr^6] GnRH (A and B) in the presence of increasing concentrations of mGnRH1 (♦), [Ser^8] GnRH1 (▲), GnRH2 (●), or GnRH3 (■) for competition binding assays or (C and D) labeled with [^{3}H]myoinositol and incubated with increasing concentrations of peptides in the presence of LiCl for peptide-stimulated IP production.

the IP second-messenger cascade. GnRH2 and GnRH3 exhibited the highest potency (EC_{50} values 8.11 ± 2.63 and 11.0 ± 2.5 nM, respectively), whereas [Ser^8] GnRH1 and mGnRH1 had low potency (Table 1 and Fig. 2). In contrast, only GnRH2 exhibited high potency at the GnRH-R2^{PEY} (EC_{50} 0.208 ± 0.034 nM) (Table 1 and Fig. 2).

In vitro analyses show that the *A. burtoni* GnRH-Rs have similar affinities for each of the GnRH forms in *A. burtoni*. However, comparison of IC_{50} and EC_{50} values indicates that the two receptors differ in their efficiency in coupling binding of different GnRH peptides to intracellular signaling. This is evident from the similar potencies of GnRH2 and GnRH3 (EC_{50} values 8.11 ± 2.63 and 11.0 ± 2.5 nM) at the GnRH-R1^{SHS}, even though GnRH3 has considerably lower affinity (IC_{50}, 1143 ± 578 nM) at this receptor than GnRH2 (IC_{50}, 53.5 ± 24.6 nM). To quantify these observations, we have calculated an efficiency quotient (IC_{50}/EC_{50}, Table 1). Clearly, the GnRH-R1^{SHS} signaling response is particularly sensitive to GnRH3 and poorly responsive to GnRH2, whereas the GnRH-R2^{PEY} is highly responsive to GnRH2 (Table 1). It is notable that neither receptor is highly responsive to [Ser^8] GnRH1.

Tissue distribution and localization of A. burtoni GnRH-R1^{SHS} and GnRH-R2^{PEY}

We mapped the distribution of both GnRH-Rs in *A. burtoni* using RT-PCR for peripheral tissue and *in situ* hybridization for expression in the brain. GnRH-R1^{SHS} is significantly more widespread in the body than is GnRH-R2^{PEY}, being expressed in muscle, intestine, liver, and heart in addition to brain, retina, pituitary, gill, kidney, testes, and ovary where both forms are expressed (Fig. 3).

To localize GnRH-Rs relative to the gonadotropes and somatotropes, we compared GnRH cell type locations visualized using immunocytochemistry (Fig. 4) with each receptor type mapped using *in situ* hybridization (Fig. 5). The organization of the teleost pituitary requires that it be sectioned parasagittally to see these relationships clearly. In sagittal sections of the pituitary, the distributions of cells containing GH (Fig. 4A) or LH (Fig. 4B) and a schematic representation (Fig. 4D) are shown. GH- and LH-containing cells are in nonoverlapping regions of the pituitary. Distribution of FSH-containing cells was similar to that of LH-containing cells (not shown). Figure 4D shows the location of the plane of the coronal section through the pituitary used in Fig. 5. Based on *in situ* hybridization, GnRH-R1^{SHS} is localized in a crescent around the ventral border of the pituitary, and GnRH-R2^{PEY} is located dorsally, directly adjacent to the attachment of the pituitary to the brain, confirming previous results for *A. burtoni* (33). Comparing the *in situ* hybridization results with those of GH and LH distribution in the pituitary of *A. burtoni*, it is clear that the distribution of the GnRH-R1^{SHS} correlates with gonadotropes and GnRH-R2^{PEY} with somatotropes (Fig. 6).

