BIOLOGY OF REPRODUCTION **70,** 1545–1551 (2004)
Published online before print 28 January 2004.
DOI 10.1095/biolreprod.103.021998

Regulation of Gonadotropin-Releasing Hormone (GnRH)-Receptor Gene Expression in Tilapia: Effect of GnRH and Dopamine[1]

Berta Levavi-Sivan,[2,3] Helena Safarian,[3] Hanna Rosenfeld,[4] Abigail Elizur,[4,5] and Ayelet Avitan[3]

Department of Animal Sciences,[3] Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University, Rehovot 76100, Israel
National Center of Mariculture,[4] Israel Oceanographic and Limnological Research, Eilat 88112, Israel
Bribie Island Aquaculture Research Centre,[5] Department of Primary Industries, Brisbane, Queensland 4507, Australia

ABSTRACT

The present work was designed to study certain aspects of the endocrine regulation of gonadotropin-releasing hormone receptor (GnRH-R) in the pituitary of the teleost fish tilapia. A GnRH-R was cloned from the pituitary of hybrid tilapia (ta-GnRH-R) and was identified as a typical seven-transmembrane receptor. Northern blot analysis revealed a single GnRH-R transcript in the pituitary of approximately 2.3 kilobases. The ta-GnRH-R mRNA levels were significantly higher in females than in males. Injection of the salmon GnRH analog (sGnRHa; 5–50 μg/kg) increased the steady-state levels of taGnRH-R mRNA, with the highest response recorded at 25 μg/kg and at 36 h. At the higher dose of sGnRHa (50 μg/kg), taGnRH-R transcript appeared to be down-regulated. Exposure of tilapia pituitary cells in culture to graded doses (0.1–100 nM) of seabream (sbGnRH = GnRH I), chicken II (cGnRH II), or salmon GnRH (sGnRH = GnRH III) resulted in a significant increase in taGnRH-R mRNA levels. The highest levels of both LH release and taGnRH-R mRNA levels were recorded after exposure to cGnRH II and the lowest after exposure to sbGnRH. The dopamine-agonist quinpirole suppressed LH release and mRNA levels of taGnRH-R, indicating an inhibitory effect on GnRH-R synthesis. Collectively, these data provide evidence that GnRH in tilapia can up-regulate, whereas dopamine down-regulates, taGnRH-R mRNA levels.

dopamine, gonadotropin-releasing hormone, gonadotropin-releasing hormone receptor, pituitary

INTRODUCTION

Regulation of the biosynthesis and secretion of the gonadotropins FSH and LH is critical for normal reproductive function in fish, as in other vertebrates. The synthesis and release of these two pituitary glycoproteins is controlled by the complex interaction of multiple brain and gonadal factors, among which the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) plays a cardinal role [1, 2]. In fish, which do not have a hypophysial portal system, GnRH reaches the pituitary directly through hypothalamic nerve fibers where the GnRH binds to specific high-affinity receptors [3]. To date, 16 distinct molecular forms of GnRH have been identified in vertebrates and a protochordate [4–6]. They are distributed in a wide range of tissues in vertebrates, where they apparently have diverse functions, including neuroendocrine (e.g., gonadotropins, growth hormone [GH] or prolactin release), paracrine (e.g., placenta and gonads), autocrine (e.g., GnRH neurons, immune cells), and neuromodulatory roles in the central and peripheral nervous system (e.g., sympathetic ganglion and midbrain) [7]. At least two forms of GnRH are present in most vertebrate species [8]: the chicken GnRH II (cGnRH II) form, which has been found to be the most conserved among taxa from jawed fish to humans [7], and GnRH I, to which gonadotropin regulation is ascribed. In several vertebrate species, a third form of GnRH (localized to the forebrain in fish) occurs and is designated GnRH III [8].

As in mammals, fish GnRH receptor (GnRH-R) is a member of the G protein-coupled receptor family. However, unlike in mammals, the piscine GnRH-R counterpart possesses a long cytoplasmic C-terminal tail [9]. In tilapia, GnRH stimulates the secretion of LH from the pituitary both in vivo [10] and in vitro [11, 12] and increases its mRNA levels [13]. The GnRH also stimulates the secretion of GH from the pituitary both in vivo and in vitro [14]. Moreover, GnRH stimulates the release of prolactin in vitro [15] and in vivo [16]. In addition to its effect as a secretagogue, GnRH also increases the synthesis of LH and FSH, but not that of GH (for review, see [1, 2]).

Perciform fish, such as seabream and tilapia, possess three forms of GnRH in the brain. The cGnRH II (cGnRH II = GnRH II) occurs in neurons within the midbrain tegmentum, and salmon GnRH (sGnRH = GnRH III) is present in the terminal nerve. However, only GnRH I (seabream GnRH [sbGnRH]) is encountered in the preoptic area (POA) of the hypothalamus and in the pituitary [17–19]. The GnRH I is thought to be the principal form inducing gonadotropin release, because it is the most abundant form of GnRH in the pituitary [5, 20, 21] and is expressed in neurons in the POA with axons terminating in the pituitary.

Binding of GnRH to GnRH-Rs on gonadotrophs initiates a cascade of events necessary for the synthesis and secretion of LH and FSH (for review, see [1, 2]). Studies concerning the endocrine regulation of gonadotropin synthesis and secretion in fish have emphasized the regulatory role of GnRH in controlling seasonal changes of gonadotropin level [22, 23]. However, other studies have indicated that the responses to GnRH vary in conjunction with the season, sexual maturity, gender, and age [24–27]. This would indicate that the changes in GnRH levels to which the gonadotrophs are exposed are not sufficient to modulate go-

[1]Supported by grant 775/01 from the Israel Science Foundation.
[2]Correspondence: Berta Levavi-Sivan, Department of Animal Sciences, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University, P.O. Box 12, Rehovot 76100, Israel.
FAX: 972 8 9465763; e-mail: sivan@agri.huji.ac.il

Received: 6 August 2003.
First decision: 27 August 2003.
Accepted: 14 January 2004.

ISSN: 0006-3363. http://www.biolreprod.org

TABLE 1. GnRH-R primers used in present study.

