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ORIGINAL ARTICLE

Gonadotrophin-Inhibitory Hormone in the Cichlid Fish *Cichlasoma dimerus*: Structure, Brain Distribution and Differential Effects on the Secretion of Gonadotrophins and Growth Hormone

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The role of gonadotrophin-inhibitory hormone (GnIH) in the inhibition of the reproductive axis has been well-established in birds and mammals. However, its role in other vertebrates, such as the teleost fish, remains controversial. In this context, the present study aimed to evaluate whether GnIH modulates the release of gonadotrophins and growth hormone (GH) in the cichlid fish *Cichlasoma dimerus*. First, we partially sequenced the precursor polypeptide for GnIH and identified three putative GnIH peptides. Next, we analysed the expression of this precursor polypeptide via a polymerase chain reaction in the reproductive axis of both sexes. We found a high expression of the polypeptide in the hypothalamus and gonads of males. Immunocytochemistry allowed the observation of GnIH-immunoreactive somata in the nucleus posterioris periventricularis and the nucleus olfacto-retinalis, with no differences between the sexes. GnIH-immunoreactive fibres were present in all brain regions, with a high density in the nucleus lateralis tuberis and at both sides of the third ventricle. Finally, we performed *in vitro* studies on intact pituitary cultures to evaluate the effect of two doses (10^{-6} M and 10^{-8} M) of synthetic *C. dimerus* (cd-) LPQRFa-1 and LPQRFa-2 on the release of gonadotrophins and GH. We observed that cd-LPQRFa-1 decreased β-luteinising hormone (LH) and β-follicle-stimulating hormone (FSH) and also increased GH release to the culture medium. The release of β-FSH was increased only when it was stimulated with the higher cd-LPQRFa-2 dose. The results of the present study indicate that cd-LPQRFa-1, the cichlid fish GnIH, inhibits β-LH and β-FSH release and stimulates GH release in intact pituitary cultures of *C. dimerus*. The results also show that cd-LPQRF-2 could act as an β-FSH-releasing factor in this fish species.

Key words: gonadotrophin-inhibitory hormone, growth hormone, follicle-stimulating hormone, luteinising hormone, fish

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In 2000, Tsutsui *et al.* (1) discovered a hypothalamic neuropeptide that directly inhibits gonadotrophin release in Japanese quail and termed it gonadotrophin-inhibitory hormone (GnIH). The discovery of GnIH changed the classical view of the regulation of vertebrate reproduction, which assumed that gonadotrophin-releasing hormone (GnRH) was the only hypothalamic neuropeptide regulating gonadotrophin release in vertebrates. The GnIH precursor encodes one GnIH and two GnIH-related peptides (GnIH-RP-1 and GnIH-RP-2), which possess an LPXRFamide (X = L or Q) motif at their C-terminus in birds (2–4). All of the identified and putative GnIH peptides in vertebrates possess a common LPXRFamide (X = L or Q) motif at the C-terminus, making them members of the RFamide peptide family. Subsequent to the discovery of GnIH, it has been demonstrated that this peptide inhibits reproduction by decreasing gonadotrophin release and synthesis in birds and mammals (5–10). However, the functional role of GnIH in the regulation of reproduction in other vertebrates, in particular in fish, remains controversial (11).

In teleost fish, GnIH has been shown to have both stimulatory and inhibitory effects on gonadotrophin synthesis and release. Some studies have shown that the administration of GnIH

stimulates luteinising hormone (LH) (gene: *lh*) and follicle-stimulating hormone (FSH) (gene: *fsh*) mRNA expression in goldfish (*Carassius auratus*) (12) and the release of both gonadotrophins in tilapia (*Oreochromis niloticus*) (13). By contrast, other *in vivo* studies have revealed that GnIH injection inhibits LH release in goldfish (12,14) and decreases *lh* and *fsh* mRNA expression levels in goldfish (15) and in the orange-spotted grouper (*Epinephelus coioides*) (16). *In vitro* studies have shown that GnIH stimulates LH and FSH release into the culture medium in sockeye salmon (*Oncorhynchus nerka*) (17) and tilapia (13), and increases the mRNA levels of both gonadotrophins in grass puffer (*Takifugu niphobles*) (18). In addition, Moussavi *et al.* (12) observed that this neuropeptide inhibits *lh* and *fsh* mRNA expression levels in primary cultures of goldfish pituitary cells. Moussavi *et al.* (12) and Qi *et al.* (15) observed that these positive and negative effects could depend on the reproductive stage, probably because of the action of steroids on the expression of GnIH receptor.

Besides the modulation of gonadotrophins, some studies have revealed that GnIH could be involved in the regulation of growth hormone (GH) release (gene: *gh*) and *gh* mRNA levels. For example, in sockeye salmon, Amano *et al.* (17) observed that GnIH stimulates its release into the culture medium. By contrast, Moussavi *et al.* (19) found that *in vivo* administration of GnIH inhibits GH release and increases *gh* mRNA levels in goldfish but, when administered *in vitro* to static cultures of dispersed pituitary cells, GnIH can have either stimulatory or inhibitory effects, depending on the gonadal stage and duration of the exposure (19).

GnIH receptor was first identified in quail by Tsutsui *et al.* (20) and in mammals by Hinuma *et al.* (21). GnIH receptor, named GPR147 or neuropeptide FF receptor 1, is a member of the G-protein coupled receptor superfamily (5–10). GnIH receptor has also been identified in some fish species. Three sequences have been identified in goldfish (15) and zebrafish (*Danio rerio*) (14) but only one in tilapia (13) and the orange-spotted grouper (16). In all cases, the expression of GnIH receptor was observed throughout the reproductive axis and other tissues (13,14,16,22).

