Dual Actions of Mammalian and Piscine Gonadotropin-Inhibitory Hormones, RFamide-Related Peptides and LPXRFamide Peptides, in the Hypothalamic–Pituitary–Gonadal Axis

Takayoshi Ubuka* and Ishwar Parhar

Jeffrey Cheah School of Medicine and Health Sciences, Brain Research Institute Monash Sunway, Monash University Malaysia, Sunway, Malaysia

Gonadotropin-inhibitory hormone (GnIH) is a hypothalamic neuropeptide that decreases gonadotropin synthesis and release by directly acting on the gonadotrope or by decreasing the activity of gonadotropin-releasing hormone (GnRH) neurons. GnIH is also called RFamide-related peptide in mammals or LPXRFamide peptide in fishes due to its characteristic C-terminal structure. The primary receptor for GnIH is GPR147 that inhibits cAMP production in target cells. Although most of the studies in mammals, birds, and fish have shown the inhibitory action of GnIH in the hypothalamic–pituitary–gonadal (HPG) axis, several in vivo studies in mammals and many in vivo and in vitro studies in fish have shown its stimulatory action. In mouse, although the firing rate of the majority of GnRH neurons is decreased, a small population of GnRH neurons is stimulated by GnIH. In hamsters, GnIH inhibits luteinizing hormone (LH) release in the breeding season when their endogenous LH level is high but stimulates LH release in non-breeding season when their LH level is basal. Besides different effects of GnIH on the HPG axis depending on the reproductive stages in fish, higher concentration or longer duration of GnIH administration can stimulate their HPG axis. These results suggest that GnIH action in the HPG axis is modulated by sex-steroid concentration, the action of neuroestrogen synthesized by the activity of aromatase stimulated by GnIH, estrogen membrane receptor, heteromerization and internalization of GnIH, GnRH, and estrogen membrane receptors. The inhibitory and stimulatory action of GnIH in the HPG axis may have a physiological role to maintain reproductive homeostasis according to developmental and reproductive stages.

Keywords: gonadotropin-releasing hormone, GPR147, aromatase, neuroestrogen, GPR30, receptor heteromerization, receptor internalization, sex steroids

INTRODUCTION

Gonadotropin-inhibitory hormone (GnIH) is a hypothalamic neuropeptide that was initially isolated from the brain of Japanese quail, which decreases luteinizing hormone (LH) concentration in the culture medium of the anterior pituitary gland (1). In vivo administration of quail GnIH also decreases gonadotropin synthesis as well as gonadal development and maintenance in quail (2).
The C-terminal of GnIH peptides has an LPXRFamide (LPXRFa, X = L or Q) motif. Therefore, peptides orthologous to GnIH are also called RFamide-related peptide (RFRP) in mammals and LPXRFa peptides in non-mammalian and non-avian vertebrates (3). Most of the studies in mammals, birds, and fish have shown inhibitory effects of GnIH on the hypothalamic–pituitary–gonadal (HPG) axis; however, several in vivo and in vitro studies in mammals and fish show their stimulatory effects (3, 4). Here, we highlight studies that show stimulatory effects of GnIH on the HPG axis and investigate their physiological or pharmacological mechanisms.

### ENDOGENOUS MATURE GnIH PEPTIDES

Human RFRP-1 and -3 (5), macaque RFRP-3 (6), Siberian hamster RFRP-1 and -3 (7), rat RFRP-3 (8), bovine RFRP-1 (9) and -3 (10), European starling GnIH (11), zebra finch GnIH (12), chicken GnIH (13), quail GnIH (1), quail GnIH-related peptide (RP) 2 (14), red-eared slider LPXRFamide-1, 2, 3 (15), frog growth hormone-releasing hormone (fGRF), fGRF-RP-1, fGRF-RP-2, and fGRF-RP-3 (16, 17), Japanese red-bellied newt LPXRFa-1, -2, -3, -4 (18), and goldfish LPXRFa-3 (19) are identified as endogenous mature LPXRFa peptides by cDNA sequencing, immunoaffinity chromatography, and mass spectrometry in gnathostomes (3). Lamprey is a jawless fish that is one of the most primitive among vertebrates. Lamprey LPXRFamide peptide precursor gene encompasses C-terminal QPQRFamide (LPXRFa-1a, 1b) and RPQRFamide peptides (LPXRFa-2) that have been identified by mass spectrometry (20). LPXRFamide peptide precursor gene is also found in amphioxus, one of the most primitive chordates (protochordates), which encompasses three mature C-terminal RPQRFamide peptides (PQRFa-1, PQRFa-2, and PQRFa-3) (21). Identified and putative amino-acid sequences of GnIH peptides are summarized in Table 1. Although the C-terminal LPXRFa structure is key for binding of GnIH to its receptor (22), the N-terminal structure may modify the action of GnIH. Studies are needed to investigate the function of the N-terminal of GnIH and the differential effect of orthologous LPXRFa peptides encoded in the precursor polypeptide (Table 1).

### GnIH RECEPTOR

Yin et al. characterized the binding activity of quail GnIH and GnIH-RPs to a G-protein-coupled receptor (GPCR) GPR147. The membrane fraction of COS-7 cells transfected with quail GPR147 cDNA specifically bound GnIH and GnIH-RPs that have a C-terminal LPXRFa motif with similar affinities (22). Hinuma et al. identified a specific receptor for GnIH (RFRP) in mammals, which was identical to GPR147 and named it OT7T022 (28). In the same year, Bonini et al. reported two GPCRs for neuropeptide FF (NPFF), a neuropeptide that has a PQRFamide (PQRFa) motif at its C-terminal that modulates pain, and designated as NPFF1 (identical to GPR147) and NPFF2 (identical to GPR74) (29). LPXRFa peptide precursor gene and PQRFa peptide precursor gene are thought to have diverged from a common ancestral gene through gene duplication (20, 21). GPR147 and GPR74 genes are also paralogous (30). The binding affinities of RFRPs to GPR147 and GPR74 and their signal transduction pathways show their higher affinity to GPR147 than NPFF that has a potent agonistic activity on GPR74 (10, 29, 31), suggesting that GPR147 (NPFF1, OT7T022) is the primary receptor for GnIH (3). However, this may not apply to teleost fishes as they generally have several subtypes of GPR147 and/or GPR74 (32).

### INTRACELLULAR SIGNALING OF GnIH RECEPTOR

Gonadotropin-inhibitory hormone peptides suppress the production of cAMP by binding to GPR147 on the cells, suggesting that GPR147 couples to Gα protein that inhibits adenyly cyclase (AC) (28, 33). Son et al. investigated the precise mechanism of GnIH cell-signaling pathway in a mouse gonadotrope cell line, LβT2 (34). Mouse RFRPs (mRFRPs) suppress GnRH-induced cAMP signaling. mRFRPs also inhibit GnRH-stimulated extracellular signal-regulated kinase (ERK) phosphorylation and gonadotropin subunit gene transcription by inhibiting the protein kinase A (PKA) pathway. Therefore, mRFRPs function as GnIH to inhibit GnRH-induced gonadotropin subunit gene transcription by inhibiting AC/cAMP/PKA-dependent ERK activation in gonadotropes (34) (Table 2).

Son et al. further investigated the signal transduction pathway that conveys the inhibitory action of GnIH in GnRH neurons by using a mouse GnRH neuronal cell line, GT1–7 (46). Although GnIH significantly suppressed the stimulatory effect of kisspeptin on GnRH release in hypothalamic culture, GnIH had no inhibitory effect on the protein kinase C (PKC) pathway that conveys the inhibitory action of GnIH in GnRH neurons. On the other hand, GnIH eliminated the stimulatory effect of vasoactive intestinal polypeptide (VIP) on AC activity, p38 and ERK phosphorylation, and c-Fos mRNA expression in GT1–7. This shows the specific inhibitory mechanism of GnIH action on AC/cAMP/PKA pathway, and demonstrates a common mechanism of GnIH action in gonadotropes and GnRH neurons (34, 46) (Table 2).