No.	Primer number	Position[a]	Primer sequence
1	GnR-TM3-F		5′ctggatccaatgtggaayrtnacwgtkcartgg3′
2	GnR-TM6-R		5′accgaattccdaggasrtartasggmgtccagca3′
3	GnR-6F	390	5′gctgcaggcgatgtactcctgcgcc3′
4	GnR-7R	822	5′cagagttctcatccgggctctcggg3′
5	GnR-5F	481	5′gccagaaagaggaacagaat3′
6	GnR-2R	858	5′gatgatgaaagacagaacaatcac3′
7	GnR-8F	1001	5′tattcaccatccacttcaga3′
8	GnR-9R	1207	5′ttagaaagctgctcctcggt3′
9	18S-R	897	5′gcaccaccacccacagaatc3′
10	18S-F	660	5′cgaccataaacgatgccaatag3′

[a] Position refers to the beginning of the receptor's open reading frame.

nadotropin response. An alternative or additional candidate for such modulation would be the prevalence of GnRH-Rs.

Regulation of GnRH-R gene expression by endocrine factors is a potential way of modulating the synthesis and/or release of pituitary hormones with or without a concomitant change in the level of the peptide. The present work was designed to study the endocrine regulation of GnRH-Rs in the pituitary of tilapia by determining their mRNA level following certain endocrine manipulations.

MATERIALS AND METHODS

Fish

The fish used in the present study were tilapia hybrids (*Oreochromis aureus* × *O. niloticus*), the predominant tilapia in Israeli aquaculture. They were collected from local fish farms and then housed at the University's fish facility in 500-L tanks at 26°C under natural photoperiod. Fish were fed every morning ad libitum with commercial pellets and flakes (Zemach, Zemach, Israel) containing 50% protein and 6% fat. All experimental procedures were in compliance with the Animal Care and Use Guidelines at the Hebrew University and were approved by the local Administrative Panel on Laboratory Animal Care Committee.

Fish were anesthetized with 2-phenoxyethanol (Sigma, St. Louis, MO) at a concentration of 1 ml/L before being weighed and injected i.p. with various doses of sGnRH analog ([D-Ala^6,Pro^9-NEt]-mammalian GnRH; 5–50 μg/kg; sGnRHa; Bachem, Inc., Torrance, CA) dissolved in 0.85% saline, and tilapia GnRH-R (taGnRH-R) mRNA levels were measured after 24 h. Controls were injected with fish saline. Fish received sGnRHa at an injection volume of 1 μl/g body weight (BW). In another experiment, fish were injected i.p. with sGnRHa, and taGnRH-R mRNA was measured 1, 2, 4, 8, 12, 24, 36, and 48 h later. Blood was collected from the caudal vasculature into heparinized syringes from anesthetized fish. After centrifugation, the plasma was stored at −20°C until analysis for LH by RIA.

Culture of Dispersed Pituitary Cells

Primary cultures of pituitary cells were prepared as described previously [11, 28]. Briefly, cells from 100 to 200 fish were collectively dispersed and then plated at 1.5×10^6 cells/well in medium (M199; 10% fetal calf serum, 10 mM Hepes, 1% antibiotics [a pen-strep-nystatin suspension]; Biological Industries, Bet Ha'emek, Israel). After 4 days of culture at 28°C under 5% CO_2, cells were exposed for 24 h to sGnRH, sbGnRH, or cGnRH II (0.1–100 nM; Bachem) or to quinpirole (1 or 10 μM; Sigma).

RNA Extraction, Blotting, and Hybridization

The RNA was extracted from each well using a scaled-down modification of the guanidinium thiocyanate-phenol:chloroform method as modified previously [13, 29]. The samples were dissolved in diethyl pyrocarbonate-treated water (45.2%, v/v), formaldehyde (4.8%, v/v), and formamide (50%, v/v) and were then transferred by capillary to a nylon membrane (Nytran N; Schleicher and Schull, Dassel, Germany) using a slot-blot apparatus from the same manufacturer.

The GnRH-R sequence was amplified from RNA obtained from four mature female tilapia pituitaries (mean ± SEM, 89.20 ± 3.54 g BW; gonadosomatic index [GSI; gonadal weight as a percentage of BW], mean ± SEM, 1.54% ± 0.23%) using rapid amplification of cDNA ends-polymerase chain reaction (PCR). Degenerate primers, based on catfish GnRH-R sequences (GnR-TM3-F and GnR-TM6-R) (Table 1), were used to amplify a PCR product. Gene-specific primers were designed (GnR-6F and GnR-7R) (Table 1), and the complete coding region of the mature peptide as well as the 3′ and 5′ untranslated regions were cloned, sequenced, and sent to GenBank (accession no. AY381299). The full coding region of the receptor was cloned using an Advantage 2 PCR kit (Clontech, Palo Alto, CA) according to the manufacturer's recommendations: A total volume of 50 μl of reaction mixture included 1 μl of cDNA, 5 μl of 10× Advantage buffer, 1 μl of dNTPs (final concentration, 200 μM each), 1 μl of Advantage Polymerase Mix, and 0.2 μM of each primer. The PCR conditions were set as follows: initial denaturation at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 30 sec and annealing at 68°C for 60 sec, followed by a final extension reaction at 68°C for 1 min.

The sequence encoding amino acids 481–858 containing transmembrane domains (TMs) III, IV, and the beginning of V from taGnRH-R was used as a probe. Another type of GnRH-R sequence was also cloned from the pituitary of tilapia. Its GenBank accession number is AY381298. The percentage homology, in the sections limited by the probe used, between the two types of GnRH-R sequenced in tilapia was less than 50%. A more detailed description of the isolation and characterization of the tilapia GnRH-R cDNA will be published elsewhere.