Our experimental model comprises the social cichlid fish *Cichlasoma dimerus*, which has been established as an excellent model in neuroendocrinology and endocrinology for studies of somatic growth and behaviour (23–27). This fish, similar to other cichlids, displays highly organised reproductive and breeding activities, which can be observed in laboratory conditions. The dominant pair aggressively defends the prospective spawning site and starts to display stereotyped pre-spawning activities. Moreover, spawning is followed by a period of parental care during which both parents cooperatively guard the eggs (28).

Previous studies in our laboratory have allowed us to gain knowledge of the brain anatomy and regulation of pituitary hormones (24,25,27,29–33). Because the main objective in our laboratory is to study the relationship between feeding, reproduction and somatic growth, investigations of GnIH as a possible regulator are needed to clarify this complex system.

Thus, the present study aimed to evaluate whether *C. dimerus* LPXRFamide peptides modulate the release of LH, FSH and GH in this fish. As a first step, we partially sequenced the precursor polypeptide for GnIH and identified the three putative GnIH peptides possessing an LPXRFamide motif or an MPLRFamide motif at their C-terminus. Because knowledge of the distribution of GnIH in the brain allows us to infer possible functions, we analysed the distribution of GnIH-immunoreactive somata and fibres in the brain and pituitary gland of *C. dimerus*. Finally, we performed *in vitro* studies aiming to evaluate the effects of two peptides: *C. dimerus* LPQRFa-1 and LPQRFa-2, as putative GnIHs, on the release of gonadotrophins and GH.

Materials and methods

Animals

Reproductive adults of both sexes of *C. dimerus* (total length 9.91 ± 0.37 cm; weight 20.84 ± 2.5 g) were captured in Esteros del Riachuelo, Corrientes, Argentina (27°12′50″S, 58°11′50″W), and then transferred to the laboratory where they were maintained. Fish were acclimated to a 14 : 10 h light/dark cycle at a constant temperature of 25 ± 2 °C in 400 l of fresh water aquaria for 1 month prior to the experiments. They were fed daily with commercial pellets (Tetra Pond Variety Blend; Tetra Werke, Melle, Germany).

Animals were handled in accordance with the Principles of Laboratory Animal Care (guidelines on the care and the use of fish in research, teaching and testing, Canadian Council on Animal Care, 2005), which were approved by the Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina (Protocol #26).

Sequencing of the GnIH precursor polypeptide

Adults of *C. dimerus* of both sexes were anaesthetised with benzocaine 0.1% (w/v) and their hypothalami were collected after euthanasia by decapitation. Total RNA was obtained using 500 μl of TRI-Reagent (Molecular Research Center (MRC), Inc., Cincinnati, OH, USA) in accordance with the manufacturer's instructions. RNA quantification and purity were determined by ultraviolet spectrophotometry. Then, 1 μg of total RNA was treated with DNAse I (Sigma-Aldrich, St Louis, MO, USA) to eliminate possible genomic DNA contamination. First-strand cDNA was synthesised using random primers (Genbiotech, Buenos Aires, Argentina) and Moloney murine leukemia virus enzyme (Promega, Madison, WI, USA), as described previously by Delgadin *et al.* (26). Degenerated consensus primers (Table 1) for the GnIH precursor polypeptide were designed by comparing cDNA sequences of goldfish (AB078976.1) and zebrafish (GU290218.1), as well as the tilapia predicted hypothetical RNA sequence for a LPXRF peptide (XM_003448903.1). The sequences of orange-spotted grouper (16) and European sea bass (34) were not included in the alignment because these were reported during the preparation of the present study (16,34). The primers that were designed amplified partial and overlaid regions of the GnIH precursor polypeptide transcript.

Polymerase chain reaction (PCR) amplifications were performed in 10-μl reaction volumes using a GoTaq FlexiDNA polymerase (Promega). After an initial denaturation at 94 °C for 3 min, the PCR cycle was repeated 40 times, comprising: denaturation at 94 °C for 30 s, annealing at 51–54 °C (depending on the primer pairs) for 30 s and elongation at 72 °C for 30 s, with a final extension step at 72 °C for 10 min. PCR products were visualised after electrophoresis on 1% (w/v) agarose gel. An expected size band assumed to be the precursor polypeptide for GnIH was purified from agarose gel using an AccuPrep gel purification kit (Bioneer, Alameda, CA, USA). The purified PCR products were sequenced (Servicio de Secuenciación y Genotipificado, EGE, FCEN-UBA, Buenos Aires, Argentina) and confirmed to

Table 1. Primers Used for Sequencing the Precursor Polypeptide for Gonadotrophin-Inhibitory Hormone (GnIH) and Acidic Ribosomal Phosphoprotein P0 (ARP).

Name	Sequence (5′- to 3′)
GnIH degFw1	CAATMTACAYGTRGCYCC
GnIH Fw2	GGTAACAATGATACTGTCTGC
GnIH degRv1	CAGGATCTKCCAAACCKCTG
GnIH Rv2	AGATGAGTTCGGTGTCCTTTC
ARP Fw	TTTGAAAATCATCCAACTTTTGGAT
ARP Rv	GCAGGGACAGACGGATGGT

M: A/C; Y: T/C; R: G/A; K: G/T.

be a partial sequence of the precursor polypeptide for GnIH using BLASTN (http://blast.ncbi.nlm.nih.gov/blast.cgi/).

The precursor polypeptide cleavage sites were predicted using NEUROPRED (http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py) (35).

mRNA expression of the GnIH precursor polypeptide in the reproductive axis

The distribution of the GnIH precursor polypeptide in the reproductive axis was studied by PCR. Telencephalons, hypothalami, pituitaries and gonads were collected from adult males and females of *C. dimerus* after decapitation under anaesthesia (benzocaine 0.1%, w/v). Total RNA was obtained using TRI-Reagent (MRC, Inc.) as described above. After cDNA was synthesised, PCR amplifications were performed with specific primers (Table 1) in 10-μl reaction volumes using a GoTaq FlexiDNA polymerase (Promega) under the same conditions as those described previously. The expression of acidic ribosomal phosphoprotein P0 (ARP) mRNA was used as an internal control (Table 1). The PCR products were separated after electrophoresis on 1% (w/v) agarose gel and visualised with SYBR safe (Invitrogen, Carldbad, CA, USA) in a Luminescent Image Analyser LAS-1000 plus (Fuji Film, Tokyo, Japan). A negative control was performed by omission of the template (data not shown).