### EXISTENCE OF GnIH AND GnIH RECEPTOR IN THE HPG AXIS

Gonadotropin-inhibitory hormone precursor mRNA is expressed in the hypothalamus of all vertebrates investigated (3). GnIH neuronal axons terminate on GnRH1 neurons in the preoptic area (POA) that terminate at the median eminence and stimulate gonadotropin secretion from the anterior pituitary gland in birds (11, 12, 52–55) (Figure 1). In situ hybridization of GPR147 mRNA combined with GnRH immunocytochemistry shows expression of GPR147 mRNA in GnRH1 neurons in birds (11). GnIH (RFPR) axons also terminate on the hypophysiotropic type of GnRH neurons in humans (5), monkey (6), sheep (56), hamsters (7, 45), rats...
Table 1: Amino-acid sequences of RFRPs, GnIHs, and LPXRFa peptides in chordates.

| Animal          | Name         | Sequence                        | Reference |
|-----------------|--------------|---------------------------------|-----------|
| Mammals         | Human        | RFRP-1                           | MHSFAHLPLRFa (5) |
|                 | Macaque      | RFRP-1                           | MHSFYTLPLRFa (5) |
|                 | RFRP-2       | SGNMVEGVLQIVMLPLRFa (9)          |           |
|                 | Bovine       | RFRP-1                           | SLTHFVEKNLKKMTHVPLVPNTSAALPLRFa (9) |
|                 |              | RFRP-3                           | AMAHLLFLRLKNEDELMSKLVNLPLRFa (10) |
|                 | Horse        | RFRP-3                           | IPNLPLRFa (23) |
|                 | Rat          | RFRP-1                           | SYFQELKNDKCDKIDSAPANKVPHSAANLPLRFa (8) |
|                 |              | RFRP-3                           | AMHGCTMHPHPFLRFa (8) |
|                 | Siberian hamster | RFRP-1  | SPAANKVPHSAANLPLRFa (7)   |
|                 | Syrian hamster | RFRP-3  | TLRVFPLPLRFa (7)   |
|                 |              | RFRP-4                           | VPHSAANLPLRFa (8) |
| Birds           | Quail        | GnIH                             | SIKPKAYLPLRFa (1) |
|                 |              | GnIH-RP-1                        | SLNFEMEKNLKKMTHVPLVPNTSAALPLRFa (14) |
|                 |              | GnIH-RP-2                        | SLNKFEEMKDWGSKNIIKMSTPTVNKVPNSVANLPLRFa (14) |
|                 |              | White-crowned sparrow            | SIKPKAYLPLRFa (14) |
|                 |              | GnIH-RP-1                        | SLNFEMEKNLKKMTHVPLVPNTSAALPLRFa (14) |
|                 |              | GnIH-RP-2                        | SLNKFEEMKDWGSKNIIKMSTPTVNKVPNSVANLPLRFa (14) |
|                 | European starling | GnIH | SIKPKAYLPLRFa (11) |
|                 |              | GnIH-RP-1                        | SLNFEMEKNLKKMTHVPLVPNTSAALPLRFa (11) |
|                 |              | GnIH-RP-2                        | SLNKFEEMKDWGSKNIIKMSTPTVNKVPNSVANLPLRFa (11) |
|                 | Zebra finch  | GnIH                             | SIKPKAYLPLRFa (12) |
|                 |              | GnIH-RP-1                        | SLNFEMEKNLKKMTHVPLVPNTSAALPLRFa (12) |
|                 |              | GnIH-RP-2                        | SLNKFEEMKDWGSKNIIKMSTPTVNKVPNSVANLPLRFa (12) |
| Reptiles        | Anole lizard | GnIH                             | SIKPKAYLPLRFa (16, 26) |
|                 |              | GnIH-RP-1                        | SIKPKAYLPLRFa (16, 26) |
|                 |              | Red-eared slider turtle          | SIKPKAYLPLRFa (15) |
|                 |              | Chinese softshell turtle         | SIKPKAYLPLRFa (15) |
| Amphibians      | Bullfrog     | IGFR-RPa                         | SLCKPAHLPLRFa (16, 26) |
|                 |              | IGRP-RPa                         | SLCKPAHLPLRFa (16, 26) |
|                 |              | Red-bellied newt                 | SLCKPAHLPLRFa (16, 26) |
| Teleost fish    | Goldfish     | gLXRFa-1                         | PTLHANLPLRFa (19) |
|                 |              | gLXRFa-2                         | AKSNLPLRFa (19) |
|                 |              | gLXRFa-3                         | SGTLSLTLPLRFa (19) |
|                 | Medaka       | mlXRFa-1                         | PLKIHMMPLRFa XM_004073848 |
|                 |              | mlXRFa-2                         | VNHMPMPLRFa XM_004073848 |
|                 |              | mlXRFa-3                         | EAPSYVLPLRFa XM_004073848 |
| Grass puffer    | LPXRFa-1     | SLKARIVQYKDPVSGLVPLPLRFa (19)    |           |
|                 |              | LPXRFa-2                         | DQVGGQDVMVPLNPMPQRFa (19) |
|                 |              | RYα                             | SMKVRLEDSCSXKVPLKQKVRAYa (79) |
| Tiger puffer    | LPXRFa-1     | SLKARIVQYKDPVSGLVPLPLRFa (19)    |           |
|                 |              | LPXRFa-2                         | DQVGGQDVMVPLNPMPQRFa (19) |
|                 |              | RYα                             | SMKVRLEDSCSXKVPLKQKVRAYa (79) |
| Agnathans       | Sea lamprey  | ILXRFa-1a                        | SVGDQGRRQSSKILPLRFa (20) |
|                 |              | ILXRFa-1b                        | AALRGQVRQQSSKILPLRFa (20) |
|                 |              | ILXRFa-2                        | SEFPRHRTRPLRFa (20) |
| Protochordates  | Amphioxus    | PQRFa-1                          | WDDEKRPQRFa (21) |
|                 |              | PQRFa-2                          | GNDKTDWQPRFa (21) |
|                 |              | PQRFa-3                          | GNQGHWQPRFa (21) |

Ensembl or Genbank accession numbers are cited for some reptile GnIHs or medaka LPXRFa peptides. C-terminal LPXRFa (X = L or Q) sequences are underlined.

*Putative peptides hypothesized from mRNA and deduced amino-acid sequences.
| In vivo (animal) or in vitro (pituitary or cell line) | Concentration or dose of peptides | Rout of administration, culture medium | Administration time, sample collection, measurement | Effect | Reference |
|-------------------------------------------------|----------------------------------|----------------------------------------|-----------------------------------------------|-------|-----------|
| Postmenopausal women                             | 50-µg/kg/h human RFRP-3          | iv                                     | Continuous administration for 3 h             | LH secretion was decreased during RFRP-3 administration | George et al. (35) |
| Estrous ewes                                     | 1-mg/h human RFRP-3             | iv                                     | 2-h infusion                                 | LH secretion was decreased during and after RFRP-3 administration | Clarke et al. (36) |
| Ovariectomized ewes treated with EB to induce LH surge | 1-mg bolus + 0.5 mg/h human RFRP-3 | iv                                     | 8-h infusion                                 | EB-induced LH surge was blocked by RFRP-3 | Clarke et al. (36) |
| Hypothalamo-pituitary disconnected ovariectomized ewes | 50, 100, 200 ng GnRH during 400-µg/h human RFRP-3 | iv                                     | Blood was collected –5, 5, 10, 15, 20, 30 min after GnRH administration | RFRP-3 decreased 100-ng GnRH-induced LH secretion | Smith et al. (37) |
| Castrated male calves                            | 90-µg bovine RFRP-3             | iv                                     | 6 injections at 10-min intervals             | LH pulse frequency was decreased during 1-h injection period | Kadokawa et al. (38) |
| Male rats                                        | 10, 100, 500 ng rat RFRP-3      | icv                                    | Blood was collected 20 min after administration | LH concentration was decreased by administration of 10-, 100-, or 500-ng RFRP-3 | Johnson et al. (39) |
| Male rats                                        | 0.1, 0.5, 1, 5 nmol rat RFRP-3  | icv                                    | Blood was collected 15–120 min after administration | Total LH secretion until 120 min after administration was decreased by 5-nmol RFRP-3. FSH concentration was decreased at 15 min by 5-nmol RFRP-3. Total FSH secretion until 120 min after administration was decreased by 5-nmol RFRP-3 | Pineda et al. (40) |
| Gonadectomized male rats                         | 0.1, 0.5, 1, 5 nmol rat RFRP-3  | icv                                    | Blood was collected 15–120 min after administration | LH concentration was decreased at 15 min by 5-nmol RFRP-3. Total LH secretion until 120 min after administration was decreased by 1- and 5-nmol RFRP-3. Total FSH secretion until 120 min after administration was decreased by 5-nmol RFRP-3 | Pineda et al. (40) |
| Gonadectomized male rats                         | 10-nmol rat RFRP-3              | iv                                     | Blood was collected 15–120 min after administration | LH concentration was decreased at 60 min. Total LH secretion until 75 min after administration was decreased. FSH concentration was decreased at 60 and 75 min after administration | Pineda et al. (40) |
| Ovariectomized rats                              | 1, 5 nmol rat RFRP-3            | icv                                    | Blood was collected 15–120 min after administration | LH concentration was decreased at 15 min by 1-nmol RFRP-3. Total LH secretion until 120 min after administration was decreased by 5-nmol RFRP-3 | Pineda et al. (40) |
| Ovariectomized rats                              | 1-µg rat RFRP-3                 | iv                                     | Blood was collected 30, 60, 120 min after administration | LH concentration was decreased 120 min after administration | Murakami et al. (41) |
| Ovariectomized rats with E2 + P4 to induce LH surge | 2.5, 25 ng/h rat RFRP-3 using osmotic pump | icv | Brains were collected 2 days later at the surge peak | 25-ng/h 25-ng/h RFRP-3-reduced c-Fos expression in GnRH neurons and anteroventral periventricular region that provides stimulatory input to GnRH neurons | Anderson et al. (42) |
| Prepubertal female mice                          | 100, 500, 1,000 ng RFRP-3       | icv                                    | Hypothalamus and blood was collected 4 h after administration | GnRH mRNA, Kiss1 mRNA, and LH concentration was decreased by 500- and 1,000-ng RFRP-3 | Xiang et al. (43) |
| Ovariectomized or E2-treated ovariectomized prepubertal or adult female mice | 20-nmol RFRP-3                  | icv                                    | Blood was collected 4 h after administration | RFRP-3 decreased LH concentration in only E2-treated ovariectomized prepubertal female mice but both E2-treated or not treated ovariectomized adult female mice | Xiang et al. (43) |