The GnRH-R mRNA values were normalized against those of tilapia 18S ribosomal RNA (rRNA; GenBank accession no. AF497908; nucleotides 450–923) in the same sample. After prehybridization of the membrane at 65°C for 4 h in 1% SDS, 10% dextran sulfate, 5.8% NaCl, and 100 μg/ml of calf thymus DNA (Sigma), RNA was hybridized overnight with the GnRH-R cDNA probe labeled with [^{32}P]dCTP (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) using the Megaprime DNA labeling system (Amersham). The membranes were washed twice with a solution of 2× SSC (1× SSC: 0.15 sodium chloride and 0.015 M sodium citrate) at 25°C for 5 min, twice with a solution of 2× SSC/1% SDS at 60°C for 30 min, and twice with 0.5× SSC/0.1% SDS at 25°C for 15 min. The membranes were exposed to the imaging plate of a phosphoimager (BAS 1000; Fuji, Kanagawa, Japan), and the data were analyzed using the TINA (PC BAS; Fuji) program.

Northern Blot Analysis

Total RNA was extracted from the pituitaries of 10 male fish (53.00 ± 0.34 g BW; GSI, 0.23% ± 0.0078%) by Trizol reagent (Gibco BRL, Paisley, U.K.) according to a protocol supplied by the manufacturer. The RNA (40 μg) was run through a 1% agarose-formaldehyde gel and transferred to positively charged nylon membranes (GeneScreen*Plus*; DuPont, Wilmington, DE) according to the manufacturer's instructions. The RNA was fixed by baking for 2 h at 80°C.

The membrane was prehybridized at 50°C in 50% formamide, 5× SSC, 2× Denhardt's reagent, 0.1% SDS, and 250 μg/ml of denatured calf thymus DNA (Sigma) for 4 h. Hybridization was carried out at 60°C for 18 h in prehybridization buffer containing 50 ng/ml of digoxigenin (DIG)-labeled DNA probe. The same probe that was used for the slot-blot hybridization was created by PCR using primers GnR-5F and GnR-2R (Table 1), corresponding to nucleotides 481–500 and 834–858 for forward and reverse primers, respectively. The PCR product was used as a DNA probe and was labeled with the PCR DIG Probe Synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany). The membrane was washed twice with 2× SSC/0.1% SDS for 5 min at room temperature and then twice with 0.5× SSC/0.1% SDS for 15 min at 55°C.

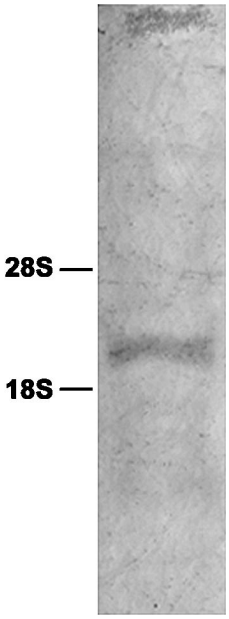

FIG. 1. Northern blot analysis of taGnRH-R. Total RNA from male tilapia pituitary was separated by agarose-formaldehyde gel electrophoresis, transferred, and hybridized with the taGnRH-R probe at high stringency. The positions of the 28S and 18S rRNAs are indicated on the left.

Real-Time PCR

To compare gender differences, the relative abundance of taGnRH-R mRNA was normalized to the amount of an endogenous reference, the 18S subunit of rRNA, by the comparative threshold cycle (C_T) method. The relative amount of taGnRH-R mRNA was calculated by the formula $2^{-\Delta C_T}$, where ΔC_T corresponds to the difference between the C_T measured for taGnRH-R and that measured for 18S rRNA. To validate this method, serial dilutions were prepared from a pituitary cDNA sample (0.5, 0.1, 0.05, 0.02, 0.01, and 0.005), and the efficiencies of taGnRH-R and 18S rRNA amplifications were compared by plotting ΔC_T versus log(template) according to the method described by PE Applied Biosystems (Perkin-Elmer, Foster City, CA). Linear regressions of the plots showed an R^2 value and slope of 0.978 and −3.37, respectively, for 18S rRNA and of 0.972 and −3.36, respectively, for taGnRH-R.

Pituitaries were collected from male (59.1 ± 6.45 g BW; GSI, 0.70% ± 0.13%; n = 5) and female (69.33 ± 5.25 g BW; GSI, 1.09 ± 0.45%; n = 5) tilapia. Total RNA was prepared from individual pituitaries using Trizol (Gibco), and each sample was reverse-transcribed at 37°C using MMLV reverse transcriptase (Promega, Madison, WI) and oligo-dT primer (Promega) according to the manufacturer's protocols.

Gene-specific primers used for the real-time PCR were designed using Primer Express 2.0 software (Perkin-Elmer). The primers used for taGnRH-R amplified a 206-base pair (bp) product and corresponded to nucleotides 1001–1020 and 1188–1207 (GnR-8F and GnR-9R, respectively) (Table 1). Primers for 18S rRNA (18S-F and 18S-R, accession no. AF497908) (Table 1) amplified a 256-bp product. The PCR mixture consisted of 5 μl of diluted cDNA sample, 300 nM of each primer, and 10 μl of Mastermix for Syber Green I (Eurogentec, Seraing, Belgium) in a final volume of 20 μl. Amplification was carried out in an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer) under the following conditions: initial denaturation at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 sec and annealing-extension at 60°C for 1 min, and then a final extension at 60°C for 20 min. Amplification of taGnRH-R and 18S rRNA cDNAs was performed simultaneously in separate tubes and in duplicates, and results were analyzed with the ABI Prism 5700 Sequence Detection System using version 1.6 software (Perkin-Elmer). Dissociation curve analysis was run after each real-time experiment to ensure only one product. To control for false positives, a reverse-transcriptase negative control was run for each template and primer pair. To verify amplification of the correct sequences, the PCR product amplified with taGnRH-R primers was sequenced and its derivation from taGnRH-R cDNA confirmed.