Peptide synthesis

Cichlasoma dimerus LPQRFa-1 (TPNSSPNLPQRF-NH_2) and LPQRFa-2 (APNQVLPQRF-NH_2) were synthesised by Bio Basic Canada Inc. (Ontario, Canada). The C-terminus of each peptide was amidated and the purity was > 98.24% for both peptides, as determined by high-performance liquid chromatography. The peptides were dissolved in autoclaved bi-distilled water in accordance with the manufacturer's recommendations, and stored at −75 °C. The final concentration was achieved by dissolving each peptide in culture medium for the *in vitro* experiments.

Immunohistochemical localisation of GnIH

Both adult males and females of *C. dimerus* were anaesthetised with benzocaine 0.1% (w/v) and euthanised by decapitation, and then brains with the pituitary attached were fixed for 18 h in Bouin's solution as described previously by Pérez Sirkin *et al.* (33). Next, they were embedded in paraplast (Fisherbrand; Fisher Scientific Company, Pittsburg, PA, USA) and cut in coronal sections at 10-μm intervals. Sections were mounted on gelatin-coated slides, deparaffinised in xylene and rehydrated through a graded ethanol gradient with phosphate-buffered saline (PBS) (pH 7.4). All sections were incubated for 5 min in 0.3% (v/v) H_2O_2 to block endogenous peroxidase activity and then washed in PBS and finally incubated with PBS containing 5% (w/v) nonfat dry milk (blocking solution) at room temperature (RT) for 1 h. After washing with PBS, slides were incubated with rabbit anti-GnIH antisera (dilution 1 : 3000 in PBS, raised against bullfrog peptide closely related to GnIH in the laboratory of K. Tsutsui) at RT overnight. Later, sections were washed in PBS and incubated with biotinylated anti-rabbit immunoglobulin (Ig)G (dilution 1 : 500 in PBS; Sigma-Aldrich) at RT for 1 h. Afterwards, they were incubated with IgG peroxidase-conjugated streptavidin (dilution 1 : 500 in PBS; Invitrogen) at RT for 1 h. The final reactive products were visualised with 0.3% (w/v) 3,3-diaminobenzidine in Tris buffer (pH 7.6) and 0.02% (v/v) H_2O_2. The sections were then slightly counterstained with haematoxylin, mounted with DPX and then examined with a Microphot FX microscope (Nikon, Tokyo, Japan) and photographed digitally. The anatomical location of the somata and fibres was evaluated using an atlas published for sea bass (36) as a result of anatomical brain similarity between this species and *C. dimerus*. To further characterise GnIH cell populations, 15 cells were randomly selected and measured with a micrometer to determine the mean cell diameter. Because brains were sectioned at 7-μm intervals, cells larger than 7 μm were counted only if their nucleus was clearly visible. The values expressed represent the mean ± SE cell diameter (μm) (31).

Specificity controls were performed by preadsorption of primary antisera with an excess of cd-LPQRFa-1 or cd-LPQRFa-2 (5 μg/500 ml) independently. An additional control was performed by omitting the primary antisera.

Pituitary cultures

Adults of both sexes (n = 25) were euthanised by decapitation under anaesthesia [benzocaine 0.1% (w/v)] and pituitaries were removed from the animals. Sampling procedures were similar to those described previously by Cánepa *et al.* (32). Briefly, five glands were removed and placed individually in a 96-multiwell plate with 100 μl of culture medium containing: 80% (v/v) Leibovitz L15 medium (Gibco, Invitrogen, Carlsbad, CA, USA) (pH 7.4), 10% (v/v) foetal bovine serum, 10 mM Hepes, 100 IU/ml penicillin and 100 μg/ml of streptomycin, and maintained in a dark incubator at 27 °C. After 3 h of preincubation, the medium was replaced by fresh medium and pituitaries were incubated for 24 h under the same conditions (day 1). Later, the medium was recovered and stored at −20 °C, with 1 μl of protease inhibitor cocktail (Sigma-Aldrich) to establish basal conditions of hormone release. Pituitaries were then randomly incubated in the absence or presence of cd-LPQRFa-1 or cd-LPQRFa-2 at 10^{-8} or 10^{-6} M. After 24 h (day 2), each medium was removed and stored frozen with 1 μl of protease inhibitor cocktail (Sigma-Aldrich) to establish the effects of these putative GnIHs on the release of gonadotrophins and GH. Data shown in Fig. 4 represent the means obtained from five independent cultures (n = 5).

Western blotting

Western blot analysis with heterologous antisera was used to determine hormone release into the culture medium, as described in detail previously by Cánepa *et al.* (32) and Di Yorio *et al.* (27). Briefly, samples (15 μl) from each pituitary culture medium diluted in 5 × sample buffer (120 mM Tris-Base (pH 6.8), 3% (w/v) dodecylsulfate, 10% (v/v) glycerol and 10% β-mercaptoethanol) were subjected to 15% (w/v) sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins and molecular markers (Genbiotech) were then transferred onto a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) at 75 V for 1 h. Membranes were washed in Tris-buffered saline with Tween (TBST) at pH 7.5 [100 mM Tris-HCl, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20] and blocked with TBST containing 5% (w/v) nonfat dry milk at RT for 1 h. Then, they were incubated with GH antiserum (anti-chum salmon GH; dilution 1 : 2000 in TBST), β-LH antiserum

(*Fundulus heteroclitus*; dilution 1 : 1000 in TBST) or β-FSH antiserum (*Fundulus heteroclitus*; dilution 1 : 1000 in TBST) at 4 °C overnight. After three washes in TBST, membranes were incubated with a biotinylated anti-rabbit IgG (dilution 1 : 2000 in TBST; Sigma-Aldrich) at RT for 1 h, washed again and then incubated with IgG peroxidase-conjugated streptavidin (dilution 1 : 3000 in TBST; Invitrogen) at RT for 1 h. Immunoreactive bands were visualised using chemiluminescence detection reagents (Sigma-Aldrich) and Luminescent Image Analyser LAS-1000 plus (Fuji Film). In all cases, blotting and developing conditions were repeated twice.