TABLE 1. Summary of ligand binding and intracellular signaling of *A. burtoni* GnRH-R1^{SHS} and GnRH-R2^{PEY}

Peptide	Cichlid GnRH-R1^{SHS}			Cichlid GnRH-R2^{PEY}		
	Binding, IC_{50} (nM)	IP production, EC_{50} (nM)	Efficiency (IC_{50}/EC_{50})	Binding, IC_{50} (nM)	IP production, EC_{50} (nM)	Efficiency (IC_{50}/EC_{50})
[Ser^8] GnRH1	20300 ± 2690	679 ± 140	29.9	20700 ± 4120	1930 ± 837	10.7
GnRH2	53.5±24.6	8.11 ± 2.63	6.6	27.3 ± 24.0	0.208 ± 0.034	131
GnRH3	1140 ± 578	11.0 ± 2.56	103.2	1510 ± 964	68.3 ± 20.6	22.2
mGnRH1	3060 ± 1110	48.2 ± 4.99	63.6	3380 ± 1270	443 ± 40.7	7.6

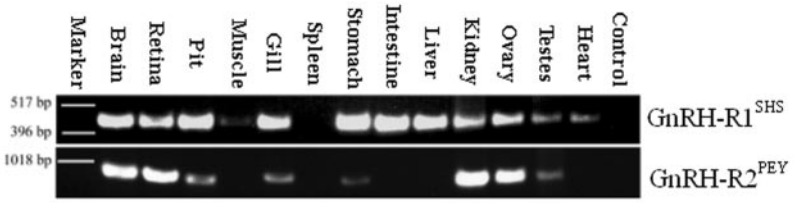

FIG. 3. Tissue distribution of GnRH-Rs. Distribution of mRNA from both GnRH-R types in *A. burtoni* tissue was assessed using PCR. Note that both receptor types are distributed throughout the body, but GnRH-R1^{SHS} is found more widely than GnRH-R2^{PEY}, particularly in the muscle, intestine, liver, and heart. Neither form is found in spleen. The first lane contains markers for 517 and 396 bp for GnRH-R1^{SHS} and 1018 bp for GnRH-R2^{PEY}. Material for the control lane was reverse transcribed without RNA and then used as the PCR template.

Diurnal rhythm and coregulation of GnRH-R mRNA levels

In *A. burtoni*, reproductive behavior in T males and activity in non-T males show distinct daily rhythms (28). To determine whether there might be a corresponding rhythm at the GnRH-R mRNA level, we collected tissue from three animals at 3-h intervals over a 24-h period and performed quantitative RT-PCR to determine the levels of receptor expression (Fig. 7). Although previous reports suggested GnRH-R mRNA levels change with a diurnal rhythm (49), we found no statistically significant nonlinearity as would be consistent with a diurnal rhythm in either GnRH-R1^{SHS} or GnRH-R2^{PEY} expression.

However, we did find significant coregulation of particular forms of GnRH and GnRH-R mRNA in data collected around the clock from the brain and pituitary. There is a highly significant positive linear relationship between each receptor and all three ligands. ICA showed that two independent sources contribute to changes in all the variables in the same direction. This means that when values in the first underlying source's distribution increase, expression of all the mRNAs increases. A third underlying source, however, shows GnRH-R2^{PEY} changing opposite of all other variables. Interestingly, the third underlying source shows GnRH3 is expressed in the same direction as receptor 2 but in the opposite direction of GnRH-R1^{SHS}.