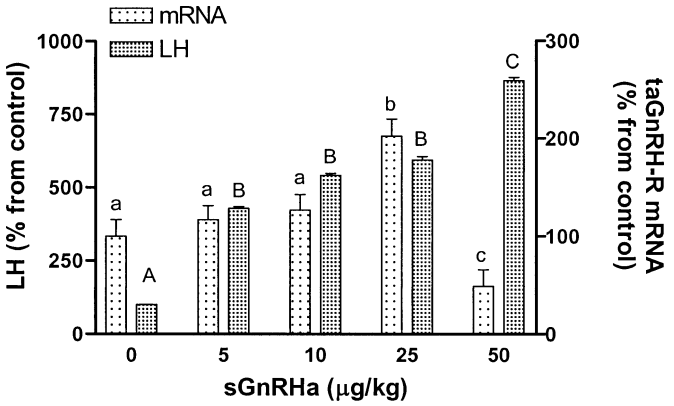

FIG. 2. Effect of sGnRHa on the levels of LH and taGnRH-R mRNA in early maturing male tilapia: dose-dependence study. Fish were injected with sGnRHa (5–50 μg/kg) and examined 24 h later. The results are expressed as the ratio to the control (mean ± SEM, n = 10–12, from three independent experiments). Means marked by different letters differ significantly ($P < 0.05$).

Radioimmunoassay

The LH was measured by homologous RIA [11, 28]. The sensitivity of the assay was 0.5 ng/tube; the intra- and interassay coefficients of variation were 7.3% and 14.0%, respectively.

Statistical Analysis

One-way ANOVA was employed to compare mean values, followed by an a posteriori Student Newman Keuls-test using PRISM 3.02 software (GraphPad, San Diego, CA). Three independent experiments were carried out, each in triplicate. The results are presented as means ± SEM of the three experiments.

RESULTS

The size of the taGnRH-R mRNA was determined by Northern blot analysis on total RNA extracted from tilapia pituitary. This analysis revealed the presence of a single mRNA of approximately 2.3 kilobases (kb) (Fig. 1). The DIG-labeled probe used for the Northern blot analysis was the same as that used for the slot-blot analysis in subsequent experiments. The presence of only one band verified that this probe recognizes only one type of taGnRH-R.

GnRH and GnRH-R mRNA Levels: Dose and Time Studies

For the dose and time studies (Figs. 2–4), we used early maturing males (50.67 ± 5.53 g BW; GSI, 0.12% ± 0.02%). To elucidate whether GnRH can modulate GnRH-R gene expression, early maturing males were injected with graded doses of sGnRHa (5–50 μg/kg), and taGnRH-R mRNA levels were measured after 24 h. The taGnRH-R mRNA levels exhibited a dose-dependent increase, which peaked (at twofold the control level) in fish injected with 25 μg/kg of sGnRHa. At the dose of 50 μg/kg, a decrease in taGnRH-R mRNA levels was noted. The LH levels in the plasma, used as a measure of GnRH potency in the fish pituitary, increased in a dose-dependent manner up to the dose of 50 μg/kg (Fig. 2).

In a time study, early maturing males were injected with the optimal dose of sGnRHa (25 μg/kg), and the concentrations of LH in the plasma and taGnRH-R mRNA were measured at different time points later. The highest plasma LH level was recorded after 2 h and then declined. However, taGnRH-R mRNA in the pituitary increased in a time-

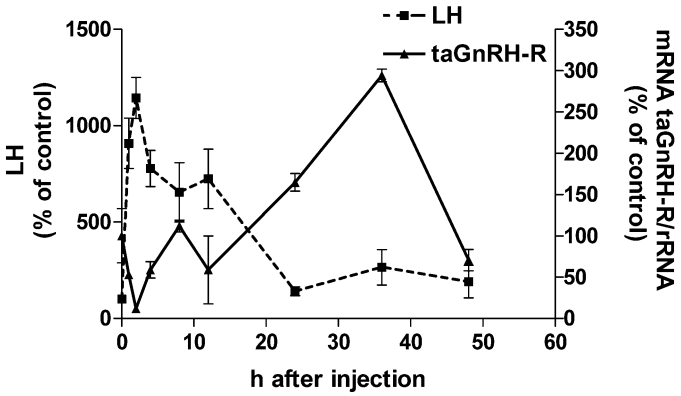

FIG. 3. Time-course changes in levels of taGnRH-R mRNA and plasma LH in early maturing male tilapia injected with sGnRHa (25 μg/kg). Steady-state levels of the GnRH-R transcripts were standardized in relation to 18S rRNA in the same sample. Means marked by different letters differ significantly ($P < 0.05$).

dependent manner, with a maximal effect (threefold) at 36 h (Fig. 3).

The release of LH and the change in mRNA levels of taGnRH-R in tilapia pituitary cells after the addition of sbGnRH, sGnRH, or cGnRH II is shown in Figure 4. All the forms used in the present study are found in the pituitary or brain of tilapia [18] and stimulate LH release in a dose-dependent manner. The lowest concentration tested (0.1 nM) significantly elevated LH secretion above basal values. However, cGnRH II was consistently more effective than either sGnRH or sbGnRH at almost all the concentrations tested (Fig. 4A). All three forms also stimulated increased mRNA levels of taGnRH-R. The lowest effective concentration for sbGnRH and cGnRH II was 0.1 nM, whereas that of sGnRH was 10 nM. The mRNA levels of taGnRH-R in response to sbGnRH increased dose-dependently and reached a peak at 10 nM, whereas in response to sGnRH, they peaked at 10 nM, with a lower response noted at 100 nM. The mRNA levels of taGnRH-R in response to cGnRH II reached their highest level at as little as 0.1 nM, and this hormone was consistently more effective at raising the mRNA levels than either sGnRH or sbGnRH (Fig. 4B).