The GH, β-LH and β-FSH released to the medium were semi-quantified as described by Cánepa *et al.* (32). Briefly, immunoreactive-protein bands from different treatments were semi-quantified by densitometric analysis with IMAGEJ (NIH, Bethesda, MD, USA) and normalised against a 148-kDa protein only present in the culture medium to correct possible variations in SDS-PAGE loading. The 148-kDa band was visualised by Ponceau-S in the nitrocellulose membrane, digitised, and its optical density quantified with IMAGEJ.

Hormone release from each pituitary was then evaluated as follows: On days 1 and 2 culture media from each treatment were loaded into adjacent lanes for SDS-PAGE. Day 1 was considered as the basal release condition of the gland; thus, the optical density values of each immunoreactive-band from day 2 were normalised to those from day 1. This normalisation enabled the comparison of values obtained from different pituitaries.

Statistical analysis

To compare the hormone release in the culture medium between groups, means were obtained by using one-way ANOVA followed by Dunnett's multiple comparison test. Normality and homoscedasticity assumptions were tested prior to the analysis. $P < 0.05$ was considered statistically significant. Data are reported as the mean ± SEM.

Results

Partial sequence of the GnIH precursor polypeptide encoding cd-LPQRFa-1 and cd-LPQRFa-2 and its mRNA expression in the reproductive axis in males and females of *C. dimerus*

The specific product for the precursor polypeptide for GnIH was amplified from hypothalamic cDNA. The partial sequence of the precursor of *C. dimerus* consisted of 555 bp, encoding 185 amino acids, and included one MPLRFa and two LPQRFa sequences (Fig. 1A). The length of cd-MPLRFa was 44 amino acids, whereas the lengths of the cd-LPQRFa peptides were 12 and 10 amino acids, respectively. Because GnIH is mainly involved in the regulation of reproductive function, we studied the expression of the GnIH precursor peptide along the reproductive axis of males and females of *C. dimerus* by PCR. We detected GnIH precursor mRNA in the hypothalamus, with apparently higher levels in males than in females, as well as in the testes. No expression was found in the telencephalon, ovary or pituitary (Fig. 1B).

GnIH distribution in the brain of males and females of *C. dimerus*

Immunohistochemistry demonstrated the presence of GnIH-immunoreactive somata in the nucleus posterioris periventricularis (NPPv) at both sides of the third ventricle (Fig. 2, label 2), as well as in a position more ventrolateral to the NPPv, with an average diameter of 10 ± 4 μm. (Fig. 2, label 3). Additionally, some GnIH-immunoreactive cells were detected in the nucleus olfacto-retinalis (NOR), characterised by their large size, with a mean diameter of 22 ± 6 μm (Fig. 2, label 1). No difference was found between the sexes regarding the location and mean size of GnIH-immunoreactive cells. GnIH-immunoreactive fibres were present in all the brain regions examined, with a high density at both sides of the third ventricle and in the nucleus lateralis tuberis (NLT) (Fig. 2, labels 2 and 4). At the pituitary level, no GnIH-immunoreactive fibres or GnIH-immunoreactive cells were detected. Preadsorption tests incubating the primary antiserum with an excess of cd-LPQRFa-1 synthetic peptide resulted in a complete loss of immunostaining in the somata and fibres (Fig. 3C). Although most of the immunostaining was lost when the antibody was preadsorbed with synthetic cd-LPQRFa-2, there were still some cells and fibres presenting weak staining (Fig. 3D).

In vitro effects of cd-LPXQFa-1 and cd-LPQRFa-2 on the release of gonadotrophins and GH

Because the 44-amino-acid cd-MPLRFa was too long relative to most of the known GnIHs and its related peptides across vertebrates, we decided to only evaluate the effect of the two LPQRFa peptides on the release of LH, FSH and GH. Accordingly, intact pituitary glands from adult *C. dimerus* were challenged against different cd-LPQRFa concentrations. Pituitary β-LH and β-FSH release showed a decrease at 10^{-6} M of cd-LPQRFa-1 ($P < 0.05$, Fig. 4A; $P < 0.01$, Fig. 4B). Although no effect on β-LH release was observed when pituitaries were challenged against the same dose of cd-LPQRFa-2 (Fig. 4A), β-FSH release increased at 10^{-6} M of cd-LPQRFa-2 compared to the control ($P < 0.001$) (Fig. 4B). GH release into the culture medium increased in the presence of 10^{-8} and 10^{-6} M of cd-LPQRFa-1 compared to the controls ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 4C).

Discussion

GnIH was first identified in the quail hypothalamus-hypophysial system as a neuropeptide capable of inhibiting gonadotrophin secretion (1) and, currently, GnIH peptides have been identified in all of the vertebrates that have been studied (8). By analysing their sequences, it is evident that GnIH peptides share low sequence identity, except for the common and highly conserved C-terminal LPXRFamide (X = L or Q) (5–10). Considering that teleost fish represent the group of vertebrates with the highest level of diversity in GnIH sequences and its precursor, it is expected that GnIH peptides can exert a diversity of functions in this group (14). Thus, it is necessary to continue investigating the role of GnIH in different fish species.

