Seasonal effects of GnIH on basal and GnRH-induced goldfish somatotrope functions

M Moussavi1, M Wlasichuk1,2, J P Chang1,2 and H R Habibi3

1Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta, Canada T2N 1N4
2Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

Abstract

To understand how gonadotropin-inhibitory hormone (GnIH) regulates goldfish GH cell functions, we monitored GH release and expression during early, mid-, and/or late gonadal recrudescence. In vivo and in vitro responses to goldfish (g) GnIH were different, indicating direct action at the level of pituitary, as well as interactions with other neuroendocrine factors involved in GH regulation. Injection of gGnIH consistently reduced basal serum GH levels but elevated pituitary gh mRNA levels, indicating potential dissociation of GH release and synthesis. Goldfish GnRH (sGnRH and cGnRHII) injection differentially stimulated serum GH and pituitary gh mRNA levels with some seasonal differences; these responses were reduced by gGnIH. In contrast, in vitro application of gGnIH during 24-h static incubation of goldfish pituitary cells generally elevated basal GH release and attenuated sGnRH-induced changes in gh mRNA, while suppressing basal gh mRNA levels at mid- and late recrudescence but elevating them at early recrudescence. gGnIH attenuated the GH release responses to sGnRH during static incubation at early, but not at mid- and late recrudescence. In cell column perifusion experiments examining short-term GH release, gGnIH reduced the cGnRHII- and sGnRH-stimulated secretion at late recrudescence but inhibited the action of cGnRHII only during mid-recrudescence. Interestingly, a reduction of basal GH release upon perifusion with gGnIH during late recrudescence was followed by a rebound increase in GH release upon gGnIH removal. These results indicate that gGnIH exerts complex effects on basal and GnRH-stimulated goldfish GH cell functions and can differentially affect GH release and mRNA expression in a seasonal reproductive manner.

Key Words
- gonadotropin-inhibitory hormone and RF amides
- somatotropin production and secretion
- serum GH
- seasonality

Introduction

Growth hormone (GH) secretion is regulated by two major hypothalamic neuropeptides, GH-releasing hormone (GHRH) and somatostatin 14 (SS14) (Tannenbaum et al. 2003, Gahete et al. 2009). In teleosts, however, a unique component of the multifactorial control of somatotropes is gonadotropin-releasing hormone (GnRH), which directly stimulates GH secretion and mRNA expression both in vivo and in vitro (Canosa et al. 2007, Chang & Wong 2009, Chang et al. 2012). Multiple forms of GnRH are present in the brain of single species from teleosts to mammals, although rats, mice, and chimpanzees only possess a single isoform of GnRH (luteinizing hormone-releasing hormone (LHRH) or GNRH1; Morgan et al. 2006, Roch et al. 2011). Goldfish possess two isoforms of GnRH,
chicken (c) GnRHII (or GnRH2) and salmon (s) GnRH (or GnRH3) (Yu et al. 1988). Furthermore, these GnRH neurons directly innervate the pituitary as teleosts lack a hypothalamo-hypophyseal portal system (Ball 1981).

The discovery of a novel hypothalamic RFamide, named gonadotropin-inhibitory hormone (GnIH), added another player to the multifactorial control of the pituitary, and GnIH peptides have now been identified in most vertebrates including teleosts, amphibians, avians, and mammals (Tsutsui et al. 2012, Tsutsui & Ubuka 2014). The majority of research on GnIH has been directed toward its effects on gonadotropes and its inhibition of the reproductive axis at the level of the hypothalamus and pituitary (Clarke et al. 2008, Tsutsui et al. 2010). Based mainly on evidence from studies on mammals and birds, the inhibitory influence of GnIH on reproduction is manifested, in part, through modulation of GnRH neuronal firing, GnRH-induced gonadotropin release, and inhibition of gonadotropin gene expression (Smith & Clarke 2010, Ubuka et al. 2012). Immunoreactive GnIH has also been detected in the pituitary of Indian major carp (Biswa et al. 2014); however, recent work on teleosts has revealed that GnIH control of gonadotrope activities is complex and not always inhibitory. Goldfish (g) GnIH (LPXRFaxime 3) exerts both stimulatory and inhibitory influences on basal goldfish gonadotrope functions in a season-dependent manner, as well as differentially modulating the effects of sGnRH- and cGnRHIII-induced gonadotrope functions (Moussavi et al. 2012, 2013). On the other hand, zebrafish GnIH reduced serum LH levels in adult goldfish (Zhang et al. 2010), whereas gGnIH elevated LH and FSH release from salmon pituitary cell cultures (Amano et al. 2006).

