Changes in brain mRNA levels of gonadotropin-releasing hormone, pituitary adenylate cyclase activating polypeptide, and somatostatin during ovulatory luteinizing hormone and growth hormone surges in goldfish

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Canosa LF, Stacey N, Peter RE. Changes in brain mRNA levels of gonadotropin-releasing hormone, pituitary adenylate cyclase activating polypeptide, and somatostatin during ovulatory luteinizing hormone and growth hormone surges in goldfish. Am J Physiol Regul Integr Comp Physiol 295: R1815–R1821, 2008. First published September 24, 2008; doi:10.1152/ajpregu.00166.2008.—In goldfish, circulating LH and growth hormone (GH) levels surge at the time of ovulation. In the present study, changes in gene expression of salmon gonadotropin-releasing hormone (sGnRH), chicken GnRH-II (cGnRH-II), somatostatin (SS) and pituitary adenylate cyclase activating polypeptide (PACAP) were analyzed during temperature- and spawning substrate-induced ovulation in goldfish. The results demonstrated that increases in PACAP gene expression during ovulation are best correlated with the GH secretion profile. These results suggest that PACAP, instead of GnRH, is involved in the control of GH secretion during ovulation. Increases of two of the SS transcripts during ovulation are interpreted as the activation of a negative feedback mechanism triggered by high GH levels. The results showed a differential regulation of sGnRH and cGnRH-II gene expression during ovulation, suggesting that sGnRH controls LH secretion, whereas cGnRH-II correlates best with spawning behavior. This conclusion is further supported by the finding that nonovulated fish induced to perform spawning behavior by prostaglandin F2α treatment increased cGnRH-II expression in both forebrain and midbrain, but decreased sGnRH expression in the forebrain.

teleost; neuroendocrine control; spawning behavior; gene expression

SPONTANEOUS OVULATION IN MANY TELEOST species is preceded by a surge of LH release from the pituitary gland that initiates the final maturation of oocytes and the rupture of the ovarian follicle (for a review, see Refs. 38, 58, 64). In fish, gonadotropin-releasing hormone (GnRH) positively regulates LH synthesis and release. The presence of either two or three forms of GnRH in teleost fishes has been well documented (for a review, see Ref 17). The so-called GnRH-I system is regarded as a species-specific form and includes mammalian GnRH (mGnRH), seabream GnRH (sBGnRH), chicken GnRH-I (cGnRH-I), and pejerrey GnRH (pGnRH), among others (17, 30, 34). The GnRH-I system is generally localized in the forebrain and is considered to exert the neuroendocrine control over LH secretion. On the other hand, [His5 Trp7 Tyr8] GnRH (cGnRH-II) also designated as GnRH-II (17, 46, 55) has been reported in all major vertebrate groups, including mammals and is mainly expressed in the midbrain (30, 49). cGnRH-II appears to have direct effects on sexual behavior in mammals, birds, and fish (35, 36, 42, 54, 61), and this effect is believed to be its primary function. Finally, GnRH-III is represented by salmon GnRH (sGnRH) and is found in the forebrain either alone or together with GnRH-I depending on the species (17, 30, 34).

Goldfish (Carassius auratus) represent a unique animal model since cGnRH-II is also present in the forebrain (18). Moreover, cGnRH-II fibers have been found in the pituitary gland of goldfish (18), the European eel (Anguilla anguilla), the African catfish (Clarias gariepinus), tilapia (Oreochromis mossambicus), the striped bass (Morone saxatilis), the European sea bass (Dicentrarchus labrax), and herring (Clupea harengus) (5, 9, 14, 32, 43, 45, 62). Furthermore, cGnRH-II induces the release of LH, GH, and prolactin in vitro from pituitary glands of several fish species (5, 25, 29, 62), indicating that cGnRH-II may also have a neuroendocrine role. In addition, both sGnRH and cGnRH-II affect reproductive behavior in the goldfish (61).

It was previously shown in goldfish that, along with LH, circulating growth hormone (GH) levels undergo a surge increase during the night prior to ovulation (66). In parallel, a rapid depletion in GnRH total content in telencephalon and hypothalamus occurs, coincident with the surge releases of LH and GH, which is interpreted as an indication of peptide release (38, 66). Taking into account that GnRH peptides stimulate both LH and GH secretion, it was suggested that the activation of GnRH neuronal systems might be a common pathway in the stimulation of both LH and GH during spawning (38).

In goldfish, GH secretion is regulated by multiple neuroendocrine factors (for a review, see Ref 1). Most of these regulators are hypothalamic factors that can exert actions on LH and GH release simultaneously (6). For example, gonadotropin-releasing hormone (GnRH) and pituitary adenylate cyclase-activating polypeptide (PACAP) stimulate LH and GH release directly from goldfish pituitary cells (7, 63).

