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

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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 tha 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

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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,

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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 (Biswas *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 (LPXRFamide 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 cGnRHII-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 (SGTGLSATLPQRF-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.

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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 (GTCTCAAACAGCCTGACCGTCG), *gh* forward 2 (GTCTCAAACAGCCTGACCGCCG), *gh* reverse 1 (CAGCGGCAGGGAGTCGTTATCATC), 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 perifusion 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 perifusion protocol followed was as described previously using identical experimental paradigms and protocols (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 somatotrope 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

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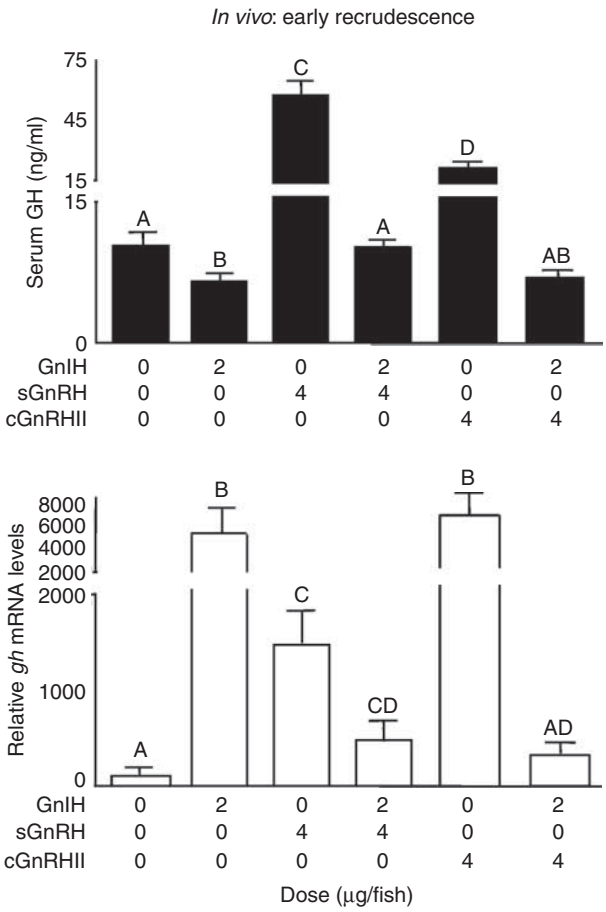

Figure 1
The effect of gGnIH on serum GH (upper panel) and pituitary *gh* mRNA levels (lower panel) during early recrudescence (October) *in vivo*. Goldfish received two injections of 2 μg gGnIH, at time 0 and 12 h; fish that had received injections of PBS served as controls. Pituitaries and blood samples 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.

the gGnIH or control groups (Fig. 3), demonstrating clear seasonal effects on GnIH-mediated GH secretion and mRNA expression. Co-injection of gGnIH with cGnRHII brought both serum GH levels and *gh* mRNA expression back to levels not different from controls that had received injections of PBS (Fig. 3).