In addition to modulating the reproductive axis, GnIH orthologs affect growth and metabolism by actions on hypothalamic areas regulating food intake and by modulation of GH secretion (Clarke 2014); however, information currently available is limited and the physiological role of GnIH in GH release remains yet to be firmly established. In frogs, a GnIH ortholog (termed frog growth hormone-releasing peptide (fGRP)) stimulates GH release in vitro and in vivo (Koda et al. 2002, Ukena et al. 2003). Similarly, the rat GnIH ortholog RFRP3 increases the levels of GH in plasma (Johnson et al. 2007). In teleosts, gGnIH and the other two putative RFamide peptides stimulate the release of GH from cultured pituitary cells from male sockeye salmon (Amano et al. 2006).

On the basis of the limited results from other vertebrates, GnIH may be involved in the multifactorial control of GH expression and secretion in goldfish. However, the effect of GnIH and its relationship with the actions of GnRH on somatotrope functions have not been investigated previously in teleosts and are the main focus of this study.

Materials and methods

Injection studies and primary static pituitary cell cultures

Animals Male and female goldfish (Carassius auratus; ~23 g and 7–12 cm) were purchased from Aquatic Imports (Calgary, AB, Canada). Goldfish acclimatization protocol, water temperature, diet, gonadal somatic index, and handling have been described previously by our group (Moussavi et al. 2012, 2013). All protocols involving animals were approved by the appropriate university animal care committees in accordance with the principles and guidelines of the Canadian Council on Animal Care. Pituitaries from both males and females were collected and analysis was performed on mixed-sex samples.

Hormones and injection protocol Synthetic gGnIH peptide (SGTLSATLPQRF-NH2) was synthesized by the University of Calgary’s Peptide Services (Calgary, AB, Canada). sGnRH and cGnRHII were purchased from American Peptide (Sunnyvale, CA, USA). Stock solutions of gGnIH or GnRH were made up in PBS (1 × PBS) and stored in aliquots at −20 °C until use. Fish were anesthetized in tricaine methanesulfonate (MS-222, Sigma–Aldrich; 200 mg/l) for all injections, as well as before tissue collection. For in vivo studies, control goldfish received sham injections of 1 × PBS (0 μg). Individual goldfish each received two injections according to the established procedure (Chang & Peter 1983), initially at 0-h and later at 12-h as described previously (Moussavi et al. 2012, 2013). At 2-h post-second-injection, blood was collected and serum samples were stored at −20 °C for later GH RIA, as described previously (Marchant et al. 1987). Fish were killed immediately afterwards and pituitaries collected for RNA extraction.

Primary pituitary cell static culture The cell culture protocol and methodology were consistent with those used in our previous investigations (Moussavi et al. 2012, 2013). Cells in static incubation cultures were treated continuously with stimuli for 12-h. At the end of incubation, medium samples were collected and frozen at −20 °C for RIA, and cells were used for RNA extraction. Each experiment was repeated twice (separate cell preparations) with four replicate wells per cell preparation.

Research GnIH affects GH expression and release M MOUSSAVI and others 223:2 192
Pituitary RNA extraction and quantitative real-time PCR  Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer’s protocol as described previously (Moussavi et al. 2012, 2013). Owing to the low RNA concentrations obtained from a single pituitary, two to three individual pituitaries were pooled for RNA extraction and subsequently used for quantitative real-time PCR (qPCR), using β-actin as an internal control. The qPCR amplification was conducted as described previously (Moussavi et al. 2012, 2013) using two sets of forward and reverse primers simultaneously to amplify both GHI and GHII (Mahmoud et al. 1998), using an annealing temperature of 60 °C. The sequences for the primers are as follows: gh forward 1 (GTCTCAACAGCCTGACCGTCG), gh forward 2 (GTCTCAACAGCCTGACCGCCG), gh reverse 1 (CAGCGGAGGGAGTCGTTATCATC), and gh reverse 2 (CAGTGGTAGGGAGTCGTTATCATC). The amplified product included both GHI and GHII. Statistical analyses were performed using one-way ANOVA on log-transformed data, followed by post-hoc Tukey’s test with the level of significance set at P<0.05 (Prism Statistical Software; GraphPad Software, Inc., La Jolla, CA, USA).