The GnIH precursor has been recently sequenced in late evolved fish species as *O. niloticus* (13) and *E. coioides* (16) and predicted in *Oryzias latipes* (14). As in these fish, the sequence of *C. dimerus* GnIH precursor encodes a peptide that contains an MPLRFa

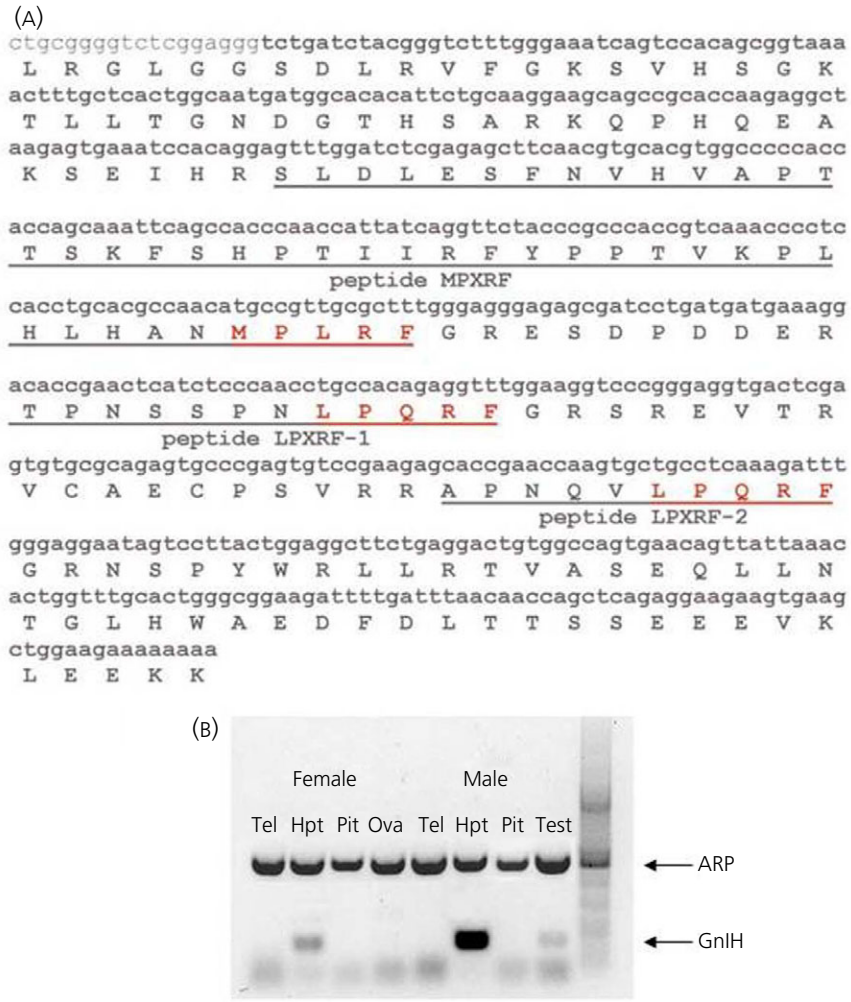

Fig. 1. Partial sequence of the precursor polypeptide for gonadotrophin-inhibitory hormone (GnIH) and polymerase chain reaction tissue distribution of GnIH mRNA in the reproductive axis of adult males and females of *Cichlasoma dimerus*. (A) Partial nucleotide and deduced amino acid sequence of the precursor polypeptide for GnIH, showing the three putative GnIH peptides (underlined) with the characteristic motif in the C-terminal (red). (B) Representative tissue distribution of GnIH mRNA in the reproductive axis of females (left) and males (right) of *C. dimerus*. Acidic ribosomal phosphoprotein P0 (ARP) was used as the reference gene. Tel, telencephalon; Hpt, hypothalamus; Pit, pituitary; Ova, ovary; Test, testis.

sequence and two subsequent peptides containing LPQRFa at the C-terminus. By contrast, in goldfish (37) and zebrafish (14), three putative LPXRFamide peptides have been characterised.

The distribution of GnIH neurones and fibres in the brain of *C. dimerus* was determined using an antiserum raised against a bullfrog peptide closely related to GnIH. This antiserum was demonstrated to be specific because it can be used to carry out affinity purifications (37) and immunoassays of GnIH in different fish species (17,37,38). Moreover, the preadsorption test with an excess of cd-LPQRF-1, a putative *C. dimerus* GnIH, resulted in a complete loss of GnIH staining, whereas, with cd-LPQRFa-2, most of the immunostaining was lost, although not completely. These results indicate that the antibody can be used in our species to recognise putative GnIHs. However, it is important to clarify that the affinity for cd-LPQRFa-1 is greater than for cd-LPQRFa-2. GnIH-immunoreactive somata were located in NPPv, whereas GnIH-immunoreactive fibres were widespread throughout the brain, although the highest density was found in the hypothalamus, particularly bordering the third ventricle and in the NLT. This pattern of distribution was also observed in other fish species, suggesting that GnIH is involved in other central functions (14,16,17,34,37,38). It is particularly interesting to note that GnIH distribution is similar to that of neuropeptide Y (NPY), orexin (33) and melanin-concentrating hormone (MCH) neurones (30), which all comprise peptides involved in feeding regulation (39). These results are in accordance with those reported for birds and mammals describing the presence of GnIH-immunoreactive somata in the dorsomedial and paraventricular nuclei, which are centres involved in feeding regulation (40). Furthermore, the administration of GnIH increases feeding behaviour in rats (41,42) and birds (43,44), as well as the expression of NPY, pro-opiomelanocortin and MCH in chicks (44). For these reasons, it was recently