Somatostatin (SS) is the major inhibitor of GH secretion (1), and its regulation and function have been reviewed elsewhere (19, 22, 47, 60). In goldfish, three cDNAs encoding for pro-SS (PSS) have been cloned (21); PSS-I encoding for SS-14, PSS-II encoding for gSS-28 that has [Glu1, Tyr7, Gly10] SS-14 and is considered to exert the neuroendocrine control (30, 34).

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at the C-terminus, and PSS-III that encodes [Pro2] SS-14 at its C-terminus (21). The expression PSS-I and PSS-III are affected by in vivo sex steroid treatments (2), while PSS-II appears to regulate GH in relation with food intake and gastrointestinal function (5).

PACAP belongs to the glucagon/vasoactive intestinal peptide superfamily of peptides (for a review, see Refs. 48 and 59). PACAP is the most conserved member of the family and represents the ancestral molecule. Although PACAP is not considered a GH-releasing factor (41) in mammals, PACAP is highly effective in releasing GH from pituitary cells of fish (1, 11, 33, 63), in which it is suggested to be a physiological GH-releasing factor (1, 63).

The objective of this study was to better characterize the neuroendocrine control of ovulatory LH and GH surges in goldfish by attempting to discriminate between the functions of its two endogenous GnRH forms. Therefore, in addition to measuring periovulatory changes in blood LH and GH, we analyzed changes in expression of sGnRH, cGnRH-II, SS, and PACAP genes.

MATERIALS AND METHODS

Animals. Sexually mature comet goldfish (25 to 40 g) were purchased from Mount Parnell Fisheries (Mersersburg, PA) and maintained in 300-liter flow-through aquaria at 12°C under a simulated natural photoperiod of Edmonton (AB, Canada), for at least 2 wk before experiments. The fish were fed with commercially prepared Unifeed NU-Way trout ration (United Feeds, Calgary, Canada). Goldfish were anesthetized with 0.05% tricaine methanesulfonate (MS-222, Syndel Laboratories, Vancouver, BC, Canada) for 2 min before checking ovulation and tissue collection (2, 4). The experimental procedures, approved by the University of Alberta, are based on the standards of the Canada Council on Animal Care.

Experiment 1: temperature- and spawning substrate-induced ovulation. Mature females were acclimatized to a 16:8-h light-dark cycle and 12°C for at least 2 wk in 300-liter aquaria, and nonovulatory control (CTR) females (n = 6 per aquarium) remained in these conditions throughout the experiment. Temperature- and spawning substrate-induced ovulation was achieved according to Stacey et al. (51, 52). Briefly, experimental (EXP) conditions (n = 6 females per aquarium) were applied at 1600 on day 1 of the experiment (D1, 1600) by adding floating artificial vegetation (spawning substrate), increasing the water temperature to 20°C over a period of 8 h, and introducing mature males (n = 6 per aquarium) on D2 at 0900. The experiment was conducted twice, and the data were combined for statistical analysis.

At five times over a 3-day period, fish were killed by anesthesia (0.05% MS-222); ovulation was checked by lightly squeezing the abdomen to eject oocytes through the ovipore; forebrain (telencephalon and hypothalamus) and midbrain tissues were collected (2, 4); and blood was sampled from the caudal vein with a 26 gauge syringe on the following schedule: D1 at 1600 (used as a reference for data normalization), D2 at 1600, D2 at 2200, D3 at 0600, and D3 at 1000. Forebrain and midbrain tissue was maintained at −80°C until RNA extraction. Blood was allowed to clot overnight at 4°C, centrifuged to collect serum, and stored at −20°C until LH and GH determination by RIA as previously described (26, 37).

As previously shown (51, 52), the EXP conditions induce a LH surge and ovulation in the majority of females. EXP females were classified as nonovulatory if they had serum LH in the range of CTR females (confirmed in D3, at 0600 and D3, at 1000 samples by the absence of ovulated eggs) and were removed from the experiment.