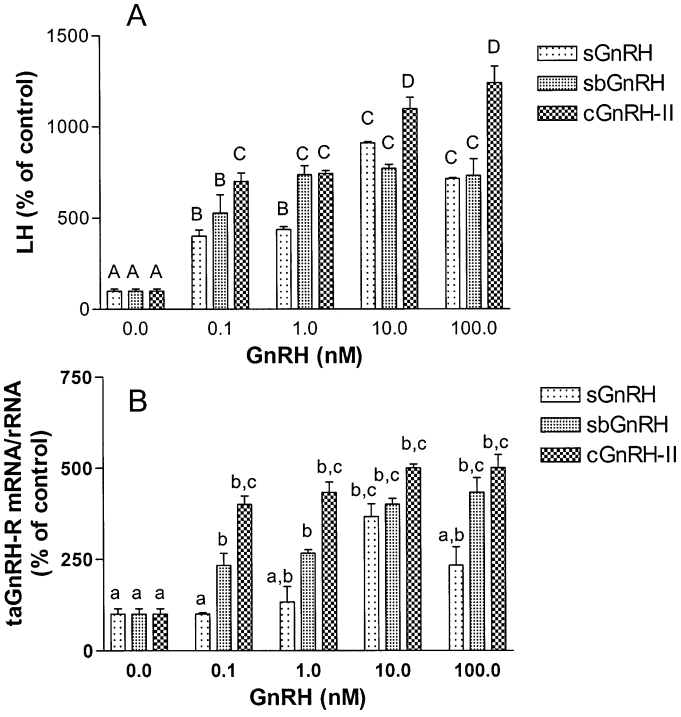

FIG. 4. LH levels (**A**) and taGnRH-R mRNA levels (**B**) in dispersed pituitary cells of early maturing male tilapia exposed to various doses of sGnRH, cGnRH II, or sbGnRH. Steady-state levels of the taGnRH-R transcript were standardized in relation to 18S rRNA in the same sample. The results are expressed as the ratio between the mRNA levels in exposed cells and those of the control. Means marked by different letters differ significantly ($P < 0.05$).

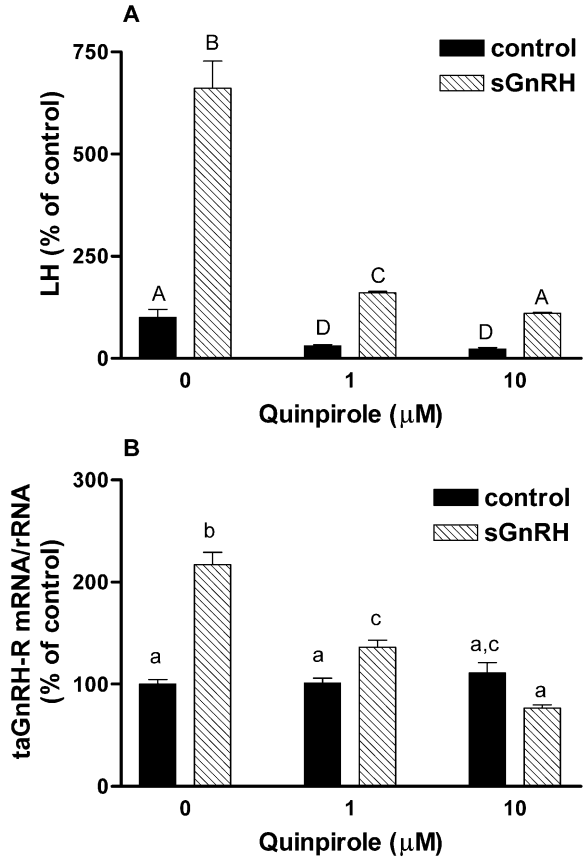

FIG. 5. LH release (**A**) and taGnRH-R mRNA levels (**B**) in dispersed pituitary cells of maturing male tilapia exposed to sGnRH (10 nM) for 24 h in the presence or absence of quinpirole (1 and 10 μM; mean ± SEM, n = 3). Steady-state levels of the transcripts were standardized in relation to 18S rRNA in the same sample. The results are expressed as the ratio between the mRNA levels in exposed cells and those of the control. Means marked by different letters differ significantly ($P < 0.05$).

Effect of Quinpirole on GnRH-R mRNA Levels

To elucidate the effect of quinpirole on sGnRH-stimulated taGnRH-R mRNA levels, maturing males (183.83 ± 10.34 g BW; GSI, 0.3% ± 0.075%) were used. Both basal and GnRH-stimulated LH release were suppressed in the presence of quinpirole. The stimulatory effect of sGnRH (10 nM) on taGnRH-R mRNA levels was also attenuated in the presence of the dopamine agonist (Fig. 5).

Gender Differences in GnRH-R mRNA Levels

To determine any differences in the levels of taGnRH-R mRNA between females and males, we compared maturing males (59.1 ± 6.45 g BW; GSI, 0.45% ± 0.13%) with maturing females (69.33 ± 5.25 g BW; GSI, 1.09% ±

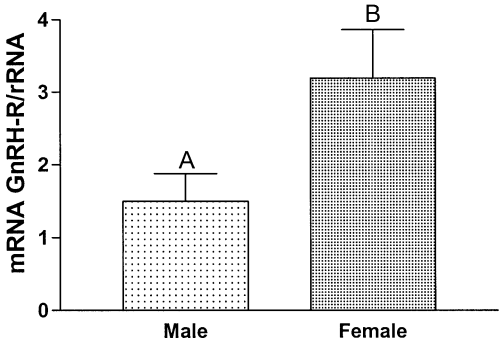

FIG. 6. Gender differences in taGnRH-R mRNA levels in the pituitary of tilapia. Total RNA was reverse-transcribed, and a 1/25th fraction of the reaction was used for quantitative real-time PCR. The relative abundance of taGnRH-R mRNA was normalized to the amount of 18S rRNA by the C_T cycle method, where $2^{-\Delta C_T}$ reflects the relative amount of taGnRH-R transcript. Results are the average ± SEM (n = 5). Means marked by different letters differ significantly (*t*-test, $P < 0.05$).

0.25%) by real-time PCR. The taGnRH-R mRNA levels in female pituitaries were twice as high as those found in males (Fig. 6).

DISCUSSION

The present study is an attempt to elucidate the effects of GnRH and dopamine on the expression of the GnRH-R gene in the pituitary of tilapia both in vivo and in vitro.

Northern blot analysis of total RNA revealed a single GnRH-R mRNA species in the pituitary of male tilapia, of approximately 2.3 kb. This mRNA is similar in size to other piscine GnRH-Rs described for rainbow trout (*Oncorhynchus mykiss*) [30], in which GnRH-R mRNA was 1.9–2 kb, and for striped bass (*Morone saxatilis*) [31], in which GnRH-R mRNA was 2.5 kb, but it is shorter than those described for mammals, in which a major band of 4–5 kb has been detected [32, 33].