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

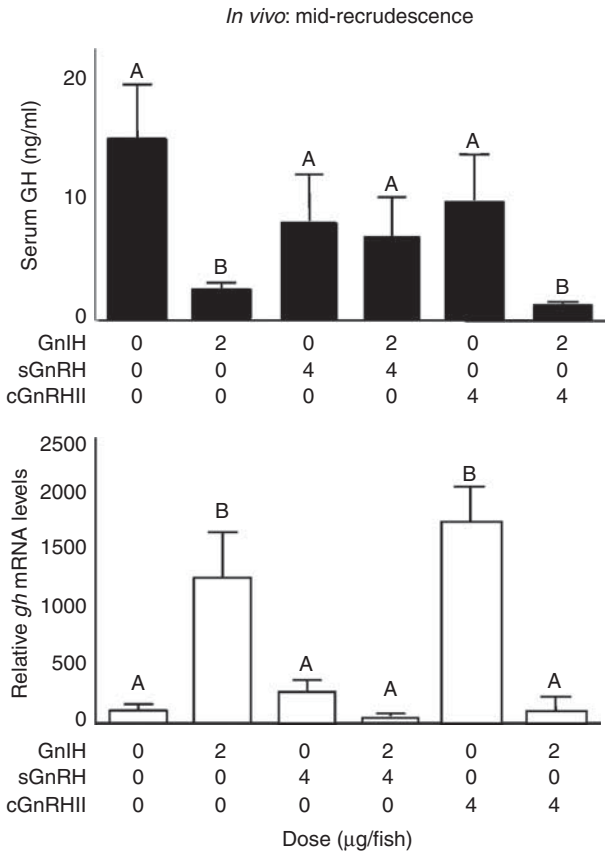

Figure 2
The effect of gGnIH on serum GH (upper panel) and pituitary *gh* mRNA levels during mid-recrudescence (December) *in vivo*. Goldfish received two injections of 2 μg gGnIH, at time 0 and 12 h; fish that had received injections of PBS served as controls. Pituitaries and blood samples for serum 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.

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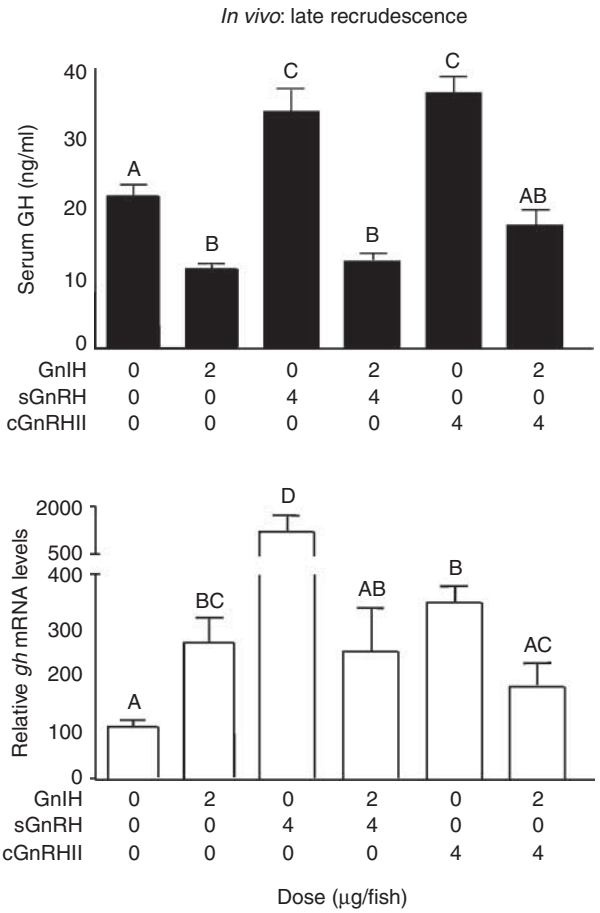

Figure 3
The effect of gGnIH on serum GH (upper panel) and pituitary *gh* mRNA (lower panel) levels during late recrudescence (March) *in vivo*. Goldfish received two injections of 2 μg gGnIH at time 0 and 12 h; fish that had received injections of PBS served as controls. Pituitaries and blood samples 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.

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 isoform, 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 recrudescent at prespawning stage (March), gGnIH alone stimulated the release of GH at

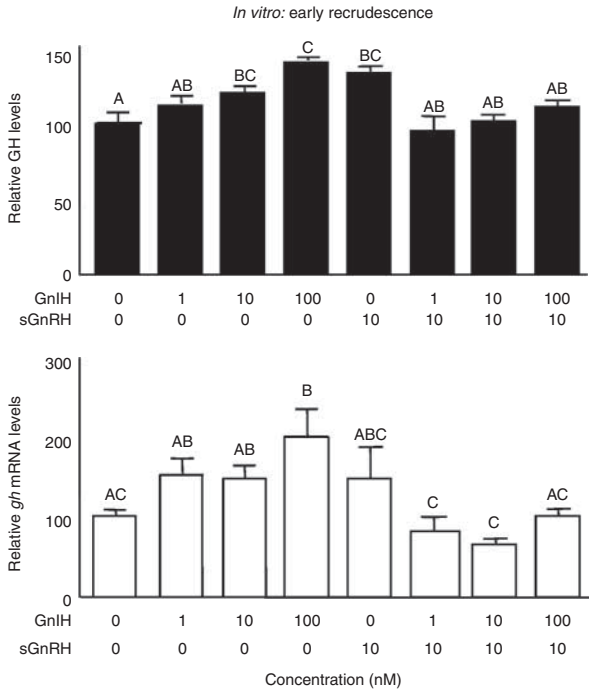

Figure 4
The dose-related effects of gGnIH on GH release (upper panel) and *gh* mRNA (lower panel) levels in primary cultures of dispersed goldfish pituitary cells prepared from fish at early gonadal recrudescence (October). After 12 h of static treatment, a medium sample was removed for GH level analysis 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, $P<0.05$).