(Continued)
**TABLE 2** Continued

| In vivo (animal or in vitro (pituitary or cell line)) | Concentration or dose of peptides | Rout of administration, culture medium | Administration time, sample collection, measurement | Effect | Reference |
|----------------------------------------------------|----------------------------------|----------------------------------------|-------------------------------------------------|----------------|----------|
| Male Syrian hamsters                              | 150, 500, 1,500, 5,000-ng Syrian hamster RFRP-3 | icv                                    | Blood was collected 30 and 120 min after administration | LH concentration was increased 30 min after administration of 500- 15,000-ng RFRP-3. FSH concentration was increased 30 min after administration of 1,500-ng RFRP-3. Testosterone concentration was increased 120 min after administration of 1,500-ng RFRP-3 | Ancel et al. (44) |
| Male Syrian hamsters acclimatized to SD            | 12-µg/day Syrian hamster RFRP-3 | icv using osmotic pump                  | Blood was collected after 5 weeks of continuous administration | Testosterone concentration and paired testicular weight were increased to LD levels | Ancel et al. (44) |
| Ovariectomized Syrian hamsters                    | 100, 300, 500 ng GnIH (icv), 600-ng GnIH (ip) | icv, ip                               | Blood was collected 5 (icv), 30 (icv and ip) min after administration | LH concentration was decreased 5 and 30 min after icv administration of 500-ng GnIH, and 30 min after ip administration of 600-ng GnIH. | Kriegsfeld et al. (45) |
| Male Siberian hamsters acclimatized to LD or SD    | 100- and 500-pmol Siberian hamster RFRP-1 or RFRP-3 | icv                                    | Blood was collected 5 and 30 min after administration | LH concentration was decreased 5 and 30 min after administration of 500-pmol RFRP-1, 100- and 500-pmol RFRP-3, 30 min after administration of 100-pmol RFRP-1 in LD. LH concentration was increased 30 min after administration of 500-pmol RFRP-1 or 500-pmol RFRP-3 in SD. | Ubuka et al. (7) |

**In vitro**

| Hypothalamic tissue of male mice                    | 10⁻¹, 10⁻² M RFRP-3 with 10⁻⁸ M kisspeptin | Medium 199 | After 1-h incubation medium was collected | 10⁻⁶ M RFRP-3 suppressed 10⁻⁸ M kisspeptin-induced GnRH release | Son et al. (46) |
| Hypothalamic tissue of female mice                  | 10⁻² M RFRP-3 with 10⁻⁸ M VIP | Medium 199 | After 1-h incubation medium was collected | 10⁻⁶ M RFRP-3 suppressed 10⁻⁸ M VIP-induced GnRH release | Son et al. (46) |
| GFP labeled GnRH neurons of transgenic mice         | 0.01-1-µM GnIH or RFRP-3 | aCSF | 15-s application | GnRH and RFRP-3 produced a non-desensitizing hyperpolarization [IC₅₀: 34 nM (GnIH), 37 nM (RFRP-3)] via a direct postsynaptic Ba²⁺-sensitive K⁺ current mechanism | Wu et al. (47) |
| GFP labeled GnRH neurons of transgenic mice         | 1-µM RFRP-3 | aCSF | 5-min application | RFRP-3 exhibited rapid and repeatable inhibitory effects on the firing rate of 41% of GnRH neurons. RFRP-3 increased the firing rate of 12% of GnRH neurons | Ducret et al. (48) |
| Mouse GnRH neuronal cell line (GT1–7)               | 10⁻⁴, 10⁻³, 10⁻², 10⁻¹ M RFRP-3 | DMEM | 6 (CRE assay) or 1 (p38, ERK assay) h application | 10⁻⁶ M VIP-induced CRE activity was suppressed by 10⁻⁴, 10⁻³, 10⁻² M RFRP-1, 3. 10⁻¹ M VIP-induced p38 and ERK phosphorylation was suppressed by 10⁻⁷, 10⁻⁸ M RFRP-3 | Son et al. (46) |
| Mouse GnRH neuronal cell line (mHypoA-GnRH/GFP)     | 10⁻⁴, 10⁻³, 10⁻², 10⁻¹ M human RFRP-3 | DMEM | 1-, 2-, 4-h application | GnRH mRNA expression was decreased by 100-nM RFRP-3 at 1-, 2-, 4-h application | Gojška et al. (49) |
| Ewe dispersed pituitary cells                       | 10⁻⁴, 10⁻³, 10⁻², 10⁻¹ M human RFRP-3 | DMEM | Medium was collected after 2-h incubation | GnRH-induced LH release was decreased by 10⁻¹², 10⁻¹⁰, 10⁻⁸ M RFRP-3. GnRH-induced FSH release was decreased by 10⁻¹⁰, 10⁻⁸ M RFRP-3 | Clarke et al. (50) |
| Gonadectomized ewe and ram dispersed pituitary cells | 10⁻⁴, 10⁻³, 10⁻² M human RFRP-3 with 10⁻⁴ M GnRH | DMEM with 10% fetal calf serum | Medium was collected 8, 16, 24 h during incubation and finally pituitary cells were collected | GnRH-induced LH release was decreased by 10⁻¹², 10⁻¹⁰, 10⁻⁸ M RFRP-3 at 8-, 16-, 24-h in ewe pituitary cells. GnRH-induced LH release was decreased by 10⁻¹², 10⁻¹⁰ M RFRP-3 at 8-, 16-h in ram pituitary cells, GnRH-induced FSH release was decreased by 10⁻¹², 10⁻¹⁰ M RFRP-3 at 16-, 24-h in ewe pituitary cells. GnRH-induced FSH release was decreased by 10⁻¹², 10⁻¹⁰ M RFRP-3 at 8-, 16-h in ram pituitary cells. GnRH-induced LH, FSH, expression, ERK phosphorylation were decreased by 10⁻¹², 10⁻¹⁰ M RFRP-3 in ewe and ram pituitary cells | Sari et al. (51) |

(Continued)
Table 2 (Continued)

| Effect | In vivo (peripheral) or in vitro (cell line) | Method/Condition | Reference |
|---|---|---|---|
| Concentration or dose of peptides | Medium was collected after 2 h | 10^{-7} M bovine RFRP-3 | Kadokawa et al. (68) |
| | After 2-h incubation medium was collected | 10^{-7} M bovine RFRP-3 | Moussawi et al. (69) |
| | After 1-h incubation medium was collected | 10^{-7} M bovine RFRP-3 | Sugawara et al. (70) |
| | After 24-h incubation medium was collected | 10^{-7} M bovine RFRP-3 | Son et al. (71) |
| | Basal LH concentration was decreased by 10^{-6} M bovine RFRP-3 | 10^{-7} M bovine RFRP-3 | Son et al. (71) |
| | LH concentration increased by 10^{-6} M bovine RFRP-3 | 10^{-6} M bovine RFRP-3 | Son et al. (71) |
| | LH concentration increased by 10^{-7} M bovine RFRP-3 | 10^{-7} M bovine RFRP-3 | Son et al. (71) |
| | LH concentration increased by 10^{-8} M bovine RFRP-3 | 10^{-8} M bovine RFRP-3 | Son et al. (71) |
| | LH concentration increased by 10^{-9} M bovine RFRP-3 | 10^{-9} M bovine RFRP-3 | Son et al. (71) |
| | LH concentration increased by 10^{-10} M bovine RFRP-3 | 10^{-10} M bovine RFRP-3 | Son et al. (71) |
| | LH concentration increased by 10^{-11} M bovine RFRP-3 | 10^{-11} M bovine RFRP-3 | Son et al. (71) |
| | LH concentration increased by 10^{-12} M bovine RFRP-3 | 10^{-12} M bovine RFRP-3 | Son et al. (71) |

**STIMULATORY EFFECTS OF GnIH ON THE HPG AXIS**

An electrophysiological study has shown that RFRP-3 exhibits rapid and repeatable inhibitory effects on the firing of 41% of GnRH neurons in adult mice (48). However, stimulatory effect of RFRP-3 was observed in 12% of GnRH neurons (Table 2). No stimulatory effect of RFRP-3 on the firing of GnRH neurons was observed in diestrus mice but 18% of GnRH neurons were stimulated by RFRP-3 in proestrus female mice (48).

To understand the physiological roles of GnIH in mammalian reproduction, GnIH precursor cDNA and endogenous mature peptides have been identified in the Siberian hamster brain (7). GnIH mRNA expression and number of GnIH-ir perikarya, fibers that innervate GnRH neurons are higher in long days (LD), breeding season, compared with short days (SD), non-breeding season. Intracerebroventricular (icv) administration of hamster RFRP-1 or RFRP-3 to male Siberian hamster inhibits plasma LH concentration 5 and 30 min after administration in LD but stimulates plasma LH concentration 30 min after administration in SD (Table 2). It has been also shown that central chronic administration of RFRP-3 to male Syrian hamsters adapted to SD fully restores testicular weight and plasma testosterone concentration (44, 70) (Table 2).