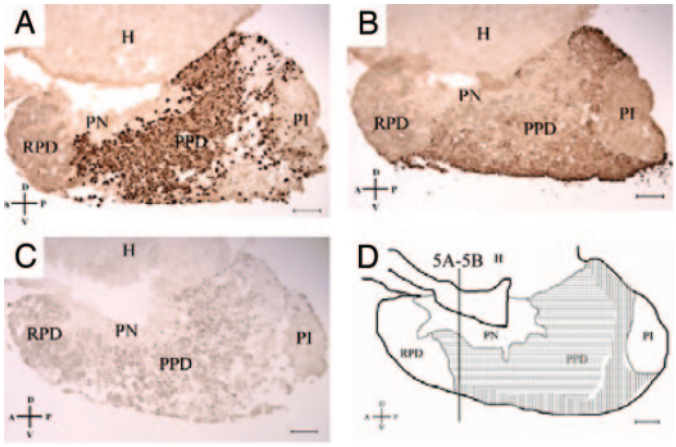

FIG. 4. Localization of gonadotropes and somatotropes. Fixed sagittal sections of *A. burtoni* pituitary from T males were incubated with anti-GH antibody (A) anti-LH (B), or no antibody (C). Bound antibodies were visualized with 3′,3′-diaminobenzidine, and nuclei were counterstained with cresyl violet. A schematic illustration (D) shows the distribution of GH cells (*horizontal shading*) and LH cells (*vertical shading*). The *line* labeled 5A-5B shows the position of the coronal sections of *in situ* staining shown in Fig. 5. *Scale bar*, 100 μm. A, Anterior; D, dorsal; P, posterior; V, ventral; H, hypothalamus; RPD, rostral pars distalis; PN, pars nervosa; PI, pars intermedia.

Comparison of vertebrate GnRH-R expression and responsiveness to different GnRH forms

The physiological role(s) of GnRH-Rs have been assessed by expression in appropriate cells of the pituitary, measurement of expression after physiological perturbations, and measurement of high affinity for GnRH1. To determine whether data in other species support the identification of GnRH-R1^{SHS} as the reproductive receptor in *A. burtoni*, despite its low affinity, we reviewed prior research. We asked whether receptor amino acid sequence correlates with ligand responsiveness and with evidence of expression in gonadotropes or responses to perturbation of the HPG axis in non-mammalian vertebrates. We found no correlation of expres-

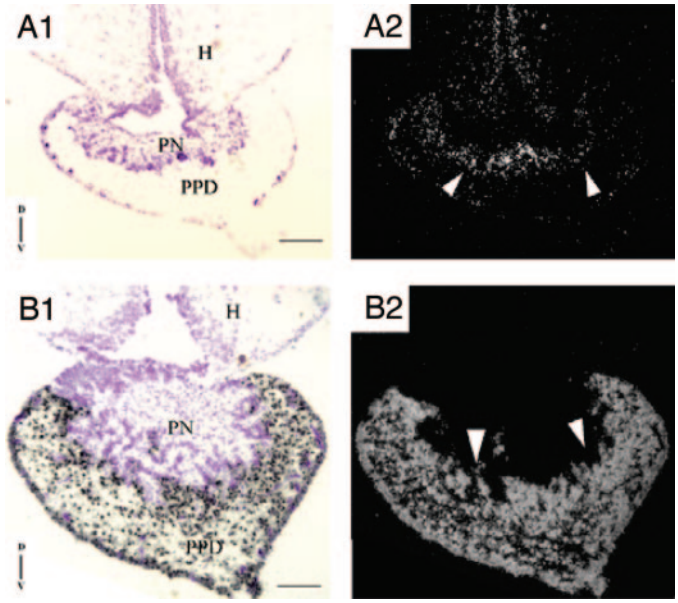

FIG. 5. Localization of GnRH-R1^{SHS} and GnRH-R2^{PEY} in the pituitary of *A. burtoni* using *in situ* hybridization with ^{35}S-labeled antisense mRNA probes. Photomicrographs show coronal sections through an *A. burtoni* pituitary showing localization of GnRH-R2^{PEY} mRNA (A) and GnRH-R1^{SHS} mRNA (B). A1, GnRH-R2^{PEY} mRNA as radioactively labeled silver grains (*black spots*) in bright field with cresyl violet counterstain (*purple*), showing that it is concentrated in the dorsal part of the pituitary in the proximal pars distalis (PPD) adjacent to the pituitary stalk; A2, dark-field photomicrograph of the same field of view where silver grains appear *silver*. *Arrowheads* mark the boundary of the staining that lies between the proximal pars distalis (PPD) and the pars nervosa (PN). B1, GnRH-R1^{SHS} mRNA as silver grains (*black spots*) in bright field with cresyl violet counterstain (*purple*), showing that it is concentrated in ventral crescent of the PPD; B2, dark-field photomicrograph of the same field of view where silver grains appear *silver*. *Arrowheads* mark the boundary of the staining that lies along the ventral border of the pituitary. *Scale bar*, 200 μm.