Experiment 2: prostaglandin (PG)-induced female spawning behavior. Female goldfish acclimatized to 12°C in 300-liter aquaria for at least 2 wk were slightly anesthetized in 0.05% MS-222, injected intramuscularly with 25 μg (5 μl) PGF2α (Lutalyse, UpJohn, Orangeville, ON, Canada), or 5 μl saline solution (PG and SAL group, respectively), and placed in 300-liter aquaria divided transversely in two by a mesh in the presence of artificial vegetation and male fish. Each Division chamber held two injected females (both injected with the same solution) and two sperminating males. Males began courtship chasing soon after the addition of PG-injected females, which remained sexually active for the duration of the experiment, resulting in numerous spawning acts (61). The occurrence of reproductive behavior was confirmed by observation, and 2 h after the PG injection, the females were removed from the aquarium and anesthetized, and the forebrain and midbrain tissues were collected. Brain tissue was kept frozen at −80°C until total RNA extraction.

The SAL and PG treatments were replicated three times (n = 6) for each treatment group, and data from each triplicate were pooled for analysis.

Quantitative slot blot quantification. Total RNA was extracted from goldfish forebrain and midbrain tissues using TRIzol reagent (Life Technologies, Gaithersburg, MD), based on the guanidinium thiocyanate-phenol-chloroform extraction method (8). The integrity of the RNA was verified in a denaturing agarose gel, stained with ethidium bromide. Slot blot analysis was used to quantify mRNA levels for the three PSS genes, according to Canosa et al. (2). Total RNA was blotted onto a Nybond-N nylon membrane (Amer sham Pharmacia Biotech, Buckinghamshire, UK) using a Bio-Dot SF blotting apparatus (Bio-Rad Laboratories, Hercules, CA). Specific cDNA probes for PSS-I, PPS-II, and PPS-III (GenBank accession numbers U40754, U60626, and U72656, respectively), and goldfish β-actin (GenBank accession number AB039726.2) were amplified by PCR according to Canosa et al. (2) and labeled with [α-32P]dCTP using Rediprime II Kit (Amer sham Pharmacia Biotech) based on the random primer method (10). The labeled probes were purified through a silica cartridge using the QIAquick Nucleotide Removal Kit (Qiagen Santa Clarita, CA). The specificity of the probes and the quantitative relationship between the amount of total RNA and the measured signal was previously demonstrated (2). The hybridization was performed as previously described (2). As an internal control, the membranes were stripped and reprobed with [α-32P]dCTP-labeled cDNA probe for goldfish β-actin. The radioactive signal was scanned using a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA) and quantified by Image Quant Software (Molecular Dynamics). Because of technical limitations, eight samples for each time point of each condition (EXP or CTR) in experiment 1 were randomly selected and processed for slot-blot analysis.

Quantitative real-time polymerase chain reaction. Two micrograms of total RNA were reverse transcribed in 20-μl reaction mixture containing 500 μM oligo(dT)12–18, 500 μM of each deoxyribonucleotide triphosphate, 10 mM DTT, 50 mM Tris-HCl (pH 8.3), 3 mM MgCl2, 75 mM KCl, and 200 U reverse transcriptase Superscript II (Invitrogen, Carlsbad, CA). The reaction mixture was incubated at 42°C for 50 min and 70°C for 10 min and then treated with 2 U of RNase H (Invitrogen) at 37°C for 30 min. PCR was performed on cDNA samples in duplicates using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Branchburg, NJ). The primers were designed using the Primer Express 2.0 software (Applied Biosystems) and were as follows: sGnRH forward 5′-AGTG-GGACGCTGAAGGTGTTG-3′; sGnRH reverse 5′-TCTTTCCTCGTCTGGTGGGA-3′; cGnRH-II forward 5′-CATCTGAGGGCTGTCTTG-3′; cGnRH-II reverse 5′-TGGCTGAGGGCTGCAAATCG-3′; PACAP forward 5′-AAGCGAGGAGAGGAGAAT-3′; PACAP reverse 5′-CCATCGGAACTGGGTTGTTG-3′. GenBank accession numbers for sGnRH is U30301 and for cGnRH-II variants are U30836.1 and U40567.1. A full-length clone of goldfish PACAP and its sequence were kindly provided by A. O. L. Wong (unpublished results).
Goldfish β-actin (GenBank accession number AB039726.2), used as endogenous control, was amplified by the following primers—actin forward 5′-GAGCTATGAGCTCCCTGACGG-3′, actin reverse 5′-AAACGCTCATTGCCAATGGT-3′—and were used to normalize variations in RNA. After optimization, PCR reactions were performed in a 7-μl volume containing 1.75 μl cDNA, 50 mM KCl, 3.5 mM MgCl₂, 7% glycerol, 0.01% Tween-20, 200 μM dNTPs, 10 mM Tris pH 8.3, 1.0× ROX (reference dye-Invitrogen), 0.3 U Jumpstart Taq polymerase (Sigma, St. Louis, MO), 0.25× SYBR Green (Molecular Probes, Carlsbad, CA) using the following condition: 95°C for 45 s, (40 cycles of 95°C for 15 s and 60°C for 1 min), then 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. The results were analyzed using the standard curve mode, according to the manufacturer’s recommendations (Applied Biosystems). Because of technical limitations, six samples for each time point of each condition (EXP or CTR) in experiment 1 and each group in experiment 2 were randomly selected and processed for quantitative RT-PCR analysis.