Moussavi et al. investigated the effect of intraperitoneal (ip) administration of goldfish LPXRFa-3 on LHβ and FSHβ subunit mRNA levels in the pituitary and serum LH concentration during gonadal cycle in goldfish (71). Circulating 17β-estradiol (E2) level is very low at early gonadal recrudescence (gr), increasing at mid-gr, very high at mid-late gr, and decreasing at late gr stages. LPXRFa-3 increased LHβ and FSHβ mRNA levels at early to mid-late and late gr, respectively. However, serum LH

---

**Table 2**

| Administration time, sample collection, measurement | Reference |
|---|---|
| In vivo (animal) or dose of peptides | In vitro (pituitary or cell line) |
| Cattle dispersed pituitary cells | DMEM Medium was collected after 2-h incubation with 10^{-8} M RFRP-3. LH concentration was decreased by 10^{-8} M RFRP-3. | Murakami et al. (38) |
| | After 24-h incubation medium was collected | Son et al. (41) |
| | DMEM 1 h (gonadotropin subunit gene expression), 2 h (LH release) application | Son et al. (34) |
| | 10^{-6} M RFRP-3 with or without 10^{-7} M GnRH | Son et al. (34) |
| | 10^{-6} M RFRP-3 with or without 10^{-7} M GnRH | Son et al. (34) |
| | 10^{-6} M RFRP-3 with or without 10^{-7} M GnRH | Son et al. (34) |
| | 10^{-6} M RFRP-3 with or without 10^{-7} M GnRH | Son et al. (34) |

---

**Figure 1**

Abundant GnIH-immunoreactive (ir) fibers exist in the median eminence of humans (5), monkey (6), sheep (50), quail (1, 25, 61), sparrow (52, 62), and turtle (15). It has been clearly shown that GnRH mRNA is expressed in the gonadotropes of human pituitary (5). GnRH-ir cells are located in the cephalic and caudal lobes of the chicken pituitary gland and they are colocalized with LHβ or FSHβ mRNA-containing cells (63). Therefore, it is likely that GnIH can directly act on the pituitary to inhibit gonadotropin synthesis and/or release from the pituitary in most birds and relatively large mammalian species (3) (Figure 1). On the other hand, GnIH may not act directly on the pituitary in some birds and rodents, as there are few or no GnIH-ir fibers in the median eminence of Rufous-winged sparrows (64), hamsters (7, 45), and rats (65). In teleost fishes, GnIH-ir fibers directly innervate the pituitary (4), which have been observed in goldfish (19), sockeye salmon (66), Indian major carp (67), sea bass (68), and tilapia (69). In the tilapia pituitary, LH cells were labeled by GnIH receptor antibody (69) (Figure 1).
Ip administration of grouper GnIH-I, II, and III decreased GnRH1 mRNA level in the hypothalamus (77). However, GnRH3 mRNA level in the hypothalamus was increased by ip administration of GnIH-III. On the other hand, LHβ mRNA level in the pituitary was decreased by GnIH-II (Table 3). Ip administration of lamprey LPXRFa-2 increased GnRH-1 and III content in the brain, gonadotropin β mRNA level in the pituitary [20], Table 3]. A study in European sea bass has shown that intramuscular administration of sea bass GnIH-2 increased GnRH2 and kiss1 receptor mRNA levels in the brain (27). On the other hand, GnIH-1, 2 decreased pituitary LHβ mRNA level and plasma LH level. Plasma FSH level was only decreased by GnIH-1 (Table 3).

In addition, 48-h incubation of grass puffer pituitary with LPXRFa-1 (10⁻⁷ M) increased LHβ and FSHβ mRNA levels [79], Table 3]. Although LH and FSH release from Cichlasoma dimerus pituitary was decreased by 24-h incubation with LPQRFa-1 (10⁻⁶ M), FSH release was increased by LPQRFa-2 (10⁻⁸ M) [(80), Table 3]. Also, 6-h incubation of Nile tilapia pituitary with pyroglutamatic-LPXRFa-2 (10⁻⁷ and 10⁻⁶ M) increased LH release and pyroglutamatic-LPXRFa-2 (only 10⁻⁶ M) increased FSH release [(81), Table 3].

Effect of goldfish LPXRFa-3 on gonadotropin synthesis and release was tested in dispersed goldfish pituitary cells collected at different gr stages (71). LHβ mRNA level was decreased by LPXRFa-3 (10⁻⁴ and 10⁻⁷ M) at early gr, but increased by LPXRFa-3 (10⁻⁹ M) at mid-gr, and decreased by LPXRFa-3 (10⁻⁸ and 10⁻⁷ M) at late gr. FSHβ mRNA levels was decreased by LPXRFa-3 (10⁻⁸ and 10⁻⁷ M) at early gr, by LPXRFa-3 (10⁻⁹, 10⁻⁸, 10⁻⁷ M) at mid-gr, and by LPXRFa-3 (10⁻⁷ M) at late gr. On the other hand, LH concentration in the media was increased by LPXRFa-3 (10⁻⁴ M) at late gr (Table 3). In dispersed pituitary cells of male sockeye salmon, LH release was increased by goldfish LPXRFa-1, 2 (10⁻⁷ and 10⁻⁵ M), and LPXRFa-3 (10⁻⁸ and 10⁻⁷ M). FSH release was increased by goldfish LPXRFa-1 (10⁻⁹ and 10⁻⁸ M), LPXRFa-2 (10⁻⁷, 10⁻⁸ M), and LPXRFa-3 (10⁻⁷ M) (66, Table 3).

### POSSIBLE MECHANISM OF THE STIMULATORY EFFECTS OF GnIH ON THE HPG AXIS

The mechanism of GnIH (RFRP-3) effect on the electrophysiological activity of GnRH neurons was studied in transgenic mice having vesicular glutamate transporter 2 (vGluT2)-GnRH neurons (47). GnIH and RFRP-3 produced a non-desensitizing hyperpolarization with Iₐ₅₀ values of 34 and 37 nM, respectively, in vGluT2-GnRH neurons via a direct postsynaptic Ba²⁺-sensitive K⁺ current mechanism (Figure 1, Table 2).

It is known that E₂ secreted from the ovary negatively and positively act on the hypothalamus and pituitary to regulate the HPG axis in females. However, it is also known that E₂ is synthesized from androgen by aromatase neurons in the hypothalamus (82). Recent studies have shown that E₂ synthesized in the brain (neurooestrogen) directly and rapidly act on GnRH neurons via membrane estrogen receptor (mER) to regulate GnRH release (83, 84). GPR30 (85, 86), ERβ (87, 88) or other membrane receptors are thought to transduce the rapid effect of E₂ on GnRH...
### TABLE 3: Effect of GnIH on the HPG axis of amphioxus, lamprey, and teleost fishes.