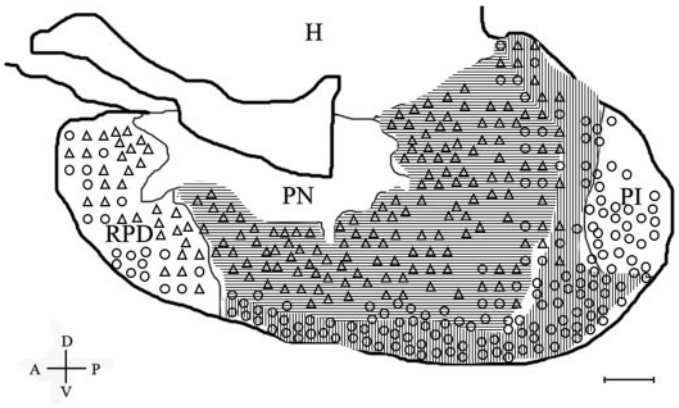

FIG. 6. Schematic illustration of a sagittal section through the *A. burtoni* pituitary showing the distribution of GnRH-R1^{SHS} (○) and GnRH-R2^{PEY} (△) receptor types relative to the GH cells (*horizontal lines*) and LH cells (*vertical lines*). *Scale bar*, 100 μm. Abbreviations are as in Fig. 4. This figure summarizes a series of sagittal and coronal sections analyzed as described for Figs. 4 and 5.

sion of either SHS- or PEY-type GnRH-Rs with reproductive function (supplemental Table B, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Because there are limited data on ligand-binding affinities of most vertebrate GnRH-Rs, we compared EC_{50} values. However, absolute EC_{50} values measured in recombinant signaling assay systems are influenced by variables such as receptor expression levels and coupling efficiencies that vary among systems. Because all nonmammalian GnRH-Rs are highly responsive to GnRH2, we compared signaling responses stimulated by various GnRH forms relative to the response stimulated by GnRH2 to calculate a ligand response index (Table 2). We included all vertebrate GnRH-Rs that fit the four defined receptor types, SDP, PEY, SHS, and PPS, for which we could find dose-response data for GnRH2 and at least one other GnRH form (Table 2). The comparison shows that ligand response is similar within each receptor type, and there is a strong correlation of receptor sequence with ligand-stimulated response relative to the response to GnRH2 (see Table 2). As expected, the mammalian, SDP-type GnRH-Rs are highly responsive to mGnRH1 relative to GnRH2 (mean EC_{50} ratio 0.15, Table 2), whereas PPS-type receptors, the classic type II GnRH2 receptors, show high responsiveness to GnRH2 (mean ratio 239, Table 2). Of the GnRH-R types that occur in teleosts, the PEY-type receptors are highly selective for GnRH2 and very poorly responsive to all other GnRH forms (mean EC_{50} ratios 140-2900, Table 2). It is notable that chicken and bullfrog PEY-type receptors are exceptions, exhibiting good responses to GnRH1. In contrast, teleost SHS-type receptors show smaller differentials in peptide-stimulated responses (mean EC_{50} ratios 3.3–58, Table 2), meaning they are much less selective for GnRH2 and suggesting that a receptor of this type may be the GnRH1 receptor in teleosts.

Discussion

Cloning and genomic analysis have identified multiple GnRH-Rs in a wide variety of nonmammalian vertebrates,