Pituitary cell column perfusion experiments  Dispersed goldfish pituitary cells were prepared as described in ‘Pituitary RNA extraction and qPCR’ section and cultured on pre-swollen Cytodex-I beads (Sigma). The perfusion protocol followed was as described previously (Moussavi et al. 2013). Briefly, in the first experimental paradigm, using fish at mid gonadal recrudescence (December through February), a 1-h 100 nM GnRH treatment was applied starting at 30 min. Then, 10 nM gGnIH treatment was applied as a 5-min pulse at 55-min (i.e. 25-min into the GnRH application). For combination treatments, gGnIH solution was made up in 100 nM GnRH. A second, reverse combination treatment paradigm, where a short 5-min GnRH pulse was applied for 25-min into a 1-h gGnIH treatment, was tested using cells obtained from fish at the late recrudescence/sexually matured stage (May–early June). Perifusate fractions were stored at −20 °C for RIA. The GH values were calculated and analyzed as described previously (Sawisky & Chang 2005, Moussavi et al. 2013). All treatments were replicated in four separate experiments using different cell preparations. Pooled results are expressed as mean ± S.E.M. Statistical analyses were performed using ANOVA followed by post-hoc Tukey’s test with the level of significance set at P<0.05.

Results  Seasonal effects of gGnIH on basal and GnRH-induced pituitary gh mRNA levels and circulating GH levels in goldfish in vivo  As an initial step toward understanding the role of gGnIH in the control of somatotroph functions, we examined the effects of i.p. injections of gGnIH, alone and in combination with sGnRH or cGnRHII, on circulating serum GH levels, as well as gh mRNA levels in the pituitary. Using goldfish at early gonadal recrudescence (October), injection of gGnIH alone significantly reduced serum GH levels, yet significantly stimulated gh mRNA expression (Fig. 1), indicating an uncoupling of GH release and expression. Injection of either sGnRH or cGnRHII alone significantly increased serum GH and gh mRNA levels. However, gGnIH injected in combination with either sGnRH or cGnRHII significantly suppressed the GnRH-induced GH levels and returned serum GH to basal levels observed in the controls that had received injections of vehicle (Fig. 1). Paradoxically, injection of gGnIH in combination with either GnRH resulted in gh mRNA levels that were significantly lower than those for fish treated with either gGnIH alone or GnRH alone (Fig. 1).

During mid-gonadal recrudescence (December), injection of gGnIH alone caused a significant decrease in serum GH levels, but significantly stimulated pituitary gh mRNA levels as observed during early gonadal recrudescence (Fig. 2). Injection of either sGnRH or cGnRHII alone did not significantly alter the serum GH levels; however, injection of cGnRHII alone, but not sGnRH alone, significantly stimulated pituitary gh mRNA expression (Fig. 2). Injection of gGnIH together with sGnRH also had no effect on either serum GH or gh mRNA levels (Fig. 2). Co-injection of gGnIH with cGnRHII did not prevent the significant gGnIH-induced inhibition of serum GH, yet prevented the significant cGnRHII-induced stimulation of gh mRNA, bringing expression levels back to basal (Fig. 2), again demonstrating differences between GnIH-mediated control of secretion and mRNA expression in the goldfish pituitary.

At late recrudescence (March), injections of gGnIH alone significantly reduced serum GH levels, but significantly stimulated gh mRNA expression (Fig. 3). At this stage of recrudescence, injection of either of the two native GnRHs alone significantly stimulated serum GH levels, as well as pituitary gh mRNA expression relative to controls that had received injections of vehicle (Fig. 3). In contrast, co-injection of gGnIH with sGnRH significantly inhibited serum GH levels and resulted in gh mRNA expression that was not significantly different from the expression for
In vivo: early recrudescence

The effect of gGnIH on serum GH (upper panel) and pituitary gh mRNA levels (lower panel) during early recrudescence (October) in vivo. Goldfish received two injections of 2 µg gGnIH, at time 0 and 12 h; fish that had received injections of PBS served as controls. Pituitaries and blood samples for serum collection were removed at 2 h following the second injection. Serum GH values were analyzed by RIA (mean ± S.E.M.; n = 12). Abundance of pituitary transcripts was determined by qPCR. gh mRNA values were normalized against β-actin mRNA and expressed relative to those for controls (mean ± S.E.M.; controls, n = 6); control groups were normalized to 100%. Different letters denote statistically significant differences (ANOVA followed by Tukey’s test, P < 0.05). Tissues that did not resemble the appropriate gonadal stage were not used.