|         | In vitro (cell line or pituitary) or in vivo (animal) | Concentration or dose of peptides | Culture medium, route of administration | Administration time, sample collection, measurement | Effect                                                                 | Reference                      |
|---------|------------------------------------------------------|----------------------------------|----------------------------------------|--------------------------------------------------|------------------------------------------------------------------------|--------------------------------|
| **In vivo** |                                                      |                                  |                                        |                                                   |                                                                        |                                |
| European sea bass | 1, 2, 4 µg sea bass GnIH-1, 2 | icv | 6 h after administration, brain, pituitary, and blood were collected | GnRH1 mRNA level in the brain was decreased by 1, 2, 4 µg GnIH-1. GnRH2 mRNA level in the brain was decreased by 1, 2, 4 µg GnIH-2. Kiss1 mRNA level in the brain was decreased by 2-µg GnIH-2. Kiss2 mRNA level in the brain was decreased by 2, 4 µg GnIH-2. Kiss1 receptor mRNA level in the brain was decreased by 2-µg GnIH-2. GnIH mRNA level in the brain was decreased by 1, 2 µg GnIH-2. GnIH receptor mRNA level in the brain was decreased by 1, 2 µg GnIH-2. LHβ mRNA level in the pituitary was decreased by 1, 2, 4 µg GnIH-2. FSHβ mRNA level in the pituitary was decreased by 2, 4 µg GnIH-2. GnRH receptor IIα mRNA level in the pituitary was decreased by 2, 4 µg GnIH-2. Plasma LH level was decreased by 4-µg GnIH-1 and 1-µg GnIH-2 | Paullada-Salmerón et al. (73) |
| Goldfish | 2-µg goldfish LPXRFa-3 | ip | Injected twice with 12-h interval and pituitaries and blood were collected 12 h after the second injection | LHβ mRNA level was increased at early to mid-late gr. FSHβ mRNA levels was increased at early to mid-late gr. Serum LH concentration was decreased at early to mid-gr | Moussavi et al. (71) |
| Goldfish | 2-µg goldfish LPXRFa-3 | ip | Injected twice with 12-h interval with or without 4-µg sGnRH or cGnRH-II and pituitaries and blood were collected 2 h after the second injection | LHβ mRNA level was increased by LPXRFa-3 at early to mid-gr. FSHβ mRNA levels was increased by LPXRFa-3 at early to mid-gr. Serum LH concentration was decreased by LPXRFa-3 at early to mid-gr. LHβ mRNA level increased by sGnRH was decreased by LPXRFa-3 at early to late gr. LHβ mRNA level increased by cGnRH-II was decreased by LPXRFa-3 at mid to late gr. FSHβ mRNA level increased by sGnRH was decreased by LPXRFa-3 at early to mid-gr. FSHβ mRNA level increased by cGnRH-II was decreased by LPXRFa-3 at mid to late gr. | Moussavi et al. (72) |
| Sexually mature female goldfish | 1-µg/g bw zebrafish LPXRFa-3 | ip | Injected twice with 3-h interval and blood was collected 1 and 3 h after the second injection | Serum LH concentration was decreased by LPXRFa-3 either at 1 and 3 h after the second injection | Zhang et al. (74) |
| Female goldfish at late vitellogenic stage | 100-ng/g bw goldfish LPXRFa-3, 3 | ip | After 12-h administration hypothalamus and pituitary were collected | sGnRH mRNA level in the hypothalamus was decreased by LPXRFa-2, 3. LHβ mRNA level in the pituitary was decreased by LPXRFa-2, 3. FSHβ mRNA level in the pituitary was decreased by LPXRFa-2, 3. | Qi et al. (75) |
| Immature, mature male and female cinnamon clownfish | 100-ng/g bw goldfish LPXRFa-3 | ip | After 0, 6, 12, and 24-h administration with or without 100-ng/g bw sbGnRH brain, pituitary and blood were collected | GnIH and GnIH receptor mRNA levels in the brain were increased at 6, 12 and 24 h. GnIH and GnIH receptor mRNA levels in the brain decreased by sbGnRH were increased at 6, 12 and 24 h. sbGnRH mRNA level in the brain, plasma GnRH, FSH, LH levels, pituitary GTHα, FSHβ, LHβ mRNA levels were decreased at 6, 12 and 24 h. sbGnRH mRNA level in the brain, plasma GnRH, FSH, LH levels, pituitary GTHα, FSHβ, LHβ mRNA levels increased by sbGnRH were decreased at 6, 12 and 24 h. | Choi et al. (76) |
| Female orange-spotted grouper | 100-ng/g bw grouper GnIH-I, II, III | ip | Injected twice with 6-h interval and hypothalamus and pituitary were collected 6 h after the second injection | GnRH1 mRNA level in the hypothalamus was decreased by grouper GnIH-I, II, III. GnRH3 mRNA level in the hypothalamus was increased by grouper GnIH-I, II, III. LHβ mRNA level in the pituitary was decreased by grouper GnIH-II | Wang et al. (77) |
| Lamprey | 50, 100 µg/kg bw lamprey LPXRFa-1a, 1b, 2 | ip | Injected twice with 24-h interval and brain and pituitary were collected 48 h after the second injection | Lamprey GnRH-I, III content in the brain, gonadotropin β mRNA level in the pituitary were increased by 100-µg/kg bw LPXRFa-2 | Osugi et al. (20) |

(Continued)
TABLE 3 | Continued

| In vitro (cell line or pituitary) or in vivo (animal) | Concentration or dose of peptides | Culture medium, route of administration | Administration time, sample collection, measurement | Effect | Reference |
|---------------------------------------------------|----------------------------------|-----------------------------------------|--------------------------------------------------|----------------------------------|-----------|
| European sea bass | 1-µg sea bass GnIH-1, 2/g bw in coconut oil | im | Injected on day 17 from October to January and blood was collected on day 22 from October to January. Brain and pituitary were collected on day 17 of February (spematogenesis stage) | Plasma testosterone and 11-ketotestosterone levels were decreased by sbGnIH-1, 2 in November and December (early and mid-spermatogenesis). GnRH2, sbGnIH, sbGnIH receptor, kiss1 receptor mRNA levels in the brain were increased by sbGnIH-2; LHβ mRNA level in the pituitary was decreased by sbGnIH-1 and -2. Plasma FSH level was decreased by sbGnIH-1. Plasma LH level was decreased by sbGnIH-1 and -2 | Paullada-Salmerón et al. (27) |
| Flatfish | 0.1, 1 µg/g bw flatfish GnIH-2, 3 | im | Injected twice with 12-h interval and brain and pituitary were collected 4 and 8 h after the second injection | GnRH3 mRNA level in the brain was decreased by 1-µg/g bw GnIH-3 at 4 h after administration. LHβ mRNA level in the pituitary was decreased by 0.1, 1 µg/g bw GnIH-3 at 4 h after administration | Aliaga-Guerrero et al. (78) |
| In vitro | | | | | |
| Primary culture of male zebrafish pituitary | 10⁻⁷, 10⁻⁸, 10⁻⁹, 10⁻¹⁰ | Culture media | After 18-h incubation pituitary was collected | Common α mRNA level was decreased by 10⁻¹¹, 10⁻¹², 10⁻¹⁰ M LPXRFa-3. LHβ mRNA level was decreased by 10⁻¹¹, 10⁻¹², 10⁻¹⁰ M LPXRFa-3 | Spicer et al. (60) |
| Primary culture of grass puffer pituitary | 10⁻³, 10⁻⁴ M goldfish LPXRFa-1 | RPMI medium | After 48-h administration pituitaries were collected | LHβ, FSHβ mRNA levels were increased by 10⁻⁷ M LPXRFa-1 | Shahjahan et al. (79) |
| Primary culture of Ochlosoma dimerus pituitary | 10⁻³, 10⁻⁴ M Ochlosoma dimerus LPQRFa-1, -2 | Leibovitz L-15 medium with 10% fetal bovine serum | After 24-h incubation medium was collected | LH and FSH concentration was decreased by 10⁻⁶ M LPQRFa-1. FSH concentration was increased by 10⁻³ M LPQRFa-2 | Di Yorio et al. (80) |
| Primary culture of male Nile tilapia pituitary | 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶ M Pyroglutamic-tilapia LPXRFa-2 | Culture medium | After 6-h incubation medium was collected | LH concentration was increased by 10⁻², 10⁻³ M pyroglutamic-LPXRFa-2. FSH concentration was increased by 10⁻³ M pyroglutamic-LPXRFa-2 | Biran et al. (81) |
| Dispersed goldfish pituitary cells | 10⁻³, 10⁻⁴, 10⁻⁵ M goldfish LPXRFa-3 | Medium 199 with 1% horse serum | After 12-h administration medium and cells were collected | LHβ mRNA level was decreased by 10⁻⁶ and 10⁻⁷ M LPXRFa-3 at early gr. increased by 10⁻² M LPXRFa-3 at mid-gr, decreased by 10⁻⁸ and 10⁻⁹ M LPXRFa-3 at late gr. FSHβ mRNA levels was decreased by 10⁻⁶ and 10⁻⁷ M LPXRFa-3 at early gr, by 10⁻⁶, 10⁻⁴, 10⁻² M LPXRFa-3 at mid-gr, by 10⁻² M LPXRFa-3 at late gr. LH concentration in the media was increased by 10⁻⁶ M LPXRFa-3 at late gr | Moussavi et al. (71) |
| Dispersed female goldfish pituitary cells | 10⁻³ M goldfish LPXRFa-2, 3 | Medium 199 with 10% fetal bovine serum | After 12-h administration with 10⁻¹ M LHRH-A cells were collected | FSHβ mRNA level increased by LHRH-A was decreased by 10⁻² M LPXRFa-3. | Qi et al. (75) |
| Dispersed male sockeye salmon pituitary cells | 10⁻³, 10⁻⁴ M goldfish LPXRFa-1, 2, 3 | MEM | After 2-h administration medium was collected | LH concentration in the media was increased by 10⁻² and 10⁻³ M LPXRFa-1, 2, and 10⁻², 10⁻⁴ M LPXRFa-3, FSH concentration in the media was increased by 10⁻⁸ and 10⁻⁴ M LPXRFa-1, 10⁻², 10⁻³ M LPXRFa-2, and 10⁻⁵ M LPXRFa-3 | Amano et al. (66) |

(Continued)
TABLE 3 | Continued

| Concentration or dose of peptides | Administration time, sample collection, measurement | Effect |
|----------------------------------|-----------------------------------------------------|--------|
| **In vitro** (cell line or animal) | **Culture medium, route of administration** | **Reference** |
| COS-7 cells | DMEM with 10% fetal bovine serum | Forskolin-induced CRE-luciferase activity was decreased by 10−10, 10−9, 10−8, 10−7, 10−6 M grouper GnIH-I, grouper GnIH-II, -III. SRE-luciferase activity was decreased by 10−9, 10−8, 10−7, 10−6 M PQRFa-1, 2, and 1 | Osugi et al. (21) |

Binding of GnRH with GnRH receptor on gonadotropes results in the activation of intracellular Gαq/11 and phospholipases and generation of the second messengers, inositol 1, 4, 5-tris-phosphate, diacylglycerol, and arachidonic acid, which stimulate Ca2+ mobilization and PKC activity. Ca2+ mobilization initiates gonadotropin release (Figure 1). PKC activates mitogen-activated protein kinases (MAPKs) such as ERK, jun-N-terminal kinase, and p38 MAPK, which initiate the transcriptional activity of gonadotropin subunit genes (95). GnRH receptor also couples with Gαs to stimulate AC/cAMP/PKA pathway, which was shown in LβT2 cells (96) and rat gonadotropes (97). Because GnIH signaling pathway triggered by Gαs does not interfere with Gαq/11 triggered pathway, GnIH may suppress gonadotropin subunit gene transcription by inhibiting AC/cAMP/PKA pathway stimulated by GnRH receptor and Gαs (34). GnIH may also suppress gonadotropin release by hyperpolarizing gonadotropes by activating K+ channel via GnIH receptor [47], Figure 1.