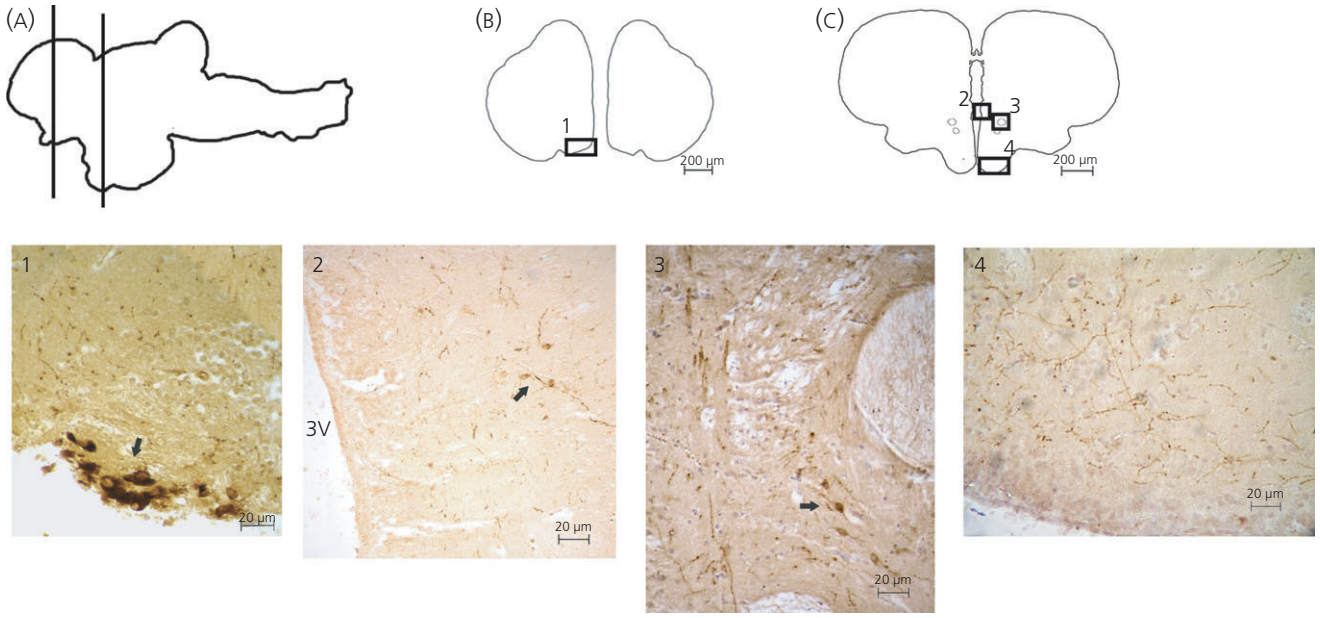

Fig. 2. Localisation of gonadotrophin-inhibitory hormone (GnIH)-immunoreactive somata and fibres detected by immunohistochemistry in coronal sections of the brain of male of *Cichlasoma dimerus*. (A) Schematic parasagittal section of *C. dimerus* brain. Lines show the position of the coronal sections of the telencephalon (B) and hypothalamus (C). (B) Camera lucida drawing of coronal section of the telencephalon, and the boxed area indicating the microphotography section showing the GnIH-immunoreactive somata (arrow) in the nucleus olfacto-retinalis (1). Scale bars = 200 µm and 50 µm, respectively. (C) Camera lucida drawing of coronal section of the hypothalamus, and the boxed area indicating the microphotography section showing GnIH-immunoreactive somata (arrow) in the nucleus posterioris periventricularis (2 and 3); fibres parallel to the third ventricle (3V) (2); and GnIH-immunoreactive fibres in the nucleus lateralis tuberis (4). Scale bar = 20 µm.

proposed that GnIH may also modulate feeding, acting as a link between feeding and reproduction in birds and mammals (8,45). Thus far, there are no reports about the possible link between GnIH and feeding in fish, although future studies conducted in *C. dimerus* might clarify this issue.

It is well known that NPPv belongs to the paraventricular organ, which is the source of pituitary afferents in goldfish (46). So, it is expected that GnIH neurones would directly affect pituitary function. However, only a few GnIH fibres have been found in the neurohypophysis of goldfish (37), salmon (17) and sea bass (34). Paullada-Salmerón *et al.* (34) also found GnIH fibres in close proximity to LH, FSH and GH cells. In the present study, we observed immunoreactive neurones in the NPPv but no immunoreaction at the pituitary level. Moreover, we found no mRNA expression of the GnIH precursor in the pituitary of *C. dimerus*, in agreement with the results obtained in the orange-spotted grouper (16) and zebrafish (14), and also with those obained in adult Indian major carp (*Labeo rohita*) by immunohistochemistry (38). However, other studies have reported GnIH precursor mRNA expression in the pituitary of tilapia (13) and grass puffer (18). It is possible that species-specific differences may exist regarding the expression of the GnIH precursor at the pituitary level or that, in *C. dimerus*, the levels of GnIH are very low and we simply failed to detect it. More extensive studies are required on this point.

In addition to NPPv, immunoreactive-somata were observed in the NOR. However, when PCRs of the anterior part of the brain including telencephalon and part of the olfactory bulbs were performed, no expression of the precursor polypeptide for GnIH mRNA was observed, suggesting that the immunohistochemical reaction was possibly a result of antibody cross-reactivity. Sawada *et al.* (37) obtained similar results with this antiserum in goldfish and suggested that their findings could be the result of a low expression of goldfish LPXRFa peptide mRNA or a cross-reaction with an unknown peptide from the RF-amide family. By contrast, a recent study conducted in sea bass suggests that GnIH is expressed in that region (34).