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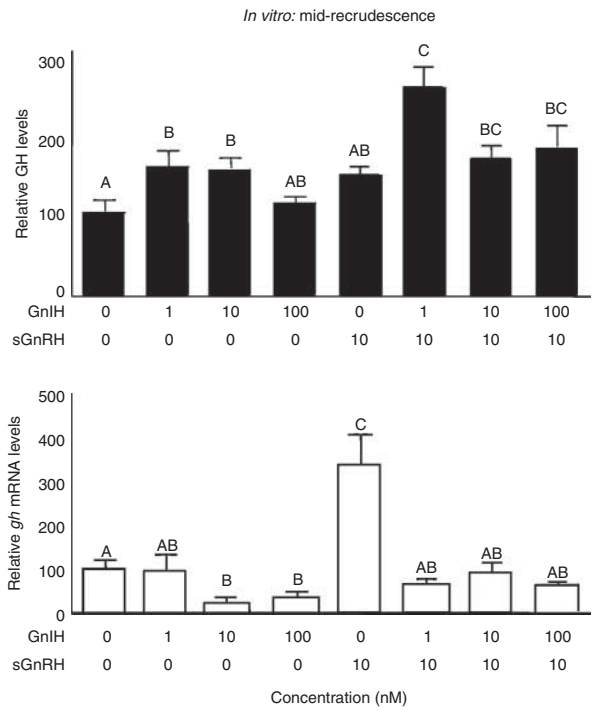

Figure 5
The dose-related effects of gGnIH on GH release (upper panel) and *gh* mRNA (lower panel) levels in primary cultures of dispersed goldfish pituitary cells prepared from fish at mid-gonadal recrudescence (December). After 12 h of static treatment, a medium sample was removed for GH level analysis 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 cGnRHII 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 cGnRHII 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 cGnRHII-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

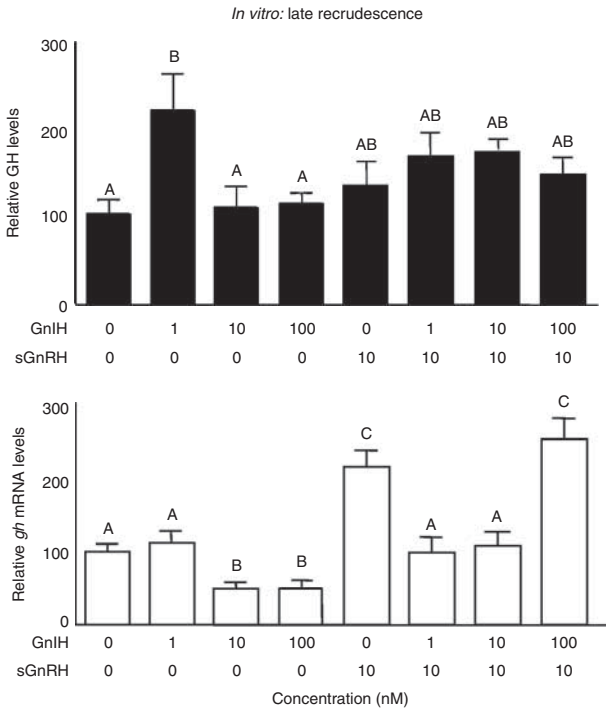

Figure 6
The dose-related effects of gGnIH on GH release (upper panel) and *gh* mRNA (lower panel) levels in primary cultures of dispersed goldfish pituitary cells prepared from fish at late gonadal recrudescence (March). After 12 h of static treatment, a medium sample was removed for GH level analysis 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, $P<0.05$).