In vivo: mid-recrudescence

The effect of gGnIH on serum GH (upper panel) and pituitary gh mRNA levels during mid-recrudescence (December) in vivo. Goldfish received two injections of 2 µg gGnIH, at time 0 and 12 h; fish that had received injections of PBS served as controls. Pituitaries and blood samples for serum collection were removed at 2 h following the second injection. Serum GH values were analyzed by RIA (mean ± S.E.M.; n = 12). Abundance of pituitary transcripts was determined by qPCR. gh mRNA values were normalized against β-actin mRNA and expressed relative to those for controls (mean ± S.E.M.; controls, n = 6); control groups were normalized to 100%. Different letters denote statistically significant differences (ANOVA followed by Tukey’s test, P < 0.05). Tissues that did not resemble the appropriate gonadal stage were not used.

Effects of gGnIH alone, sGnRH alone, and in combination on pituitary GH levels and Gh mRNA levels in static primary pituitary cell cultures in vitro

The effects of gGnIH in vivo potentially involve a combination of both direct effects on the pituitary, as well as indirect effects through actions at multiple points along the hypothalamic–pituitary hormone target axis regulating somatotrope functions and responsiveness. To gain insight into the direct effects of gGnIH on GH release and production at the level of the pituitary, we investigated the dose-related effects of GnIH alone and in combination with sGnRH using static primary goldfish pituitary cell cultures and measured the amount of GH released into the media, as well as cellular gh mRNA levels. To complement the in vivo studies, these in vitro experiments were conducted at three different time points of gonadal recrudescence: early, mid-, and late recrudescence. Owing to the small size of the pituitary and the limited number of

The effect of gGnIH on serum GH (upper panel) and pituitary gh mRNA levels during mid-recrudescence (December) in vivo. Goldfish received two injections of 2 µg gGnIH, at time 0 and 12 h; fish that had received injections of PBS served as controls. Pituitaries and blood samples for serum collection were removed at 2 h following the second injection. Serum GH values were analyzed by RIA (mean ± S.E.M.; n = 12). Abundance of pituitary transcripts was determined by qPCR. gh mRNA values were normalized against β-actin mRNA and expressed relative to those for controls (mean ± S.E.M.; controls, n = 6); control groups were normalized to 100%. Different letters denote statistically significant differences (ANOVA followed by Tukey’s test, P < 0.05). Tissues that did not resemble the appropriate gonadal stage were not used.
pituitary cells, in order to test pituitary cells from goldfish at different stages of recrudescence, we were only able to test the effects on one GnRH isof orm, and we chose sGnRH.

During early gonadal recrudescence (October), treatment with gGnIH alone resulted in a dose-dependent increase in GH release at 10 and 100 nM and significantly stimulated gh mRNA expression at 100 nM (Fig. 4). Treatment with 10 nM sGnRH alone resulted in a significant increase in GH released but had no significant effect on gh mRNA expression (Fig. 4). Treatment with increasing doses of gGnIH and 10 nM sGnRH prevented the sGnRH- and gGnIH-induced increase in GH release and had no effect on gh mRNA expression levels (Fig. 4). The apparent uncoupling of GH release and mRNA expression observed following in vivo treatment was not present in vitro.

In cells prepared with pituitaries from goldfish during the mid-recrudescence stage (December), treatment of gGnIH significantly stimulated GH release at concentrations of 1 and 10 nM (Fig. 5). Interestingly, gGnIH treatment resulted in a significant inhibition of gh mRNA expression at concentrations of 10 and 100 nM (Fig. 5). Treatment with sGnRH alone did not significantly affect GH release, but sGnRH significantly stimulated GH secretion relative to basal levels when administered in combination with gGnIH at 1, 10, and 100 nM (Fig. 5). The expression of gh mRNA was significantly increased by sGnRH alone, which was reduced when it was administered in combination with gGnIH (Fig. 5).