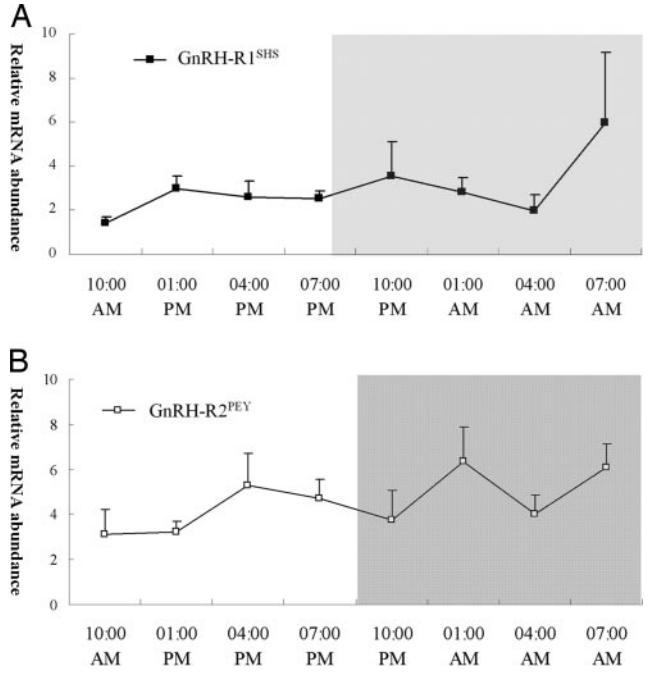

FIG. 7. GnRH-R mRNA abundance as a function of time of day over a 24-h period. Panels show the fluctuation of relative GnRH-R1^{SHS} (A) and GnRH-R2^{PEY} (B) mRNA abundance over 24 h. *A. burtoni* tissue samples were taken at 3-h intervals. mRNA abundance was normalized by mRNA levels of housekeeping genes (see *Materials and Methods*). *Gray shaded area* represents darkness. *Bars* indicate SEM. Lights were on at 0900 h and off at 2100 h (n = 24).

including chickens and several species of fish and amphibians, yet it is not known which receptor type regulates reproduction. The GnRH-R responsible for reproduction must 1) be expressed in the gonadotrope cells of the pituitary, 2) exhibit enhanced expression in animals that are reproductively competent, 3) be responsive to the endogenous GnRH ligand that regulates the HPG axis, and 4) be phylogenetically close to receptors that regulate reproductive function in related species.

To identify which GnRH-R regulates reproduction in *A. burtoni*, we cloned the GnRH-R1^{SHS} receptor, known to be up-regulated in reproductively active T males, and measured its phylogenetic and functional characteristics. We found that GnRH-R1^{SHS} is less closely related than the GnRH-R2^{PEY} to the SDP-type receptors, which are clearly established as reproductive receptors in mammals. Interestingly, neither cichlid GnRH-R has high-affinity interaction with [Ser^8] GnRH1, but GnRH-R^{SHS} expression colocalizes with gonadotropes, whereas GnRH-R^{PEY} does not.

Phylogenetic relationships of jawed vertebrate GnRH-Rs

Phylogenetic analysis identified four GnRH-R subfamilies with characteristic motifs in EC3. We have defined GnRH-R types according to their EC3 motif, indicated by a three-letter superscript, to reduce confusion of multiple contradictory nomenclature systems for GnRH-Rs (supplemental Table A) and poor correlation of assigned receptor names with receptor type. This unambiguous classification scheme placing GnRH-Rs into four types, SDP, PEY, PPS, and SHS using the