Data analysis. The mRNA levels for each PSS form were expressed as the ratio of PSS mRNA to β-actin mRNA and then normalized as a percentage of the D1, 1600 group. Levels of mRNA corresponding to PACAP, sGnRH, and cGnRH-II were calculated as relative to those of β-actin and expressed as a percentage of either the D1, 1600 or the SAL groups. The normalized data were subjected to ANOVA followed by Tukey’s multiple-comparisons tests. Differences were considered significant when P < 0.05.

RESULTS

Experiment 1: temperature- and spawning substrate-induction of ovulation and spawning. Under control conditions (CTR: 12°C, no males or spawning substrate), plasma LH concentrations did not differ among the five sample times (day 1–day 3, denoted as D1–D3 in Fig. 1) and no females ovulated (Fig. 1A). However, in experimental conditions (EXP: 20°C, males and spawning substrate), 8 to 10 of the 12 females in each group sampled on D2 at 2200 and D3 had plasma LH concentrations higher than the LH range of CTR females, and on D3, ovulation had occurred in 8 of 12 females sampled at 0600, and all females sampled at 1000 (Fig. 1A). All of the females that ovulated on D3 and 8 females with high LH concentrations sampled on D2 at 2200 were kept in the EXP group, and the remainder was excluded from further analysis. No females were excluded from EXP groups D1 or D2 at 1600.

Among the ovulatory females retained in the EXP group, the LH surge commenced by D2 at 1600 and reached peak levels from D2 at 2200 to D3 at 0600, consistent with previous descriptions of the LH surge under similar conditions (51, 52, 66). In contrast, the ovulatory GH surge occurred later than described previously (66) and was at peak levels at the final sample (D3, at 1000; Fig. 2A).

In CTR females, forebrain sGnRH mRNA expression and forebrain and midbrain cGnRH-II mRNA expression did not differ at any sample time (Fig. 1B–D). However, forebrain sGnRH expression increased significantly in EXP fish at D2 at 2200, coincident with the LH peak (Fig. 1B), whereas forebrain cGnRH-II expression did not increase significantly in EXP females until D3 at 0600 (Fig. 1C). Unlike cGnRH-II expression in the forebrain, expression in midbrain increased only nonsignificantly at D3 at 0600 and reached peak levels at D3 at 1000 (Fig. 1D).

Forebrain PACAP mRNA expression (Fig. 2B) paralleled that of serum GH (Fig. 2A), with values for EXP females being nonsignificantly greater than those for CTR females on D2 and significantly greater on D3. Expression levels of...
the SS precursors, PSS-I and PSS-III (Fig. 2, C and D), were similar to those of PACAP, with the exception that PSS-III levels were significantly lower in EXP females than CTR females at D2 at 1600 (Fig. 2D). No significant changes in

PSS-II mRNA levels were found at any time point (data not shown).

Experiment 2: prostaglandin F2α-induced female spawning behavior. After 2 h of performing spawning behavior, PG-treated females had significantly less forebrain sGnRH mRNA than SAL-treated females (Fig. 3), but significantly more cGnRH-II mRNA than SAL-treated females both in the forebrain and midbrain (Fig. 3). PG treatment had no significant effect on serum GH levels ($P = 0.254581$) and induced only a marginal nonsignificant decrease in LH ($P = 0.054345$) levels (data not shown).

DISCUSSION

In this study, changes in gene expression of sGnRH and cGnRH-II, PACAP, and SS, during temperature- and spawning substrate-induced ovulation, and PG-induced female spawning behavior in goldfish were analyzed to additionally characterize the role of several neuropeptides in the control of pituitary secretion. This study confirms previous results from our laboratory showing surge increases in serum LH and GH levels during ovulation in goldfish (51, 52, 66). In addition, we report for the first time, changes in the gene expression levels of several neuropeptides in correlation with pituitary hormone secretion, ovulation, and spawning behavior. Although we are aware that mRNA levels do not always match with protein levels and/or the physiological effects of the protein products, the regulation of mRNA levels provides an indication of the activity of a particular peptide neuronal system.