Because the main objective of our laboratory is to study the neuroendocrine control of reproduction and somatic growth in a social cichlid fish, we investigated the possible effects of putative *C. dimerus* GnIHs (cd-LPQRFa-1 and -2) on the release of gonadotrophins and GH. As a first step, we performed *in vitro* studies where entire pituitaries were exposed to different concentrations of synthetic cd-LPQRFa-1 and -2. An inhibitory effect on the release of both gonadotrophins was observed when pituitaries were exposed to cd-LPQRFa-1. These results indicate that at least cd-LPQRFa-1 may act as GnIH in this fish. The functional roles of GnIH in the regulation of reproduction in fish thus remain controversial. Both inhibitory and stimulatory effects on gonadotrophin synthesis and/or release have been reported in other fish. There is also one study (14) showing that zebrafish GnIH peptide, zfLPXRF-3, also has an inhibitory effect on gonadotrophin release. Wang *et al.* (16) observed that grouper GnIH peptides decrease

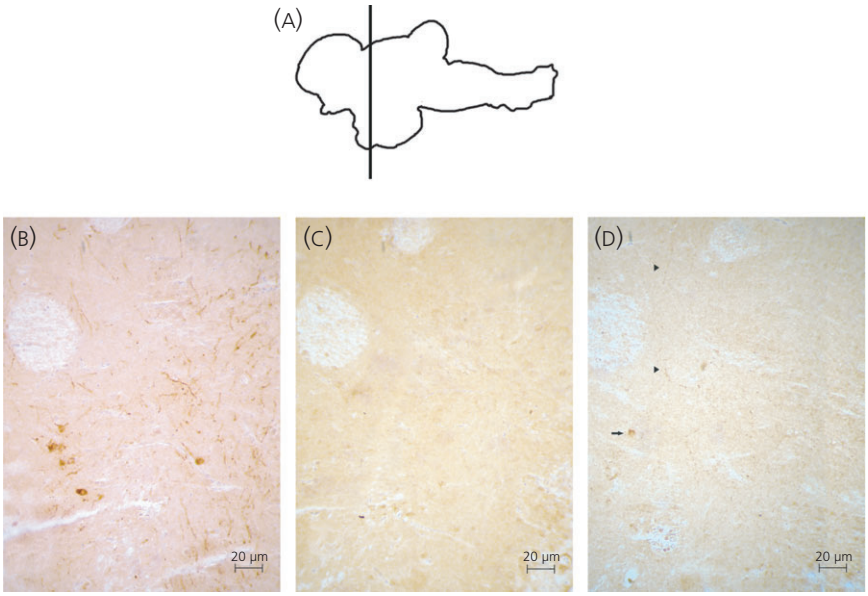

Fig. 3. Preadsorption test of anti-gonadotrophin-inhibitory hormone (GnIH) antisera with an excess of synthetic cd-LPQRFa-1 or cd-LPQRFa-2 individually in immediately serial sections of the brain of male of *Cichlasoma dimerus*. (A) Schematic parasagittal section of *C. dimerus* brain. The line shows the position of the coronal section of the hypothalamus. (B) Microphotography section showing GnIH-immunoreactive somata and fibres in the nucleus posterioris periventricularis and the corresponding preadsorption tests of anti-GnIH antisera with an excess of synthetic cd-LPQRFa-1 (C) or cd-LPQRFa-2 (D), where GnIH-immunoreactive somata (arrow) and few fibres (arrowhead) were detected. Scale bar = 20 μm.

the activity of the forskolin-induced CRE promoter in COS-7 cells transfected with grouper GnIH receptor, suggesting an inhibitory effect of GnIH. By contrast, in the few studies conducted in fish using primary pituitary cell culture, goldfish GnIH peptides, gfLPXRFa-1, -2 and -3, showed no effect or a stimulatory effect on the synthesis and/or release of LH or FSH in sockeye salmon (17), grass puffer (18), goldfish (12,47) and tilapia (in this last case with tilapia-LPXRFa-2) (13). These different effects on gonadotrophin synthesis and/or release may depend on the reproductive conditions in fish (12,47), the use of nonspecies-specific peptides (17,18) or the experimental approach used: primary cell culture (12,13,17,18,47) versus intact pituitary culture (present study). The use of *in vitro* studies with intact pituitaries allows us investigate both the direct and indirect effects of GnIH peptide, without discriminating between them, because nerve terminals remain in the neurohypophysis and the tissue structure is conserved. It is known that GnIH receptor is expressed in the pituitary in all of the fish species studied (12–14,16,18) and so it possible that this receptor is expressed in nerve terminals ending on pituitary cells. Taking into account the lack of pituitary GnIH-immunoreactive fibres reported in our model species, it is possible that the indirect effects are those that prevail with respect to the regulation of pituitary hormones in *C. dimerus*, although direct effects cannot be ruled out. Our *in vitro* data concerning cd-LPQRFa-1 are in accordance with most of the *in vivo* studies carried out in zebrafish (14), goldfish (12,47) and the orange-spotted grouper (16), as well as other vertebrates, such as birds and mammals (8,10,48), in which GnIH showed an inhibitory effect on gonadotrophin release.

Interestingly, in contrast to the inhibitory effect of cd-LPQRFa-1 on gonadotrophins, cd-LPQRFa-2 acted as a stimulatory factor with respect to FSH release in *C. dimerus*, as previously observed in sockeye salmon (17) and tilapia (13). To our knowledge, the present study is the first to show opposite effects between two GnIH peptides on FSH secretion in the same species. Accordingly, it would be important to study the *in vivo* effect of these peptides on pituitary hormones, especially on FSH secretion and/or expression.

In addition to the inhibitory effect on the release of gonadotrophins, in the present study, cd-LPQRFa-1 stimulated GH release. Similar results were obtained in goldfish (19), bullfrog (49), sockeye salmon (17) and rats (40), although not in other species such as sheep (50) or tilapia (13). However, it is important to emphasise that Moussavi *et al.* (19) partially explained the long-term stimulatory effect of GnIH on GH secretion by a rebound increase in GH secretion that follows the removal of the neuropeptide in perifused dispersed pituitary cells in goldfish.

The results reported in the present study indicate that GnIH is involved not only in the multifactorial control of gonadotrophin and GH secretion, but also probably in the cross-talk between growth and reproduction in *C. dimerus*. Moreover, because GH plays a role in reproduction, promoting gametogenesis and gonadal steroidogenesis in teleost fish (51), GnIH could also be involved in homeostasis within the reproductive function in this fish species, as previously proposed for other species (19,41).