TABLE 2. Comparison of GnRH ligand structure-function data from GnRH-Rs

Receptor name	Accession no.	EC_{50} GnRH2 (log M)	EC_{50} ratio (GnRH3/GnRH2)[a]	EC_{50} ratio (GnRH1/GnRH2)[b]	EC_{50} ratio (mGnRH1/GnRH2)	Ref.
Sheep GnRH-RI^{SDP}	NM_001009397	−8.92[a]			0.07	69
Human GnRH-RI^{SDP}	NM_000406	−8.76			0.17	19
Mouse GnRH-RI^{SEP}	NP_034453	−8.28			0.22	18
Mean ratio					0.15 ± 0.04	
Medaka GnRH-R3^{SHS}	AB083363	−7.99	1.0	10.5	1.7	29
A. burtoni GnRH-R1^{SHS}	AY705931	−8.09	1.3	83.2	5.9	Current study
Bullfrog GnRH-R-1^{SQS}	AF144063	−8.64	4.7		13.5	11
Medaka GnRH-R1^{SHS}	AB057677	−9.29	6.3	81.3	33.1	28
Mean ratio			3.3 ± 1.3	58.3 ± 20.7	13.5 ± 7.0	
Bullfrog GnRH-R-3^{PPS}	AF224277	−8.9	0.5		20.9	11
Marmoset GnRH-RII^{PPS}	AF368286	−9.4			91.2	25
Macaque GnRH-RII^{PPS}	NM_001032842	−9.1			398.1	26
Green monkey GnRH-RII^{PPS}	AF353988	−8.4			446.7	70
Mean ratio					239.2 ± 107.2	
Chicken GnRH-R1^{PEY}	AJ304414	−9.22		8.7	6.8	71
Bullfrog GnRH-R2^{PEY}	AF153913	−9.40	25		109.6	11
Goldfish GnRH-R1B^{PEY}	AF121846	−8.47	17		832	40
Medaka GnRH-R2^{PEY}	AB057676	−9.00	245	3715	1175	28
Catfish GnRH-R1^{PDY}	X97497	−8.69		603	1175	27
Xenopus laevis GnRH-R1^{PEY}	AF172330	−9.64	79		1820	72
A. burtoni GnRH-R2^{PEY}	AT028476	−9.68	324	9333	2138	Current study
Catfish GnRH-R2^{PEY}	AF329894	−8.94		955	6457	27
Goldfish GnRH-R1A^{PEY}	AF121845	−10.52	151		6918	40
Mean ratio			140 ± 51	2923 ± 1724	2292 ± 863	

To assess whether a specific GnRH-R type exhibits specificity for GnRH1, intracellular signaling stimulated by GnRH peptides is summarized.

[a] Ligand-binding affinities have been determined for very few GnRH-Rs. To compensate for this lack, EC_{50} values for ligand-stimulated signaling were compared. To compensate for variations in receptor expression and coupling efficiencies, responses relative to the GnRH2-stimulated response (peptide EC_{50}/GnRH2 EC_{50}) were compared. All GnRH-Rs for which EC_{50} values for GnRH2 and at least one other peptide were found are included.

[b] GnRH1, the reproductive form of GnRH, has variable structure. The EC_{50} for the GnRH1 form used in the cited reference is used to calculate this ratio, except where GnRH1 is mGnRH1.

EC3 motif, is based solely on receptor amino acid sequence. It has major advantages in that it includes all but a few closely related SHS- and PPS-type receptors (Fig. 1 and supplemental Fig. A) and cannot be confused with GenBank assigned receptor names.

It is likely that the four GnRH-R subfamilies identified (a1, a2, b1, and b2, corresponding to types PEY, SDP, PPS, and SHS) arose through gene duplication, an important process in evolution. Duplications allow new gene functions to evolve (50, 51), and there is good evidence in yeast that duplicated genes can provide functional compensation against null mutations (52). In this case, GnRH-R^{SHS} and GnRH-R^{PEY} show similar affinity for [Ser^8] GnRH1, but they are clearly not functionally redundant, because their expression patterns are divergent. GnRH-R subfamilies can be understood in terms of the two rounds of genome duplication that occurred early in the vertebrate lineage (known as the 2R hypothesis) (53). Because this hypothesis would predict the presence of four GnRH-Rs in all vertebrates, the phylogenetic tree predicts that GnRH-R genes have been lost independently, on multiple occasions from different branches of the vertebrate lineages. For example, SDP-type (family a2) GnRH-Rs have been identified only in mammals and thus must have been lost independently in fish, amphibians, and reptiles. Similarly, PEY-type (family a1) GnRH-Rs are not found in mammals. Amphibians have GnRH-Rs in both subfamilies of family b (PPS-type and P/SQS variations of the SHS type), whereas all fish GnRH-Rs in family b are SHS type and all bird and mammalian receptors are PPS type. Although GnRH-R^{PPS} receptors have been identified in amphibians, chicken, and nonhuman primates, this subtype is absent from the mouse and rat genome sequence data bases. The human GnRH-RII^{PPS} lacks a start codon, is truncated at the second transmembrane segment, and is nonfunctional (54–56). Similarly, a sheep GnRH-RII^{PPS} ortholog is also nonfunctional (57). Thus, this receptor has been independently lost in rodents and has become a pseudogene in humans and sheep.