During late recrudescence at prespawning stage (March), gGnIH alone stimulated the release of GH at

![Figure 3](image-url)

**Figure 3**
The effect of gGnIH on serum GH (upper panel) and pituitary gh mRNA (lower panel) levels during late recrudescence (March) in vivo. Goldfish received two injections of 2 μg gGnIH at time 0 and 12 h; fish that had received injections of PBS served as controls. Pituitaries and blood samples for serum collection were removed at 2 h following the second injection. Serum GH values were analyzed by RIA (mean ± S.E.M.; n = 14). Abundance of pituitary transcripts was determined by qPCR. gh mRNA values were normalized against β-actin mRNA and expressed relative to those for controls (mean ± S.E.M.; controls, n = 7); control groups were normalized to 100%. Different letters denote statistically significant differences (ANOVA followed by Tukey’s test, P < 0.05). Tissues that did not resemble the appropriate gonadal stage were not used.

![Figure 4](image-url)

**Figure 4**
The dose-related effects of gGnIH on GH release (upper panel) and gh mRNA levels in primary cultures of dispersed goldfish pituitary cells prepared from fish at early gonadal recrudescence (October). After 12 h of static treatment, a medium sample was removed for GH level analysis via RIA, and the RNA from cells was extracted for determination of transcript abundance. Gh transcript abundance was determined by qPCR and the results normalized against β-actin and expressed as a percentage of control values (mean ± S.E.M.; n = 8 from two independent cell preparations). Different letters denote statistically significant differences (ANOVA followed by Tukey’s test, P < 0.05).
a concentration of 1 nM, but was without effect at higher concentrations (10 and 100 nM). Treatment with gGnIH alone significantly suppressed the expression of gh mRNA at concentrations of 10 and 100 nM as was observed at the mid-recrudescence stage (Fig. 6). During the late recrudescent stage, treatments with either sGnRH alone or in combination with increasing doses of gGnIH had no effect on GH release. However, gh increase in the mRNA level was significantly stimulated by treatment with sGnRH alone, and the sGnRH-induced gh increase in the mRNA level was inhibited by 1 and 10 nM, but not 100 nM gGnIH (Fig. 6).

Effects of gGnIH alone and in combination with GnRH on in vitro acute GH release from perifused goldfish pituitary cells

To further understand the dynamics of direct actions of gGnIH on GH release, we investigated the effects of gGnIH alone and in combination with both sGnRH and cGnRHI in perifused cultured primary goldfish pituitary cells. Using pituitary cells from fish at the midstage of gonadal recrudescence (December–February), a 1-h treatment with either 100 nM sGnRH or 100 nM cGnRHI alone significantly stimulated GH release. A single acute 5-min pulse of 10 nM gGnIH did not significantly affect GH release by itself. However, when gGnIH was applied for 30-min into the 1-h GnRH treatment, the quantified total cGnRHI-induced GH release response but not the sGnRH-induced response, was reduced significantly (Fig. 7).

Using the reverse paradigm, we investigated the effects of a prolonged 1-h exposure to gGnIH on the acute effect of GnRH on stimulating GH release using pituitary cells obtained from fish at the late recrudescence/sexually matured stage (May–June). A 1-h treatment with gGnIH alone significantly reduced basal GH secretion for 15–25 min following the commencement of gGnIH treatment.
from the goldfish pituitary. Limited information is available in the literature regarding the effect of GnIH homologs on gh mRNA expression in vivo or in vitro. Herein, we investigate for the first time, to our knowledge, in any vertebrate system, both in vivo and in vitro effects of GnIH on GH release and mRNA expression during three gonadal recrudescence stages, as well as comparing the long- and short-term effects of GnIH alone and in combination with two native GnRHs on GH secretion in vitro within the same study. In addition, this study demonstrates for the first time, to our knowledge, that gGnIH differentially modulates the effect of two native GnRHs (sGnRH and cGnRHII), reveals the seasonal reproductive influences on gGnIH action, and indicates the possible differences between the direct and indirect effects of gGnIH on somatotrope functions.

**Discussion**

In an effort to clarify the possible involvement of GnIH in the neuroendocrine regulation of somatotrope functions, we have investigated the effect of GnIH on basal and GnRH-induced mRNA expression and secretion of GH exposure (average GH values in these three fractions in columns exposed to gGnIH = 73.6 ± 3.2% pretreatment, n = 32; equivalent GH values in columns not exposed to gGnIH = 100.7 ± 4.4% pretreatment, n = 16, P < 0.05 vs gGnIH-treated columns; Fig. 8). Acute 5-min applications of either sGnRH or cGnRHII significantly increased GH secretion when changes in GH contents were quantified over 20 min following GnRH application. These responses were significantly attenuated when sGnRH or cGnRHII was applied 30 min into the 1-h gGnIH exposure (Fig. 8). Interestingly, upon the removal of the prolonged gGnIH treatment, a significant increase in GH release was observed; this ‘rebound increase’ was not observed for the columns treated with an acute pulse of GnRH alone (Fig. 8).