In conclusion, the results of the present study indicate that cd-LPQRFa-1, a cichlid fish GnIH, inhibits LH and FSH release *in vitro* and stimulates GH release using intact pituitary cultures. Moreover,

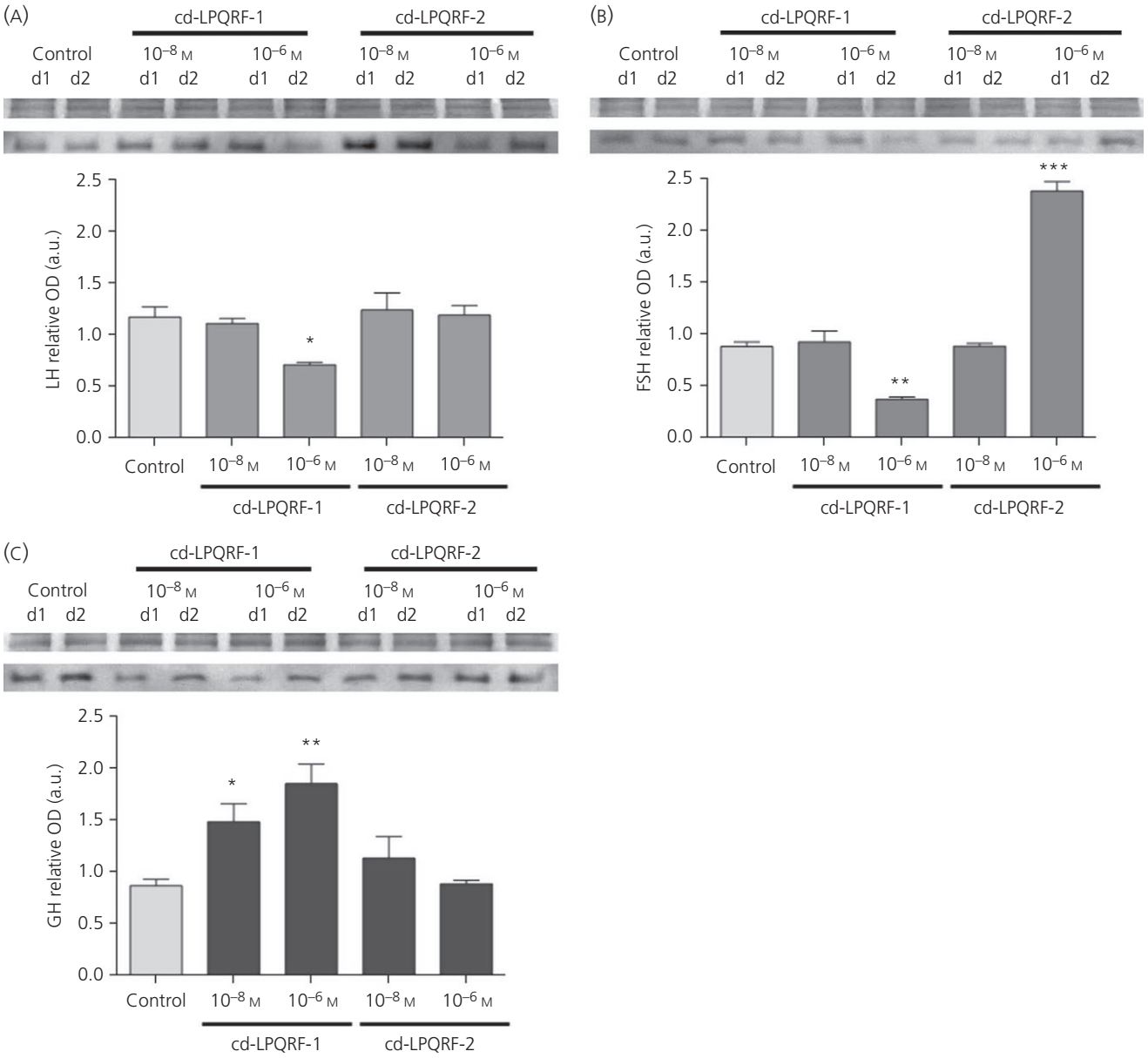

Fig. 4. *In vitro* effects of cd-LPQRF-1 and -2 on β-LH, β-FSH, and GH release from pituitaries of adults of *Cichlasoma dimerus*. (A) Representative Ponceau-S band (loading control) (upper band) and the inmunoreactive band (lower band) of one experiment. Semiquantitative analysis from five independent experiments of β-luteinising hormone (LH) release into the culture media from pituitaries from day 1 (d1) and after treatment with cd-LPQRF-1 and -2 (0, 10^{-6} and 10^{-8} M), on day 2 (d2). (B) Representative Ponceau-S band (loading control) (upper band) and the inmunoreactive band (lower band) of one experiment. Semiquantitative analysis from five independent experiments of β-follicle-stimulating hormone (FSH) release into the culture media from pituitaries from day 1 (d1) and after treatment with cd-LPQRF-1 and -2 (0, 10^{-6} and 10^{-8} M), on day 2 (d2). (C) Representative Ponceau-S band, (loading control) (upper band) and the inmunoreactive band (lower band) of one experiment. Semiquantitative analysis from five independent experiments of growth hormone (GH) release into the culture media from pituitaries from day 1 (d1) and after treatment with cd-LPQRF-1 and -2 (0, 10^{-6} and 10^{-8} M), on day 2 (d2). Values are expressed in arbitrary units (a.u.) as the mean ± SEM of optical density of hormonal release on day 2/optical density of hormonal release on day 1 (relative optical density; OD). Results are shown as the mean ± SEM (n = 5). *P < 0.05; **P < 0.01; ***P < 0.001.

cd-LPQRF-2 could act as an FSH-releasing factor in *C. dimerus*. The results of the present study comprise a starting point for future experiments investigating the functional role of cd-LPQRFa peptides in reproduction, as well as feeding behaviour, which is an aspect of fish physiology that remains to be explored.

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