The sequence used as a probe in the present study was part of a GnRH-R that was cloned from the tilapia pituitary. This part encodes amino acids 481–858, containing TMs III, IV, and the beginning of V from the GnRH-R. The cloned tilapia GnRH-R (accession no. AY381299) is 84.4% homologous to the European bass GnRH-R (accession no. AJ419594), 84.3% homologous to the striped bass GnRH-R [31], and 83.8% homologous to the amberjack GnRH-R (accession no. AJ130876). The tilapia GnRH-R showed only 51.8% homology to the goldfish GnRH-R IA or 50.8% to the cichlid (*Haplochromis burtoni*) GnRH-R [34; unpublished results]. On the basis of differences in the amino acid sequence of extracellular loop 3, two types of GnRH-R have been distinguished: I and II [35]. Because three forms of GnRH (GnRH I, II, and III; see *Introduction*) are present in many vertebrates (e.g., bony fish and amphibians), it was assumed that three types of corresponding receptors also exist. Millar [7] stated recently that only type I and type II receptors occur in mammals, reptiles, and birds, whereas only type I and type III receptors are present in fish. Based on the homology between the tilapia GnRH-R and those in other fish, it would appear that the receptor reported here bears similarity to GnRH-R type II as described by Troskie et al. [35] or GnRH-R type III as described by Millar [7]. Type II and type III receptors are closely related, suggesting more recent gene duplication [7].

Parhar et al. [36] showed that the type of GnRH-R described in the present study is found on somatotrophs and not necessarily on gonadotrophs. In the present study, LH levels served as a physiological parameter for the effectiveness of GnRH treatments in vivo and in vitro (Figs. 2–4) and for the effect of quinpirole (Fig. 5).

Injection of sGnRHa increased the steady-state levels of taGnRH-R mRNA, with the highest response recorded at 25 μg/kg. Injection of this dose was followed by an increase in the steady-state levels of taGnRH-R mRNA after 36 h, much later than the peak LH release noted as early as 2 h after challenge. When a higher dose of sGnRHa (50 μg/kg) was injected into the fish, the taGnRH-R transcript appeared to be down-regulated, although we cannot rule out the possibility that this decrease resulted from gonadal negative feedback. It should be mentioned in this context that taGnRH-R contains a C-terminus, like other piscine GnRH-Rs, and that enhanced down-regulation was achieved when the C-terminus from catfish was added to the rat GnRH-R [37].

Exposure of tilapia pituitary cells in culture to graded doses of all the GnRH forms (sGnRH, sbGnRH, and cGnRH II) resulted in a significant increase in taGnRH-R mRNA levels. These in vitro results are in line with the finding in cultured rat pituitary cells of increased GnRH-R mRNA levels on exposure to GnRH [38]. However, the peak in rat was achieved faster (6 h) than in tilapia, and the response was already evident at a lower dose of the peptide (2 nM). Similar results were also found with sheep, in which GnRH acted as a positive regulator of its own receptor [39]. All three forms of GnRH used in the present study were effective at releasing LH from dispersed pituitary cells, with cGnRH II being the most potent. The cGnRH II (i.e., GnRH II) was more efficient than the other GnRH forms in both in vitro stimulation of LH release and elevation of taGnRH-R mRNA. In herring, cGnRH II was also consistently more effective than either sGnRH, herring GnRH, or sbGnRH in increasing both LH and GH levels [5].

Our results, both in vivo and in vitro, show that GnRH up-regulates its own receptor in tilapia. Similarly, transient transfection of αT3 cells with the full-length 5′ flanking region of the mouse GnRH-R gene resulted in a 10-fold increase in response to GnRH agonist stimulation [40]. Moreover, mutational analysis and functional transfection studies localized GnRH responsiveness of the mouse GnRH-R gene promoter to novel DNA sequences designated Sequence Underlying Responsiveness to GnRH [40]. To our knowledge, no promoter of a fish GnRH-R has been cloned, and the tilapia GnRH-R promoter sequence is currently under study.

The D2-type dopamine agonist quinpirole reduced the mRNA level of taGnRH-R. These results corroborate those of early studies with goldfish, in which exposure of pituitary fragments to the dopamine-agonist apomorphine was followed by a decrease in GnRH-binding capacity [41], a phenomenon that can be explained by a decrease in the synthesis of GnRH-R.

To test the gender differences in the level of GnRH-R mRNA, we used same-age males and females with similar BW (59.10 ± 6.85 or 69.33 ± 5.25 g, respectively) and GSI (0.45% ± 0.13% or 1.09% ± 0.25%, respectively). According to these values, the fish taken for this experiment were maturing males and females. The testes of maturing male tilapia contained spermatocytes and spermatids, whereas the ovaries of maturing female tilapia were vitellogenic [25]. The taGnRH-R mRNA levels were higher in vitellogenic females than in maturing males. This may be

associated with the higher estradiol-17β levels in females at this stage and its effect on taGnRH-R mRNA [42]. In seabream, higher levels of the same type of GnRH-R have been found in females with higher GSI [31]. The GH levels have also been higher in female tilapia than in males during the spawning season [43].

Using antibodies against the sequence of extracellular loop 3 from goldfish GnRH-R type IA or IB, marmoset GnRH-R type II, and amberjack, striped bass, or medaka GnRH-R type III, it was shown that GnRH-R type IA is present in cells containing the LHβ subunit in the pituitary of tilapia; GnRH-R type IB was visualized in prolactin cells and LH cells and type III GnRH-R in GH cells [36]. Our results cannot confirm or refute the existence of taGnRH-R on somatotrophs or gonadotrophs; however, previous studies with tilapia have indicated that GH release more than doubles in response to GnRH [14]. However, the levels of GH mRNA are not affected [2, 13]. In seabream, higher levels of GnRH-R type II mRNA were found in females with high GSI [31], suggesting a role in reproduction for this GnRH-R type. Moreover, Temple et al. [44] recently showed that in the brain of the musk shrew (*Suncus murinus*), GnRH II also plays a role in controlling reproductive function. The question of which type of GnRH-R controls the reproductive function of various vertebrates remains open at this stage.

In summary, the results of the present paper indicate that a GnRH-R exists in tilapia pituitary and this type of receptor can be modulated by GnRHs, with the following order of potency: cGnRH II > sbGnRH > sGnRH. The taGnRH-R mRNA levels were higher in females than in males. Moreover, dopamine was able to down-regulate taGnRH-R mRNA levels.