Role of GnRH and PACAP peptides on the ovulatory GH surge. It has been proposed (38) that GnRH might control the periovulatory surges of both LH and GH in goldfish, because GnRH peptides stimulate LH and GH secretion both in vivo and in vitro in goldfish (6, 12, 25) and other teleosts (23, 28). However, GH secretion is controlled not only by GnRH but also by other hypothalamic peptides, including GHRH, PACAP, and SS (1). Indeed, the present results indicate that PACAP regulates the ovulatory GH surge because it is more

Fig. 2. Changes (mean ± SE) in serum GH levels (A) and forebrain mRNA levels of PACAP (B), PSS-I (C), and PSS-III (D) during temperature- and spawning substrate-induced ovulation and spawning. PACAP mRNA levels were determined by quantitative RT-PCR ($n = 6$ per time point), while PSS mRNA levels were determined by slot-blot hybridization ($n = 8$ per time point). Details of EXP and CTR conditions are described in the MATERIALS AND METHODS section. Among EXP fish, groups with the same letter are not significantly different ($P < 0.05$).
closely correlated with changes in PACAP mRNA than with changes in GnRH mRNA. PACAP could act indirectly through the activation of GnRH neurons, as demonstrated in rats (20); however, our results indicate that the activation of the PACAP neuronal system does not precede the activation of the forebrain GnRH neuronal systems. Furthermore, activation of the sGnRH and cGnRH-II neuronal systems precedes the surge increase in serum GH levels and is unlikely to play a role in the stimulation of GH secretion.

Role of SS peptides on GH surge. Although SS peptides inhibit GH secretion (1), the present results show that both PSS-I and PSS-III gene expression increases in parallel with PACAP and GH. Similar parallel changes in serum GH levels and PSS-I and PSS-III gene expression in goldfish (2, 3) were interpreted as part of a negative feedback mechanism triggered by GH as demonstrated in goldfish (1). However, the decrease in PSS-III gene expression on D2 at 1600 cannot be explained in this way, but it could be related to changes in water temperature and/or to a circadian cycle. PSS-III gene expression decreases during the first half of photophase of an 8:16-h light-dark cycle (3), but it is not known whether there is a circadian variation in expression on the 16:8-h light-dark cycle used in this study. Growth rates and GH secretion change seasonally, and the daily pattern of GH secretion changes with the temperature and day length (27); however, to the best of our knowledge, possible influences of temperature changes on expression of SS peptides have not been investigated.

Role of GnRH peptides on LH surge and spawning behavior. GnRH analogs stimulate sexual behavior in mammalian and nonmammalian species, including goldfish (24, 40, 44, 50, 61). Because of the anatomical brain distribution of cGnRH-II neurons and projections, it has been hypothesized that it is this GnRH variant that regulates sexual behavior (31). This hypothesis is supported by studies in female goldfish showing that cGnRH-II is more efficient than sGnRH in reversing the effects of a GnRH antagonist on female sexual behavior (61) and by our present results showing that sGnRH and cGnRH-II are differentially expressed during ovulation and spawning. Thus, sGnRH neurons are activated with the initial rise in serum LH levels, indicating that sGnRH is the primary regulator of the ovulatory LH surge, whereas cGnRH-II expression increases late in the night when spawning behavior begins (53), indicating that cGnRH-II regulates spawning behavior. Further evidence that sexual behavior is regulated by cGnRH-II rather than sGnRH is our finding that, during PG-induced spawning behavior, cGnRH-II expression increases, whereas sGnRH expression decreases. Notably, no significant changes in serum GH or LH levels have been found with PG treatment, although LH levels tended to be lower. Intriguingly, both midbrain and forebrain cGnRH-II expression changed in association with spawning, regardless of whether spawning was natural (temperature-induced) or PG-induced, suggesting that the GnRH-II system of goldfish is not restricted to the midbrain.

Significance and Perspectives

This study was prompted by evidence from neuroanatomical and peptide injection studies that the forebrain of goldfish contains two GnRH forms (cGnRH-II and sGnRH) that both innervate the pituitary and induce release of LH and GH, which also increase dramatically in the blood during the periovulatory period. To determine whether both forebrain GnRH peptides induce these hormone changes, we, therefore, measured changes in mRNA levels as an indication of neuronal activation during the periovulatory period, the rationale being that changes in secretory demand would at some point impact on the steady-state levels of mRNA. The results suggest that periovulatory surges of LH and GH are differentially regulated, by sGnRH and PACAP, respectively, whereas female spawning behavior is influenced by cGnRH-II. From this perspective, it will be interesting to determine in future studies whether fish in which the forebrain contains both sGnRH and a species-specific GnRH variant (e.g., sbGnRH) exhibit differential functional effects of the GnRH-I and GnRH-III systems.

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