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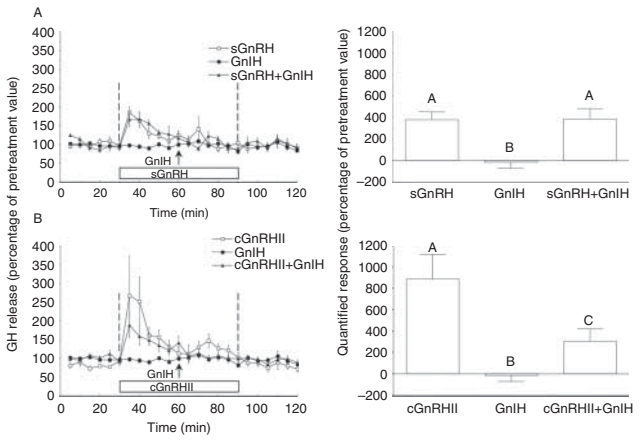

Figure 7
Effects of gGnIH (10 nM) on the GH response to (A) sGnRH (100 nM) and (B) cGnRHII (100 nM) in perifused dispersed goldfish pituitary cells prepared from goldfish of mixed sex at mid-recrudescence (December–February). gGnIH was applied as a 5-min pulse (arrow), 25 min into a 1-h treatment with GnRH (horizontal bar). GH content in perifusates collected in 5-min fractions was analyzed by RIA. GH values for each treatment column were normalized as a percentage of pretreatment values (average of the first five fractions collected before hormone treatment). Net GH responses to GnRH were quantified as 'area under the curve with baseline subtracted' (duration of quantification indicated by the vertical dotted lines; baseline defined as the average of the three fractions before the quantification period). GH release profiles are presented in the left panels and quantified net GH responses to GnRH are presented in the right panels (mean ± S.E.M., $n=8$ from four separate cell preparations). Different letters denote significant differences between treatment groups (ANOVA followed by Tukey's test, $P<0.05$).

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).

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 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.

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

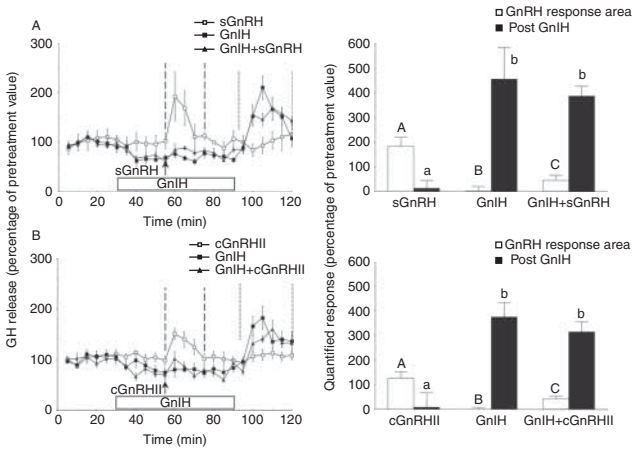

Figure 8
Effects of gGnIH (10 nM) on the GH response to (A) sGnRH (100 nM) and (B) cGnRHII (100 nM) in perifused dispersed goldfish pituitary cells prepared from goldfish of mixed sex at late recrudescence/sexually matured stages (May–early June). GnRH was applied as a 5-min pulse (arrow), 25 min into a 1-h treatment with gGnIH (horizontal bar). GH content in perifusates collected in 5-min fractions was analyzed by RIA. GH values for each treatment column were normalized as a percentage of pretreatment values (average of the first five fractions collected before hormone treatment). Net GH responses to GnRH were quantified as 'area under the curve with baseline subtracted' (duration of quantification indicated by the vertical dotted lines; baseline defined as the average of the three fractions before the quantification period). GH release profiles are presented in the left panels and quantified net GH responses to GnRH are presented in the right panels (mean ± S.E.M., $n=8$ from four separate cell preparations). Different letters denote significant differences between treatment groups (ANOVA followed by Tukey's test, $P<0.05$).

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examined. In addition, results from perifusion 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 (fGRP 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 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 (E_2) 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 E_2 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 perifusion 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 perifusion 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-elicited 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

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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 to an inhibitory *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 perifusion 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 cGnRHII 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 cGnRHII 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 cGnRHII 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,

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implantation of silastic pellet containing 17β-E_2 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 E_2 also enhances the GH release responses to sGnRH and cGnRHII (Trudeau *et al*. 1992). In addition, *in vivo* treatment with E_2 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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