However, recent studies of GPCR have shown that GPCR not only functions as a monomer or homodimer but also as a heterodimer with different GPCR resulting in modulation of ligand binding affinity, signal transduction, and internalization of the receptors (98, 99). It has been shown that Class A GPCRs form homo- and heteromers (100). As GnRH and GnIH receptors, and GPR30 all belong to Class A GPCR family (101), it is possible that they form heteromers in GnRH neurons and/or gonadotropes to modify the action of their ligands. Some of the stimulatory effect of GnIH on the HPG axis may be due to heteromerization of GnIH and GnRH receptor and GPR30 (Figure 1).

A recent study has shown that centrally administered GnIH can decrease plasma LH concentration in ovariectomized (OVX) prepubertal female mice that were treated with E2 but not in OVX mice that were not treated with E2 (43) (Table 2). E2 can abolish intracellular free Ca2+ concentration and LH release in ovine pituitary culture induced by GnRH (102). The inhibitory effect of low concentration of E2 on LH release was shown in bovine anterior pituitary mediated by GPR30 expressed on the gonadotrope (103, 104). These results suggest the modification of GnIH action by E2 in the hypothalamus and pituitary (Figure 1).

Finally, it is known for a long time that binding of GnRH with GnRH receptors is followed by aggregation, complex formation and internalization (105). Chronic administration of GnRH or antagonist administration can desensitize pituitary gonadotropes, downregulate GnRH receptor and suppress serum LH,
showed effects of GnIH peptides on gonadal activates (Tables 2 (114) and goldfish testis (118). Therefore, studies that story activity of GnIH peptides was also shown in mouse ovary (115–117) and fishes (118). Most of these studies showed inhibitory effects of GnIH on the HPG axis in an shorter time frame (73–76) (Tables 2 and 3; Figure 1).

Complex mechanism may be involved in in vivo studies that show stimulatory and inhibitory effects of GnIH on the HPG axis in addition to downregulation of receptors and changes in the number of receptors depending on reproductive and developmental stages and endogenous sex-steroid levels (Tables 2 and 3; Figure 1). It is also important to note that GnIH peptides are produced in gonads (3, 109) and it has been shown that they have direct effects on gonadal activates in mammals (110–114), birds (115–117) and fishes (118). Most of these studies showed inhibitory effects of GnIH peptides on gonadal activities, but stimulatory activity of GnIH peptides was also shown in mouse ovary (114) and goldfish testis (118). Therefore, in vivo studies that showed effects of GnIH peptides on gonadal activates (Tables 2 and 3) may include direct effects of GnIH peptides on the gonads.

**CONCLUSION**

Gonadotropin-inhibitory hormone orthologous peptides have a characteristic LPXRFamide C-terminal motif in most vertebrate species, which is critical for receptor binding. The primary receptor for GnIH is GPR147 that inhibits cAMP production in target cells. GnIH generally decreases gonadotropin synthesis and release by directly acting on the gonadotrope or by decreasing the activity of GnRH neurons. However, one study shows stimulatory effects of GnIH on the electrophysiological activity of some GnRH neurons in mice (48). Stimulatory effect of GnIH on GnRH neurons in the hypothalamus may be explained by the action of neuroestrogen synthesized in the hypothalamus by the stimulatory action of GnIH on aromatase neurons that terminate on GnRH neurons that express estrogen membrane receptor. GnIH may further stimulate LH release that was shown in hamsters by stimulating the electrophysiological activity of GnRH neurons and GnRH release (7, 44). Peripheral sex-steroid levels may also modify the action of GnIH (7, 44, 71, 72). Some of the stimulatory effects of GnIH on the HPG axis may be due to heteromerization of GnIH and GnRH receptors and GPR30 in GnRH neurons and/or gonadotropes, which modifies ligand binding and signaling transduction mechanism. Stimulatory effect of GnIH on the HPG axis may also be due to internalization of GnIH receptor by high concentration or chronic administration of GnIH or antagonistic effect of the peptides administered (20, 66, 77, 79–81). Besides pharmacological effect of administered peptides, the general inhibitory action of GnIH by decreasing cAMP concentration and inducing hyperpolarization in target cells and the additional stimulatory action of GnIH by neuroestrogen synthesis, receptor heteromerization, and internalization may have a physiological role to maintain reproductive homeostasis according to developmental and reproductive stages.

**AUTHOR CONTRIBUTIONS**

TU wrote the manuscript and IP edited the manuscript.
14. Satake H, Hisada M, Kawada T, Minakata H, Ukena K, Tsutsui K. Characterization of a cDNA encoding a novel avian hypothalamic neuropeptide exerting an inhibitory effect on gonadotropin release. Biochem J (2001) 354:379–85. doi:10.1042/bj3540379

15. Ukena K, Iwakoshi-Ukena E, Osugi T, Tsutsui K. Identification and localization of gonadotropin-inhibitory hormone (GnIH) orthologs in the hypothalamus of the red-eared slider turtle, Trachemys scripta elegans. Gen Comp Endocrinol (2016) 227:69–76. doi:10.1016/j.ygcen.2015.06.009

16. Chartrel N, Dujardin C, Leprince J, Desrues L, Tonon MC, Cellier E, et al. Isolation, characterization, and distribution of a novel neuropeptide, Rana Rfamide (R-Rfα), in the brain of the European green frog Rana esculenta. J Comp Neurol (2002) 448:111–27. doi:10.1002/cne.10253

17. Ukena K, Kodá A, Yamamoto K, Kobayashi T, Iwakoshi-Ukena E, Minakata H, et al. Novel neuropeptides related to frog growth hormone-releasing peptide: isolation, sequence, and functional analysis. Endocrinology (2003) 144:3879–84. doi:10.1210/en.2003-0359

18. Chowdhury VS, Ubuka T, Osugi T, Shimura T, Tsutsui K. Identification, localization and expression of LPXRFamide peptides, and melatonin-dependent induction of their precursor mRNA in the newt brain. J Endocrinol (2011) 209:211–20. doi:10.1530/JEO-10-0494

19. Sawada K, Ukena K, Satake H, Iwakoshi E, Minakata H, Tsutsui K. Novel fish hypothalamic neuropeptide. Eur J Biochem (2002) 269:6000–8. doi:10.1046/j.1432-1033.2002.03351.x

20. Akisato K, Usuki A, Ueno M, Kato K, Usuki A, Kusugi T, Nosaki M, et al. Evolutionary origin of the structure and function of gonadotropin-inhibitory hormone: insights from lampreys. Endocrinology (2012) 153:2362–74. doi:10.1210/en.2011-2046

21. Osugi T, Okamura T, Son YL, Ohkubo M, Ubuka T, Henni Y, et al. Evolutionary origin of GnIH and NPF in chordates: insights from novel amphioxus Rfamide peptides. PLoS One (2014) 9:e100962. doi:10.1371/journal.pone.0100962

22. Yin H, Ukena K, Ubuka T, Tsutsui K. A novel G protein-coupled receptor for gonadotropin-inhibitory hormone in the Japanese quail (Coturnix japonica): identification, expression and binding activity. Endocrinology (2005) 146:257–66. doi:10.1210/jo.1.5.09126

23. Thorson JF, Rezzotto LD, Cardoso RC, Sharpont SM, Edwards JF, Welsh TH Jr, et al. Hypothalamic distribution, adenosinophasyopeptide receptor expression, and ligand functionality of Rfamide-related peptide 3 in the mare during the breeding and nonbreeding seasons. Biol Reprod (2014) 90:28. doi:10.1095/biolreprod.113.112185

24. Ikemoto T, Park MK. Chicken RFamide-related peptide (GnIH) and two evolutionary considerations. J Endocrinol (2016) 209:395–402. doi:10.1530/JOE-16-0197

25. Paullada-Salmerón JA, Cowan M, Aliaga-Guerrero M, López-Olmeda JF, de la Hoz AM, et al. Characterization of the inhibitory roles of RFRP3, the mammalian ortholog of GnIH, in the control of gonadotropin secretion in female rats. J Endocrinol (2005) 184:159–66. doi:10.1677/joe.1.03942

26. Pineda R, Garcia-Galiano D, Sanchez-Garrido MA, Romero M, Ruiz-Pino F, Aguilar E, et al. Characterization of the inhibitory roles of RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. Horm Behav (2007) 51:171–80. doi:10.1016/j.hormbeh.2006.09.009