**GH release**

Results from i.p. injection studies indicate that gGnIH reduces basal and/or GnRH-induced elevation in serum GH levels at all three gonadal recrudescence stages.
examined. In addition, results from perfusion experiments indicate that gGnIH reduces basal and sGnRH- and cGnRHII-stimulated GH secretion from pituitary cells prepared from fish at late recrudescence and attenuates cGnRHII-induced GH release from pituitary cells prepared from fish at mid-recrudescence. These results indicate that gGnIH can reduce GH release, at least in part, by direct actions on basal GH secretion and by inhibition of GnRH-stimulated GH release at the level of the pituitary cells. These results differ from those observed for amphibians and male rats, where the GnIH homolog (iGRP and RFRP3 respectively) stimulates GH secretion in vivo (Koda et al. 2002, Johnson et al. 2007). Whether the inhibitory effects of i.p. injection of gGnIH on basal and stimulated GH secretion also involve gGnIH actions at other levels of the neuroendocrine axis regulating GH release in the goldfish is not known, but the pineal gland and gonads may be important elements, as pinealectomy resulted in decreased body growth and melatonin injection increased the body weight in goldfish in a dose-dependent manner (de Vlaming 1980). Although it is unclear whether the pineal gland and melatonin are involved in the control of gGnIH in goldfish, it is an important modulator in avians and mammals (Tsutsui et al. 2012, Chowdhury et al. 2013). Estradiol (E2) increases early gene expression in, and alters the activities of, GnIH-immunoreactive cells in hamsters (Kriegsfeld et al. 2006, Gibson et al. 2008), whereas it decreases Gnih mRNA expression in mice (Molnár et al. 2011). In goldfish, gGnIH implantation stimulated serum testosterone levels in males, although it had no effect on E2 levels in females (Qi et al. 2013). Interestingly, the gonads of many mammalian and avian species also express gGnIH (Tsutsui et al. 2012, Chowdhury et al. 2013). Hence, it is possible that the growth axis is subsequently modulated by the GnIH-induced changes in gonadal steroids (Melamed et al. 1998).

In previous experiments with goldfish pituitary cells, a rebound increase in GH release is observed following termination of inhibitory norepinephrine and epinephrine treatments (Lee et al. 2000), but not upon termination of exposure to SS14 (Marchant et al. 1987). Interestingly, cessation of the 1-h perfusion treatment with gGnIH resulted in a large increase in GH secretion (Fig. 8). This demonstrates for the first time, to our knowledge, in goldfish that, similar to the effects of SS14 in mammals (Rigamonti et al. 2002), a rebound increase in GH secretion can occur following the removal of an inhibitory neuropeptide.

Unexpectedly, the stimulatory effects of static 12-h incubation of primary goldfish pituitary cells with gGnIH on GH release differ from the inhibitory actions observed with i.p. administration and with perfusion treatments. Exposure to gGnIH reduced goldfish pituitary gGnIH receptor mRNA expression in a previous study (Moussavi et al. 2012). It is possible that gGnIH receptors desensitize and/or down-regulate over the 12-h treatment period, resulting in the diminution of gGnIH effects, the subsequent manifestation of the phenomenon of the ‘rebound increase’ in GH release, and an overall increase in accumulated GH present in the media.

Complex local cellular and paracrine interactions probably exist in goldfish pituitary cell cultures under long-term static incubation conditions. For example, the presence of sGnRH enhances the ability of gGnIH to increase GH release at mid-recrudescence while the combination of sGnRH and gGnIH treatment reduces the GH response relative to either sGnRH or gGnIH alone in experiments with cells prepared from fish at the early recrudescence stage. Although factor(s) underlying these switches in combinatory sGnRH and gGnIH effects are unknown, one scenario may be considered. Local LH secretion is known to enhance somatotrope gh gene transcription and GH release responsiveness in carp pituitary cells during static incubation (Wong et al. 2006, Chang & Wong 2009). However, gGnIH treatment increased cumulative LH release in 12-h static incubation studies only in experiments with cells collected during late recrudescence, but not at early and mid-recrudescence in previous studies with goldfish pituitary cells (Moussavi et al. 2012). Thus, changes in the local LH milieu alone are insufficient to explain this paradoxical switch in GH responses to combination treatments with gGnIH and sGnRH during static incubation.