Teleost fish have undergone an additional genome duplication since their divergence from tetrapods (58, 59), so it is not unexpected that there are multiple GnRH-Rs of a single receptor type in the *Ostariophysi* lineage including goldfish (GnRH-RA^{PEY} and GnRH-RB^{PEY}) (38), catfish (GnRH-R1^{PDY} and GnRH-R2^{PEY}) (25), and zebrafish (GnRH-R1^{PEY}, GnRH-R3^{PEY}, GnRH-R2^{SHS}, and GnRH-R4^{SHS}) (12). Similarly, in the *Neoteleostei* lineage multiple GnRH-Rs are present in pufferfish (GnRH-R1/III-1^{SHS}, GnRH-R1/III-2^{SHA}, and GnRH-R1/III-3^{SHS}) and medaka (GnRH-R1^{SHS} and GnRH-R3^{SHS}).

In the case of *A. burtoni*, the different expression patterns of the GnRH-R genes suggest that these receptors are in the process of diverging in function. Only GnRH-R2^{PEY} is colocalized on all three groups of neurons that produce GnRH, apparently specialized for feedback control of GnRH production (33). Moreover, GnRH-R1^{SHS} colocalizes with gonadotropes, whereas GnRH-R2^{PEY} colocalizes with somatotropes, revealing different roles in the pituitary. When detailed data about receptor localization and function are available from other species, it will become clear whether

these specializations are unique to teleosts and whether evolution has taken different directions in different species.

Role of GnRH-R1^{SHS} as a functional receptor for [Ser^8] GnRH1

We have previously shown, in *A. burtoni*, that [Ser^8] GnRH1 is the only GnRH peptide found in the pituitary and that it is up-regulated in reproductively active T males. The low affinity of both *A. burtoni* receptors for [Ser^8] GnRH1 indicates that high concentrations of [Ser^8] GnRH1 (in the micromolar range) would be required in the pituitary to activate either receptor. Superficially, this might suggest that neither receptor is the target of [Ser^8] GnRH1 but that some other receptor with a higher affinity for [Ser^8]-GnRH1 may exist. However, all of the nonmammalian GnRH-Rs that have been functionally analyzed have the highest affinity for GnRH2, and all fish receptors are poorly responsive to GnRH1 forms (11, 12, 25, 27, 38) (Table 2). Available information from zebrafish and pufferfish genomes does not reveal additional GnRH-Rs, making it unlikely that receptors with high similarity to currently known GnRH receptors and high affinity for GnRH1 exist.

Although [Ser^8] GnRH1 had low potency in stimulating IP production in our recombinant system, the efficiency of [Ser^8] GnRH1-stimulated activation of fish G proteins is not known. High-affinity GnRH-R binding has been reported in fish pituitary tissue (60–63) using synthetic, high-affinity ligands, before the discovery of the teleost forms of GnRH1 (see Ref. 12). However, consistent with results in recombinant systems, catfish GnRH1 was found to have low potency in stimulating gonadotropin release and had very low affinity in GnRH-R binding assays performed with catfish pituitary membranes (60). This suggests the known receptors may regulate reproduction despite their low affinity for the biologically relevant ligands, raising the possibility that synchronous release of GnRH in the pituitary may lead to transiently elevated levels triggering the receptors.

Consistent with their known functions in mammals, all SDP-type GnRH-Rs are highly selective for mGnRH1, and all PPS-type receptors are highly selective for GnRH2 (Table 2). This suggests that PPS-type GnRH-Rs may have a conserved function in amphibians and in nonhuman primates. As in *A. burtoni*, all SHS-type GnRH-Rs exhibit relatively low ligand selectivity, with similar responses to different GnRH peptides, suggesting that SHS-type receptors may have similar functions in teleost and amphibian species. In contrast, all PEY-type GnRH-Rs from fish species are highly selective for GnRH2, suggesting that GnRH2 may be their major physiological ligand. However, PEY-type receptors from chicken and bullfrog are much less selective for GnRH2 and are quite responsive to GnRH1 ([Gln^8] GnRH1 or mGnRH1, respectively) suggesting that the functions of PEY-type GnRH-Rs may differ between teleosts and other vertebrates. Indeed, there is physiological evidence that the GnRH-R1^{PEY} regulates reproduction in chickens, because its expression is up-regulated in the pituitaries of castrated cockerels (64). However, these data are not consistent with a recent study in which expression of GnRH-R2^{SHS} was shown to increase with reproductive status in male and female chickens (65).