REFERENCES

1. Yaron Z, Gur G, Melamed P, Rosenfeld H, Levavi-Sivan B, Elizur A. Regulation of gonadotropin subunit genes in tilapia. Comp Biochem Physiol B Biochem Mol Biol 2001; 129:489–502.
2. Yaron Z, Gur G, Melamed P, Rosenfeld H, Elizur A, Levavi-Sivan B. Regulation of fish gonadotropins. International Review of Cytology—A Survey of Cell Biology, vol. 225. 2003:131–185.
3. Van Der Kraak G, Chang JP, Janz DM. Reproduction. In: Evans DH (ed.), The Physiology of Fishes. New York: CRC; 1998:465–488.
4. Powell JF, Reska-Skinner SM, Prakash MO, Fischer WH, Park M, Rivier JE, Craig AG, Mackie GO, Sherwood NM. Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. Proc Natl Acad Sci U S A 1996; 93:10461–10464.
5. Carolsfeld J, Powell JFF, Park M, Fischer WH, Craig AG, Chang JP, Rivier JE, Sherwood NM. Primary structure and function of three gonadotropin-releasing hormones, including a novel form, from an ancient teleost, herring. Endocrinology 2000; 141:505–512.
6. Somoza GM, Lescheid DW, Miranda LA, Lo Nostro FL, Cepriano-Magliulo L, Montaner AD, Schreibman MP, Rivier JE, Sherwood NM. Expression of pejerrey gonadotropin-releasing hormone in three orders of fish. Biol Reprod 2002; 67:1864–1871.
7. Millar RP. GnRH II and type II GnRH receptors. Trends Endocrinol Metab 2002; 14:35–43.
8. White RB, Eisen JA, Kasten TL, Fernald RD. Second gene for gonadotropin-releasing hormone in humans. Proc Natl Acad Sci U S A 1998; 95:305–309.
9. Blomenrohr M, Bogerd J, Leurs R, Schulz RW, Tensen CP, Zandbergen MA, Goos HJ. Differences in structure-function relations between nonmammalian and mammalian gonadotropin-releasing hormone receptors. Biochem Biophys Res Commun 1997; 238:517–522.
10. Gissis A, Levavi-Sivan B, Rubin-Kedem H, Ofir M, Yaron Z. The effect of gonadotropin-releasing hormone superactive analog and dopamine antagonists on gonadotropin level and ovulation in tilapia hybrids. Israeli Journal of Aquaculture-BAMIDGEH 1991; 43:123–136.
11. Levavi-Sivan B, Ofir M, Yaron Z. Possible sites of dopaminergic inhibition of gonadotropin release from the pituitary of a teleost fish, tilapia. Mol Cellular Endocrinol 1995; 109:87–95.
12. Levavi-Sivan B, Yaron Z. Intracellular mediation of GnRH action on GtH release in tilapia. Fish Physiol Biochem 1993; 11:51–59.
13. Melamed P, Gur G, Elizur A, Rosenfeld H, Sivan B, Rentier-Delrue F, Yaron Z. Differential effects of gonadotropin-releasing hormone, dopamine and somatostatin and their second messengers on the mRNA levels of gonadotropin II beta subunit and growth hormone in the teleost fish, tilapia. Neuroendocrinology 1996; 64:320–328.
14. Melamed P, Eliahu N, Ofir M, Levavi-Sivan B, Smal J, Rentierdelrue F, Yaron Z. The effects of gonadal development and sex steroids on growth-hormone secretion in the male tilapia hybrid (*Oreochromis niloticus* × *Oreochromis aureus*). Fish Physiol Biochem 1995; 14: 267–277.
15. Weber GM, Powell JFF, Park M, Fischer WH, Craig AG, Rivier JE, Nanakorn U, Parhar IS, Ngamvongchon S, Grau EG, Sherwood NM. Evidence that gonadotropin-releasing hormone (GnRH) functions as a prolactin-releasing factor in a teleost fish (*Oreochromis mossambicus*) and primary structures for three native GnRH molecules. J Endocrinol 1997; 155:121–132.
16. Seale AP, Itoh T, Moriyama S, Takahashi A, Kawauchi H, Sakamoto T, Fujimoto M, Riley LG, Hirano T, Grau EG. Isolation and characterization of a homologue of mammalian prolactin-releasing peptide from the tilapia brain and its effect on prolactin release from the tilapia pituitary. Gen Comp Endocrinol 2002; 125:328–339.
17. Gothilf Y, Munoz-Cueto JA, Sagrillo CA, Selmanoff M, Chen TT, Kah O, Elizur A, Zohar Y. Three forms of gonadotropin-releasing hormone in a perciform fish (*Sparus aurata*): complementary deoxyribonucleic acid characterization and brain localization. Biol Reprod 1996; 55:636–645.
18. Parhar IS, Pfaff DW, Schwanzel-Fukuda M. Gonadotropin-releasing hormone gene expression in teleosts. Brain Res Mol Brain Res 1996; 41:216–227.
19. White SA, Kasten TL, Bond CT, Adelman JP, Fernald RD. Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide. Proc Natl Acad Sci U S A 1995; 92: 8363–8367.
20. Powell JF, Fischer WH, Park M, Craig AG, Rivier JE, White SA, Francis RC, Fernald RD, Licht P, Warby C, Sherwood NM. Primary structure of solitary form of gonadotropin-releasing hormone (GnRH) in cichlid pituitary: three forms of GnRH in brain of cichlid and pumpkinseed fish. Regul Pept 1995; 57:43–53.
21. Powell JFF, Standen EM, Carolsfeld J, Borella MI, Gazola R, Fischer WH, Park M, Craig AG, Warby CM, Rivier JE, ValSella MV, Sherwood NM. Primary structure of three forms of gonadotropin-releasing hormone (GnRH) from the pacu brain. Regul Pept 1997; 68:189–195.
22. Holland MC, Gothilf Y, Meiri I, King JA, Okuzawa K, Elizur A, Zohar Y. Levels of the native forms of GnRH in the pituitary of the gilthead seabream, *Sparus aurata*, at several characteristic stages of the gonadal cycle. Gen Comp Endocrinol 1998; 112:394–405.
23. Rodriguez L, Carrillo M, Sorbera LA, Soubrier MA, Mananos E, Holland MCH, Zohar Y, Zanuy S. Pituitary levels of three forms of GnRH in the male European sea bass (*Dicentrarchus labrax* L.) during sex differentiation and first spawning season. Gen Comp Endocrinol 2000; 120:67–74.
24. Hassin S, Gothilf Y, Blaise O, Zohar Y. Gonadotropin-I and -II subunit gene expression of male striped bass (*Morone saxatilis*) after gonadotropin-releasing hormone analogue injection: quantitation using an optimized ribonuclease protection assay. Biol Reprod 1998; 58:1233–1240.
25. Gur G, Melamed P, Gissis A, Yaron Z. Changes along the pituitary-gonadal axis during maturation of the black carp, *Mylopharyngodon piceus*. J Exp Zool 2000; 286:405–413.
26. Melamed P, Gur G, Rosenfeld H, Elizur A, Schulz RW, Yaron Z. Reproductive development of male and female tilapia hybrids (*Oreochromis niloticus* × *O. aureus*) and changes in mRNA levels of gonadotropin (GH) I beta and II beta subunits. J Exp Zool 2000; 286: 64–75.
27. Dickey JT, Swanson P. Effects of salmon gonadotropin-releasing hormone on follicle stimulating hormone secretion and subunit gene expression in coho salmon (*Oncorhynchus kisutch*). Gen Comp Endocrinol 2000; 118:436–449.
28. Levavi-Sivan B, Yaron Z. Involvement of cyclic adenosine monophosphate in the stimulation of gonadotropin secretion from the pituitary of the teleost fish, tilapia. Mol Cell Endocrinol 1992; 85:175–182.
29. Gur G, Bonfil D, Safarian H, Naor Z, Yaron Z. GnRH signaling pathways regulate differentially the tilapia gonadotropin subunit genes. Mol Cell Endocrinol 2002; 189:125–134.