**Steady-state gh mRNA expression**

In ovine pituitary cell cultures, RFRP3 is reported to have no effect on gh gene expression (Sari et al. 2009). In contrast, in this study, gGnIH reduces GnRH-elicted elevations in pituitary gh mRNA expression in both in vivo and in vitro experiments, barring one exception (12-h static incubation with the combination of 100 nM gGnIH with sGnRH at late gonadal recrudescence; Fig. 6). How gGnIH attenuates the increases in gh gene expression in response to GnRH is unknown but inhibition of ERK signaling is a likely mechanism. GnRH stimulation of gh gene expression in goldfish is mediated by ERK activation (Klausen et al. 2005) and GnIH targets ERK signaling to reduce gonadotropin subunit transcript levels in LβT2 cells (Son et al. 2012, Ubuka et al. 2013). The
inability of the 100 nM gGnIH to reduce sGnRH-induced elevations in gh mRNA levels in pituitary cells prepared from fish at late recrudescence is indicative of the presence of differences in dose-dependent effects of gGnIH and/or the result(s) of its interaction with other local regulatory factors over the 12-h incubation period.

Our results also reveal that the effects of gGnIH on basal gh gene expression are distinct from those affecting stimulated gh mRNA expression. In contrast to the largely inhibitory effects of gGnIH on GnRH-elicited increases in gh gene expression, gGnIH treatment alone elevates goldfish pituitary gh mRNA levels at all three gonadal recrudescence stages examined in vivo and during early gonadal recrudescence in vitro. However, during mid- and late gonadal recrudescence, gGnIH decreased gh mRNA levels in vitro indicating that differences in gGnIH influence in vitro when compared with in vivo at these reproductive stages. These differences reinforce the ideas discussed in the previous section on GH release that complex combinations of direct pituitary level actions, including local pituitary paracrine actions and indirect actions through effects on other neuroendocrine regulators of gh gene expression, interact to modulate gGnIH actions.

Our results also indicate that gGnIH often has opposite effects on GH release and gh mRNA expression following gGnIH injections in vivo (Figs 1, 2 and 3) and 12-h incubation with gGnIH in vitro (Figs 4 and 5), revealing the ability of gGnIH to uncouple the expression and secretion of GH. Uncoupling of GH expression and secretion by other hormones has been observed before. For example, goldfish kisspeptin ten stimulates GH release but has no effect on gh mRNA expression (Yang et al. 2010). Additionally, results from our previous investigations have indicated that gGnIH is also able to uncouple lhβ mRNA expression and LH secretion (Moussavi et al. 2012). Post-receptor signaling mechanisms mediating differential actions of gGnIH on GH release and gene expression remain to be studied in the future, but differential use of pharmacologically distinct intracellular Ca^{2+} pools, protein kinase C, and ERK have been indicated to be factors enabling selective modulation of GH secretion and synthesis in goldfish (Johnson et al. 2002, Chang et al. 2012).

**Influences of gonadal recrudescence stages**

Results from this study also indicate that gonadal recrudescence status exerts profound influences on the effects of gGnIH, GnRH, and the combined gGnIH plus GnRH treatments on somatotrope functions in the goldfish. While 12-h incubation with gGnIH consistently stimulates GH release, the effective dose switches from high nanomolar to low nanomolar in cell preparations from fish at early recrudescence to those at late recrudescence (Figs 4, 5 and 6), indicating a change in sensitivity to gGnIH. Accompanying this is a change from a stimulatory gh mRNA response to gGnIH application in these experiments, indicating a switch in how 12-h incubation with gGnIH affects gh gene expression with gonadal recrudescence. Furthermore, the presence of a reproductive-state-dependent, direct, isoform-selective inhibitory influence on short-term GnRH effects on GH release in perfusion experiments is also revealed (Figs 7 and 8).