Which GnRH-R regulates reproduction in A. burtoni?

Several lines of evidence suggest that the GnRH-R1^{SHS} receptor regulates reproduction in *A. burtoni*. First, GnRH-R1^{SHS} receptor mRNA transcripts are up-regulated in the pituitaries of T males, whereas GnRH-R2^{PEY} receptor mRNA transcripts are not (34). Second, GnRH-R1^{SHS} mRNA transcripts specifically localize with expression of gonadotropes, whereas GnRH-R2^{PEY} transcripts localize with somatotropes. Our results using *in situ* hybridization contrast with results using receptor immunocytochemistry in the closely related teleost *O. niloticus* (42). In *O. niloticus*, antibodies that would be predicted to recognize the GnRH-R1^{SHS} (AB111356) reacted with somatotrope cells, whereas antibodies that would be predicted to recognize GnRH-R2^{PEY} (AB111357) reacted with gonadotropes (42). Because both species are cichlids, this difference is likely to result from the different techniques used. The antibodies were raised against short peptides corresponding to the EC3 sequences of GnRH-Rs. These peptide sequences can be readily identified in diverse proteins by a BLAST search, so it is possible that the antibodies recognize the peptide sequences of other proteins besides GnRH-Rs (57). Thus, although comparing GnRH-R expression patterns in different species (supplemental Table B) does not yield a consensus on whether GnRH-R^{PEY} or GnRH-R^{SHS} receptors regulate release of gonadotropins in nonmammalian vertebrates, our additional expression data confirm that GnRH-R1^{SHS} is the receptor expressed in the gonadotropes of *A. burtoni*.

Coregulation of GnRH ligand and receptor expression

The widespread distribution of GnRH-Rs (66, 67) has led to speculation that different forms of GnRH may play roles beyond reproduction. Chen and Fernald (33) recently showed that the receptor types differ in their brain distribution in *A. burtoni* and only GnRH-R2^{PEY} colocalized with the three GnRH-producing cell types in the brain, suggesting direct feedback control of the GnRH production. The coregulation of receptors with GnRH suggests that the GnRH system has some common regulatory underpinnings. The presence of multiple GnRH forms across all vertebrates suggests they might share some functional role in reproduction with GnRH1.

GnRH-R evolution and the usefulness of the EC3 motif classification system

The poor correlation of GnRH-R sequence with a role in reproduction across species (supplemental Table B) makes it impossible to predict which GnRH-R will regulate reproductive function in any particular species. The absence of SHS-type GnRH-Rs in higher vertebrates precludes their role as the receptor responsible for reproduction in these species. Different families of duplicated receptor genes regulate reproductive function in mammals (family a, SDP-type) compared with fish (family b, SHS-type). There is insufficient evidence in other taxa (birds, reptiles, and amphibians) to conclude which receptor is used or when selection for one receptor rather than another might have occurred. Thus, it makes sense to define GnRH-R types by genotype, which,

perforce, correlates with EC3 amino acid motif and with ligand selectivity, rather than using a classification system based on physiological function in the face of insufficient and conflicting information.

In summary, we cloned the GnRH-R1^{SHS} receptor, which is up-regulated in reproductively active *A. burtoni*, and showed that it is coexpressed with LH in the pituitary. Like all teleost GnRH-Rs, it binds and responds poorly to GnRH1. Although this remains puzzling, it suggests that synchronous release of GnRH1 into the pituitary allows delivery of sufficient ligand to activate the receptor. The absence of the SHS-type GnRH-R in many taxa and the poor correlation of GnRH-R sequence with a role in reproduction across species, suggest that different families of duplicated GnRH-R genes have been selected to regulate reproduction during vertebrate evolution.

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