30. Madigou T, Mananos-Sanchez E, Hulshof S, Anglade I, Zanuy S, Kah O. Cloning, tissue distribution, and central expression of the gonadotropin-releasing hormone receptor in the rainbow trout (*Oncorhynchus mykiss*). Biol Reprod 2000; 63:1857–1866.
31. Alok D, Hassin S, Kumar RS, Trant JM, Yu KL, Zohar Y. Characterization of a pituitary GnRH-receptor from a perciform fish, *Morone saxatilis*: functional expression in a fish cell line. Mol Cell Endocrinol 2000; 168:65–75.
32. Tsutsumi M, Zhou W, Millar RP, Mellon PL, Roberts JL, Flanagan CA, Dong K, Gillo B, Sealfon SC. Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. Mol Endocrinol 1992; 6:1163–1169.
33. Brooks J, Taylor PL, Saunders PT, Eidne KA, Struthers WJ, McNeilly AS. Cloning and sequencing of the sheep pituitary gonadotropin-releasing hormone receptor and changes in expression of its mRNA during the estrous cycle. Mol Cell Endocrinol 1993; 94:R23–R27.
34. Robison RR, White RB, Illing N, Troskie BE, Morley M, Millar RP, Fernald RD. Gonadotropin-releasing hormone receptor in the teleost *Haplochromis burtoni*: structure, location, and function. Endocrinology 2001; 142:1737–1743.
35. Troskie B, Illing N, Rumbak E, Sun Y, Hapgood J, Sealfon S, Conklin D, Millar R. Identification of three putative GnRH receptor subtypes in vertebrates. Gen Comp Endocrinol 1998; 112:296–302.
36. Parhar IS, Soga T, Sakuma Y, Millar RP. Spatio-temporal expression of gonadotropin-releasing hormone receptor subtypes in gonadotropes, somatotropes and lactotropes in the cichlid fish. J Neuroendocrinol 2002; 14:657–665.
37. Lin X, Janovick JA, Brothers S, Blomenrohr M, Bogerd J, Conn PM. Addition of catfish gonadotropin-releasing hormone (GnRH) receptor intracellular carboxyl-terminal tail to rat GnRH receptor alters receptor expression and regulation. Mol Endocrinol 1998; 12:161–171.
38. Cheon M, Park D, Kim K, Park SD, Ryu K. Homologous up-regulation of GnRH receptor mRNA by continuous GnRH in cultured rat pituitary cells. Endocrine 1999; 11:49–55.
39. Turzillo AM, Nolan TE, Nett TM. Regulation of gonadotropin-releasing hormone (GnRH) receptor gene expression in sheep: interaction of GnRH and estradiol. Endocrinology 1998; 139:4890–4894.
40. Norwitz ER, Jeong KH, Chin WW. Molecular mechanisms of gonadotropin-releasing hormone receptor gene regulation. J Soc Gynecol Investig 1999; 6:169–178.
41. De Leeuw R, Habibi HR, Nahorniak CS, Peter RE. Dopaminergic regulation of pituitary gonadotrophin-releasing hormone receptor activity in the goldfish (*Carassius auratus*). J Endocrinol 1989; 121: 239–247.
42. Safarian H, Gur G, Rosenfeld H, Yaron Z, Levavi-Sivan B. Regulation of GnRH receptors in tilapia pituitary. In: Goos HJTh, Rastogi RK, Vaudry H, Pierantoni R (eds.), Perspective in Comparative Endocrinology: Unity and Diversity. Bologna: Monduzzi Editore; 2001:639–645.
43. Mol K, Byamungu N, Cuisset B, Yaron Z, Ofir M, Melard C, Castelli M, Kuhn ER. Hormonal profile of growing male and female diploids and triploids of the blue tilapia, *Oreochromis aureus*, reared in intensive culture. Fish Physiol Biochem 1994; 13:209–218.
44. Temple J, Millar R, Rissman E. An evolutionarily conserved form of gonadotropin-releasing hormone coordinates energy and reproductive behavior. Endocrinology 2003; 144:13–19.