This is the first time, to our knowledge, that the effects of GnRH-induced GH secretion have been studied in parallel with gh mRNA levels across gonadal recrudescence stages in the goldfish, as well as in any teleost species. While results from this study confirm previous findings in which both sGnRH and cGnRHIH stimulated the secretion and expression of GH (Klausen et al. 2002, Canosa et al. 2007), seasonal reproductive influences on these aspects of GnRH action are also elucidated. Although changes in the ability of sGnRH and cGnRHIH to stimulate GH secretion throughout the seasonal reproductive year in vitro and in vivo have previously been demonstrated (Peter & Chang 1999, Chang et al. 2012), serum and culture medium GH levels were not elevated, at 2-h post-injection and 12-h incubation respectively, by GnRH at times of mid-recrudescence (Figs 2 and 5). On the other hand, both GnRHs can directly elicit acute GH release responses in perifusion experiments with pituitary cells prepared from fish at this reproductive stage (Fig. 7). Interestingly, the magnitudes of the elevation in gh mRNA levels following cGnRHIH injections decreased, as fish advanced in gonadal recrudescence (Figs 1, 2 and 3; ∼ 7000, 1700, and 300% of the levels for controls at early, mid-, and late recrudescence respectively). These results, when taken together, indicate that gonadal recrudescence status affects the efficacy and/or kinetics of the GH release and gh gene expression responses to GnRH. The presence of seasonal reproductive differences in the gh mRNA responses to GnRH is unlikely to be unique to goldfish. Seasonal differences in the effects of a GnRH analog on pituitary gh gene expression have also been reported for masu salmon (Bhandari et al. 2003).

How these seasonal reproductive influences are manifested is unknown, but seasonal changes in gonadal steroids are probably involved. In female goldfish,
implantation of silastic pellet containing 17β-E2 potentiates sGnRH-induced GH secretion and elevates pituitary GH content. Application of testosterone in vitro, as well as in vivo, similarly increases pituitary gh mRNA levels in sexually matured, as well as immature, goldfish. Treatment of cultured goldfish pituitary cells in vitro with E2 also enhances the GH release responses to sGnRH and cGnRHII (Trudeau et al. 1992). In addition, in vivo treatment with E2 reduces the in vitro effects of SS14 on GH release and down-regulates the expression of pituitary SS type 2 receptor mRNA in goldfish (Cardenas et al. 2003).

Hence, seasonal differences in somatotrope control by gGnIH and GnRH may be due to changes in circulating gonadal steroids, changes in receptor levels, and other neuroendocrine factors.

**Summary perspectives**

Our findings provide the first insights, to our knowledge, into the influence of a native GnIH and its effects on somatotrope cell functions in a teleost system. One of our key findings is that gGnIH is not simply a stimulatory peptide for GH release, as accepted for GnIH and its orthologs in higher vertebrates. In goldfish, gGnIH affects both GH release and gh mRNA expression, alone and in combination with known regulators of somatotrope functions in this system, and in a complex manner that is dependent on the mode of application, and duration of exposure, as well as reproductive season/gonadal recrudescence status. Overall, gGnIH is largely inhibitory to GnRH-induced increases in GH release and pituitary gh mRNA levels. Based on observations from in vivo and acute in vitro applications, gGnIH inhibits basal GH secretion but stimulates gh gene expression. However, a net stimulatory effect on GH secretion is observed upon long-term exposure to gGnIH alone, which may be partly due to a rebound increase in GH secretion following an inhibitory episode. Seasonal reproductive influences are manifested in changes in sensitivity to gGnIH and in GnRH isoform-selective interactions.

Importantly, gGnIH may dissociate GH release episodes from gh gene expression, as opposite changes in these two parameters are often observed in its presence. In addition to the regulation of body growth, GH has been shown to enhance the gonadal steroidogenic response to LH and to promote gametogenesis in teleosts (Van der Kraak et al. 1990). It is likely that gGnIH, through its ability to differentially affect and uncouple GH release and gh gene expression, as well as through its modulatory influences on the somatotrope actions of other neuroendocrine factors such as the two native GnRHs, may contribute to the seasonal control of changes in pituitary GH production, pituitary GH release, and the resultant serum GH levels important for the regulation and partition of energy utilization between gonadal and somatic growth through the annual reproductive cycle. Interestingly, our previous findings indicated that a complex interaction between gGnIH and GnRH also exists with regard to the regulation of goldfish gonadotrope functions and that gGnIH can cause dissociation between gonadotropin gene expression and secretion (Moussavi et al. 2012, 2013). Thus, gGnIH may be an integral part of a multifactorial system that controls seasonal regulation of somatotrope and gonadotrope functions. Our results provide the basis for future studies of the neuroendocrine roles of gGnIH and other regulators in the integrated control of goldfish gonadotrope and somatotrope functions, and ultimately seasonal reproduction and growth.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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