27. Smith JT, Young JR, Veldhuis JD, Clarke IJ. Characterization of a marine GnIH ortholog in the control of gonadotropin secretion in the eel. Horm Behav (2010) 60:29–38. doi:10.1016/j.yhbeh.2009.06.001

28. Kadokawa H, Shibata M, Tanaka Y, Kojima T, Matsumoto K, Oshima K, et al. Characterization of the inhibitory roles of RFRP3, the mammalian ortholog of GnIH, in the control of gonadotropin secretion in the rat: in vivo and in vitro studies. Am J Physiol Endocrinol Metab (2010) 299:E39–46. doi:10.1152/ajpendo.00108.2010

29. Johnson MA, Tsutsui K, Fraley GS. Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. Horm Behav (2007) 51:171–80. doi:10.1016/j.hormbeh.2006.09.009

30. Murakami M, Matsuzaki T, Iwasa T, Yassu T, Irahara M, Osugi T, et al. Hypophysiotropic role of RFamide-related peptide-3 in the inhibition of LH secretion in female rats. J Endocrinol (2008) 199:105–12. doi:10.1677/JOE-08-0197

31. Liu Q, Guan XM, Martin WJ, McDonald TP, Clements MK, Jiang Q, et al. Identification and characterization of novel mammalian neuropeptide FF-like peptides that attenuate morphine-induced antinociception. J Biol Chem (2001) 276:36961–9. doi:10.1074/jbc.M015030200

32. Ubuka T, Tsutsui K. Evolution of gonadotropin-inhibitory hormone receptor and its ligand. Gen Comp Endocrinol (2014) 209:148–61. doi:10.1016/j.ygcen.2014.09.002

33. Shimizu M, Bédéccarrats GY. Activation of the chicken gonadotropin-inhibitory hormone receptor reduces gonadotropin releasing hormone receptor signaling. Gen Comp Endocrinol (2010) 167:331–7. doi:10.1016/j.ygcen.2010.03.029

34. Son YL, Ubuka T, Millar RP, Kanasaki H, Tsutsui K. Gonadotropin-inhibitory hormone inhibits GnRH-induced gonadotropin subunit gene transcriptions by inhibiting AC/cAMP/PKA-dependent ERK pathway in LPT2 cells. Endocrinology (2012) 153:2323–43. doi:10.1210/endo.2011-1904

35. George JT, Hendriks M, Veldhuis JD, Clarke IJ, Anderson RA, Millar RP. Effect of gonadotropin-inhibitory hormone on luteinizing hormone secretion in humans. Clin Endocrinol (2017) 86:731–8. doi:10.1111/cen.13308

36. Clarke IJ, Smith JT, Henry BA, Oldfield BJ, Stefanidis A, Millar RP, et al. Gonadotropin-inhibitory hormone is a hypothalamic peptide that provides a molecular switch between reproduction and feeding. Neuroendocrinology (2012) 95:305–16. doi:10.1159/000332822

37. Smith JT, Young JR, Veldhuis JD, Clarke IJ. Gonadotropin-inhibitory hormone inhibits LH secretion in the ovine hypothalamic portal system. Endocrinology (2012) 153:3368–75. doi:10.1210/endo.2012-1088

38. Kadokawa H, Shibata M, Tanaka Y, Kojima T, Matsumoto K, Oshima K, et al. Characterization of the inhibitory roles of RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. Horm Behav (2007) 51:171–80. doi:10.1016/j.hormbeh.2006.09.009

39. Johnson MA, Tsutsui K, Fraley GS. Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. Horm Behav (2007) 51:171–80. doi:10.1016/j.hormbeh.2006.09.009

40. Murakami M, Matsuzaki T, Iwasa T, Yassu T, Irahara M, Osugi T, et al. Hypophysiotropic role of RFamide-related peptide-3 in the inhibition of LH secretion in female rats. J Endocrinol (2008) 199:105–12. doi:10.1677/JOE-08-0197

41. Ancel C, Bentsen AH, Sibert ME, Tena-Sempere M, Mikkelsen JD. Dynamic switch of LH secretion in bovines. J Biol Chem (2012) 287:15368–75. doi:10.1074/jbc.M012536200

42. Kriegsfeld LJ, Mei DF, Bentley GE, Ubuka T, Mason AO, Inoue K, et al. Characterization of a mammalian gonadotropin-inhibitory hormone ortholog, regulates a mammalian gonadotropin-inhibitory hormone ortholog, regulates
gonadotropin-releasing hormone neuron firing in the mouse. Endocrinology (2009) 150:2799–804. doi:10.1210/en.2008-1623
49. Gojska NM, Friedman Z, Belsham DD. Direct regulation of gonadotrophin-releasing hormone (GnRH) transcription by RF-amide-related peptide-3 and kisspeptin in a novel GnRH-secreting cell line. mHypoA-GnRH/GFP. J Neuroendocrinol (2014) 26:888–97. doi:10.1111/jene.12122
50. Clarke IJ, Sari IP, Rao A, Smith JT, Tilbrook AJ, Clarke IJ. Effect of RF-amide-related peptide-3 on luteinizing hormone and follicle-stimulating hormone synthesis and secretion in ovine pituitary gonadotropes. Endocrinology (2009) 150:5549–56. doi:10.1210/en.2009-0775
51. Qi Y, Oldfield BJ, Clarke IJ. Projections of RFamide-related peptide-3 receptor gene expression in GnRH and kisspeptin neurons and GnRH-dependent mechanism of action. Endocrinology (2013) 154:1944–55. doi:10.1210/en.2013-1786
52. Bentley GE, Perfiso N, Ukema K, Tsutsui K, Wingfield JC. Gonadotropin-inhibitory peptide in song sparrows (Melospiza melodia) in different reproductive conditions, and in house sparrows (Passer domesticus) relative to chicken-gonadotropin-releasing hormone. J Neuroendocrinol (2003) 15:794–802. doi:10.1046/j.1365-2826.2003.01062.x
53. Ubuka T, Bentley GE. Identification, localization, and regulation of passerine GnRH-I messenger RNA. J Neuroendocrinol (2009) 21:81–7. doi:10.1111/j.1365-2826.2008.01001.x
54. Ubuka T, Bentley GE. Neuroendocrine control of reproduction in birds. In: Norris DO, Lopez KH, editors. Hormones and Reproduction of Vertebrates, Vol. 4. Birds. Academic Press (2010). p. 1–25.
55. Ubuka T, Cadigan PA, Wang A, Liu J, Bentley GE. Identification of European starling GnRH-I precursor mRNA and its seasonal regulation. Gen Comp Endocrinol (2009) 162:301–6. doi:10.1016/j.ygcen.2009.04.001
56. Qi Y, Oldfield BJ, Clarke IJ. Projections of RFamide-related peptide-3 neurons in the ovine hypothalamus, with special reference to regions regulating energy balance and reproduction. J Neuroendocrinol (2009) 21:690–7. doi:10.1111/j.1365-2826.2009.01886.x
57. Soga T, Kitahashi T, Clarke IJ, Parhar IS. Gonadotropin-inhibitory hormone promoter-driven enhanced green fluorescent protein expression decreases during aging in female rats. Endocrinology (2014) 155:1944–55. doi:10.1210/endo-2013-1786
58. Rizwan MZ, Poling MC, Corr M, Cornes PA, Augustine RA, Quennell JH, et al. RFamide-related peptide-3 receptor gene expression in GnRH and kisspeptin neurons and GnRH-dependent mechanism of action. Endocrinology (2012) 153:3770–9. doi:10.1210/endo.2012-1133
59. Pinelli C, Jadhao AG, Biswas SP, Tsutsui K, D’Aniello B. Neuroanatomical organization of the brain gonadotropin-inhibitory hormone and gonadotropin-releasing hormone systems in the frog Pelophylax esculentus (capito). Brain Behav Evol (2015) 85:15–28. doi:10.1159/000363894
60. Spicer OS, Zmora N, Wong TT, Golan M, Levavi-Sivan B, Gothilf Y, et al. The gonadotropin-inhibitory hormone (Lpxrfa) system’s regulation of reproductive energy balance and reproduction-related genes in the protandrous cinnamon clownfish, Amphiprion melanopus. Gen Comp Endocrinol (2016) 235:89–99. doi:10.1016/j.ygcend.2016.06.010
61. Wang Q, Qi X, Guo Y, Li S, Zhang Y, Liu X, et al. Molecular identification of GnIH/GnIHR signal and its reproductive function in protogynous hermaphroditic orange-spotted grouper (Epinephelus coioides). J Comp Neurol (2015) 524:2753–75. doi:10.1002/cne.23990
62. Shahjahan M, Ikegami T, Osugi T, Ukena K, Doi H, Hattori A, et al. Gonadotrophin-inhibitory hormone in the cichlid fish cichlasoma dimerus: structure, brain distribution and differential effects on the reproduction of gonadotropins and growth hormone. J Neuroendocrinol (2016) 28:12377. doi:10.1111/jne.12377
63. Choi YJ, Kim NN, Habibi HR, Choi CY. Effects of gonadotropin inhibitory hormone or gonadotropin-releasing hormone on reproduction-related genes in the platyfish, S. spleus. Fish Physiol Biochem (2017) 52(6):349–70. doi:10.1007/s10695-016-0433-9
64. Shahjahan M, Ikemoto T, Tsutsui K, Doi H, Hattori A. Somatic and neuroendocrine mechanisms of gonadotropin-inhibitory hormone and its reproductive function in protogynous hermaphroditic orange-spotted grouper. Epinephelus coioides. Gen Comp Endocrinol (2015) 216:9–23. doi:10.1016/j.ygcen.2015.04.016
65. Osaka N, Sato M, Ishihara A, Sato M, Ishihara A, et al. Oral administration of RFamide-related peptide-1/3, the mammalian gonadotropin-inhibitory hormone or gonadotropin-releasing hormone neuron firing in the mouse. J Neuroendocrinol (2011) 23:39–51. doi:10.1111/j.1365-2826.2010.02081.x
66. Amano M, Moriyama S, Igo M, Kitamura S, Amiya N, Yamamori K, et al. Novel fish hypothalamic neuropeptides stimulate the release of gonadotrophins and growth hormone from the pituitary of sockeye salmon. J Endocrinol (2005) 186:417–23. doi:10.1677/joe.1.04794
67. Biswas S, Jadhao AG, Pinelli C, Palande NV, Tsutsui K. GnIH and GnRH expressions in the central nervous system and pituitary of Indian major carp, Labeo rohita during ontogeny: an immunocytochemical study. Gen Comp Endocrinol (2015) 220:88–92. doi:10.1016/j.ygcen.2014.06.005
68. Paulallada-Salmerón JA, Cowan M, Aliaga-Guerrero M, Gómez A, Zanuy S, Mañas E, et al. LPXRFa peptide system in the European sea bass: a molecular and immunohistochemical approach. J Comp Neurol (2016) 524:176–98. doi:10.1002/cne.23833
69. Ojewole JO, Ologunajo AO, Aboderin JI, Orisakwe MO, Akinfajriyeh OA, Akinpelu MO, et al. Dual Actions of GnIH Peptides in the Piscine Ortholog of GnIH, and LPXRFa 3. J Front Endocrinol (2012) 3:136. doi:10.3389/fendo.2012.00036
70. Moussavi M, Wlaschuk M, Chang JP, Habibi HR. Seasonal effect of GnIH on gonadotrotope functions in the pituitary of goldfish. Mol Cell Endocrinol (2012) 356:53–60. doi:10.1016/j.mce.2011.11.020
71. Moussavi M, Wlaschuk M, Chang JP, Habibi HR. Seasonal effect of gonadotropin inhibitory hormone on gonadotrophin-releasing hormone-induced gonadotroph functions in the goldfish pituitary. J Neuroendocrinol (2013) 25:506–16. doi:10.1111/j.1600-0416.2012.12024
72. Shahjahan M, Ikegami T, Osugi T, Zanuy S, Mañas E, et al. Somatic and neuroendocrine mechanisms of gonadotropin-inhibitory hormone and its reproductive function in protogynous hermaphroditic orange-spotted grouper (Epinephelus coioides). J Comp Neurol (2015) 524:2753–75. doi:10.1002/cne.23990
73. Ubuka T, Bentley GE. RFamide-related peptide-3 receptor gene expression in GnRH and kisspeptin neurons. Front Endocrinol (2012) 3:136. doi:10.3389/fendo.2012.00081

Ubuka T, Haraguchi S, Tobari Y, Narihiro M, Ishikawa K, Hayashi T, et al. Rapid action of estrogens on intracellular calcium oscillations in primate luteinizing hormone-releasing hormone-1 neurons. Endocrinology (2008) 149:1155–62. doi:10.1210/en.2007-0942

Abe H, Keen KL. Estradiol stimulates cAMP-mediated attenuation of the epidermal growth factor receptor-MAPK signaling axis. Mol Endocrinol (2002) 16:70–84. doi:10.1210/mend.16.1.7058

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A (1996) 93:5925–30. doi:10.1073/pnas.93.12.5925

Filardo EJ, Quinn JA, Bland KI, Frackelton AR Jr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol (2000) 14:1649–60. doi:10.1210/mend.14.10.0532

Kenealy BP, Kapoor A, Guerriero KA, Keen KL, Garcia JP, Kurian JR, et al. Neuroestriol in the hypothalamus contributes to the regulation of gonadotropin-releasing hormone release. J Neurosci (2013) 33:19051–9. doi:10.1523/JNEUROSCI.3787-13.2013

Ubuka T, Parhar

103. Rudolf FO, Kadokawa H. Expression of estradiol receptor, GPR30, in bovine anterior pituitary and effects of GPR30 agonist on GnRH-induced LH secretion. Anim Reprod Sci (2013) 139(1–4):9–17. doi:10.1016/j. aniprodu.2013.04.003

104. Rudolf FO, Kadokawa H. Cytoplasmic kinases downstream of GPR30 suppress gonadotropin-releasing hormone (GnRH)-induced luteinizing hormone secretion from bovine anterior pituitary cells. J Reprod Dev (2016) 62:65–9. doi:10.1262/jrd.2015-1014

105. Conn PM, Staley D, Harris C, Andrews WV, Gorospe WC, McArdle CA, et al. Mechanism of action of gonadotropin releasing hormone. Annu Rev Physiol (1986) 48:495–513. doi:10.1146/annurev.ph.48.030186.002431

106. Katt JA, Duncan JA, Herbon L, Barkan A, Marshall JC. The frequency of gonadotropin-releasing hormone stimulation determines the number of pituitary gonadotropin-releasing hormone receptors. Endocrinology (1985) 116:2113–5. doi:10.1210/endo-116-5-2113

107. Conn PM, Crowley WF Jr. Gonadotropin-releasing hormone and its analogues. N Engl J Med (1991) 324:393–100. doi:10.1056/NEJM199101103240320

108. Halmsch, Schally AV. Changes in subcellular distribution of pituitary receptors for luteinizing hormone-releasing hormone (LH-RH) after treatment with the LH-RH antagonist cetrorelix. Proc Natl Acad Sci U S A (2002) 99:961–5. doi:10.1073/pnas.012598399

109. McGuire NL, Bentley GE, Ubuka T, McGuire NL, Chowdhury VS, Morita Y, Yano T, et al. Gonadotropin-inhibitory hormone and its receptor in the avian reproductive system. Gen Comp Endocrinol (2008) 156:34–43. doi:10.1016/j.ygcend.2007.10.003

110. Singh P, Krishna A, Tsutsui K. Effects of gonadotropin-inhibitory hormone on folliculogenesis and steroidalgenesis of cichlid mice. Fertil Steril (2011) 95:1395–404. doi:10.1016/j.fertnstert.2010.03.052

111. Oishi H, Klaussen C, Bentley GE, Osugi T, Tsutsui K, Gilks CB, et al. The human gonadotropin-inhibitory hormone ortholog RFamide-related peptide-3 suppresses gonadotropin-induced progesterone production in human granulosa cells. Endocrinology (2012) 153:3435–45. doi:10.1210/en.2012-1086

112. Anjum S, Krishna A, Tsutsui K. Inhibitory roles of the mammalian GnIH ortholog RFRP3 in testicular activities in adult mice. J Endocrinol (2014) 223:79–91. doi:10.1530/JEO-14-0333

113. Zheng L, Su J, Fang R, Jin M, Lei Z, Hou Y, et al. Developmental changes in the role of gonadotropin-inhibitory hormone (GnIH) and its receptors in the reproductive axis of male Xiaomeishan pigs. Anim Reprod Sci (2015) 154:113–20. doi:10.1016/j.aniprodu.2015.01.004

114. Dave A, Krishna A, Tsutsui K. Direct effects of RFRP-1, a mammalian GnIH ortholog, on ovarian activities of the cyclic mouse. Gen Comp Endocrinol (2017) 252:193–9. doi:10.1016/j.ygcend.2017.06.024

115. Maddineni SR, Ocon-Grove OM, Krysak-Walker SM, Hendrickx GI, III, Ramachandran R. Gonadotropin-inhibitory hormone (GnIH) receptor gene is expressed in the chicken ovary: potential role of GnIH in follicular maturation. Reproduction (2008) 135:267–74. doi:10.1530/REP-07-0369

116. McGuire NL, Bentley GE. A functional neuropeptide system in vertebrate gonads: gonadotropin-inhibitory hormone and its receptor in testes of field-caught house sparrow (Passer domesticus). Gen Comp Endocrinol (2010) 166:565–72. doi:10.1016/j.ygcend.2010.01.010

117. McGuire NL, Kangas K, Bentley GE. Effects of melatonin on peripheral reproductive function: regulation of testicular GnIH and testosterone. Endocrinology (2011) 152:3461–70. doi:10.1210/en.2011-1053

118. Qi X, Zhou W, Lu D, Wang Q, Zhang H, Li S, et al. Sexual dimorphism of steroidalogenesis regulated by GnIH in the goldfish, Carassius auratus. Biol Reprod (2013) 88:89. doi:10.1093/biolre/btt15114

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.