

Growth hormone, luteinizing hormone, and follicle-stimulating hormone regulation by neuropeptide Y in both sexes of the cichlid fish, *Cichlasoma dimerus*

M. P. Di Yorio · T. H. Delgadin ·
D. I. Pérez Sirkin · P. G. Vissio

Received: 25 July 2014 / Accepted: 7 April 2015 / Published online: 14 April 2015
© Springer Science+Business Media Dordrecht 2015

Abstract Neuropeptide Y (NPY) is considered the most potent orexigenic peptide, increasing before meal time and during fasting. In teleost, most studies on NPY action upon growth hormone (GH) and luteinizing hormone (LH) were conducted in females or group of animals without sex discrimination. The aim of this study was to evaluate whether NPY modulates the expression and release of GH and gonadotropins in both sexes of *Cichlasoma dimerus*. By double-label immunofluorescence, we first determined the association between NPY fibers and pituitary cells. In addition, we performed in vitro studies to evaluate the effect of NPY on GH and gonadotropins expression by real-time PCR, and release by Western blot, in males and females separately. Contacts between NPY fibers and GH and follicle-stimulating hormone (FSH)-producing cells were detected, indicating possible functional relationships. We observed an increase in GH release in the culture medium at 2 nM for males ($p = 0.043$) and 20 nM for females

($p = 0.028$). Pituitary FSH release was stimulated at 20 nM ($p = 0.026$) and 200 nM ($p = 0.033$) for males and females, respectively. Finally, NPY only increased β -LH mRNA expression at 20 nM in females ($p = 0.028$) and its release at 2 nM ($p = 0.049$) and 200 nM for males ($p = 0.005$) and 200 nM for females ($p = 0.018$). In conclusion, NPY acts as a GH-, LH- and FSH-releasing factor, in a dose- and sex-dependent way.

Keywords NPY · GH · FSH · LH · Cichlids

Introduction

In vertebrates, the nutritional status has an impact in somatic growth and reproduction. The link between energy balance and both processes is ensured by several metabolic signals such as NPY, the most potent orexigenic factor described in mammals (Kalra et al. 1999; Valassi et al. 2008). NPY shows remarkable sequence homology from fish to mammals (Larhammar 1996), and several studies strongly suggest that NPY is also an orexigenic peptide in fish (reviewed in Volkoff et al. 2010; Matsuda et al. 2012).

In addition to its major role, this neuropeptide is involved in the regulation of pituitary hormones related to somatic growth and reproduction in teleost fish, at least for females in different stages of the reproductive cycle (Kah et al. 1989; Breton et al. 1989, 1991; Danger et al. 1991; Peng et al. 1990,

M. P. Di Yorio · T. H. Delgadin · D. I. Pérez Sirkin ·
P. G. Vissio (✉)
Laboratorio de Neuroendocrinología del Crecimiento y la
Reproducción, DBBE, FCEN-UBA, Ciudad Universitaria,
C1428EHA Buenos Aires, Argentina
e-mail: pvissio@gmail.com

M. P. Di Yorio · T. H. Delgadin · D. I. Pérez Sirkin ·
P. G. Vissio
IBBEA-CONICET-UBA, Ciudad Universitaria,
C1428EHA Buenos Aires, Argentina

1993a, b, c; Cerdá-Reverter et al. 1999; Peyon et al. 2001; Gaikwad et al. 2003; Mazumdar et al. 2006; Wu et al. 2012). Several studies have reported that the administration in vivo (Breton et al. 1991; Peng et al. 1993b; Gaikwad et al. 2003; Mazumdar et al. 2006) or incubation in vitro (Kah et al. 1989; Breton et al. 1989; Danger et al. 1991; Peng et al. 1990, 1993a, c; Peyon et al. 2001) with different NPY concentrations increased growth hormone (GH) and luteinizing hormone (LH) release. Moreover, this effect is dependent on sex steroids (Breton et al. 1989, 1991; Danger et al. 1991; Peng et al. 1990, 1993c; Peyon et al. 2001; Chang and Habibi 2002) and nutritional status (Cerdá-Reverter et al. 1999), and its action is exerted mainly through the interaction with NPY Y1- or Y2-like receptors (Peng et al. 1993a; Mazumdar et al. 2006). On the other hand, only few works have studied the effect of NPY on β -LH and GH RNA messenger expression in vivo (Wu et al. 2012) and in vitro (Gur et al. 2002; Wu et al. 2012), and in both cases, NPY increased their expression. In regard to follicle-stimulating hormone (FSH), to the best of our knowledge, there is only one report on the action of NPY on β -FSH gene transcription in which no effect was observed (Gur et al. 2002). Taking into account that all these works have been conducted in females in different reproductive stages or in animals without sex discrimination, the question arises whether there is a sex-dependent response of pituitary hormones to NPY.

In previous studies, localization of pituitary cell populations were described in the South American cichlid fish *Cichlasoma dimerus* (Pandolfi et al. 2001). Particularly, somatotrophs form a layer surrounding neurohypophyseal projections in the proximal *pars distalis*. Gonadotrophs were mainly localized in central, ventral, and marginal zones in the *pars distalis*, and the external border of the *pars intermedia*. Pérez Sirkin et al. (2013) determined the distribution of NPY immunoreactive cells and fibers in the preoptic area and the hypothalamus in adult *C. dimerus*. Also, numerous NPY immunoreactive fibers were observed in the neurohypophysis at the *pars distalis* level (Pérez Sirkin et al. 2013). Thus, the aim of this study was to evaluate whether NPY differentially modulates the expression and release of LH, FSH, and GH in both sexes of *C. dimerus*. First, we studied possible morphological relationships between NPY fibers and these pituitary cells. Then, we

performed in vitro studies to evaluate the effect of NPY on GH and gonadotropins synthesis and release.

Materials and methods

Animals

Adults of *C. dimerus* were captured in “Esteros del Riachuelo”, Corrientes, Argentina (27°12'50''S, 58°11'50''W), transferred to the laboratory, and maintained in 400-l freshwater aquaria at 25 ± 2 °C under a 14 L: 10-D photoperiod where they were daily fed with commercial pellets (Tetra Pond Variety Blend) prior to the experiments.

In all the experiments, we used sexually mature fish with total length 9.38 ± 0.68 cm, weight 19.4 ± 4.7 g, and gonadosomatic index (GSI) was 0.073 ± 0.038 for males and 4.25 ± 1.75 for females.

Animals were handled according to 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), and the experimental protocols were counted with the approval from the “Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina” (Protocol number 26).

Double-label immunofluorescence

Adults of *C. dimerus* were anesthetized with benzocaine 0.1 % and euthanized by decapitation, and brains with the pituitary attached were fixed for 18 h in Bouin’s solution as it was previously described by Cánepa et al. (2006). Then, they were embedded in paraplast (Fisherbrand, Fisher, WA, USA) and cut at the pituitary level into serially consecutive parasagittal sections at 10- μ m intervals. Sections were mounted on charged slides, deparaffinized in xylene, rehydrated through a graded ethanol gradient to phosphate-buffered saline (PBS, pH 7.4). For β -FSH immunodetection, sections were pretreated for epitope unmasking with citrate buffer 10 mM, pH 6 at 90 °C for 10 min, cooled at room temperature (RT), and washed in PBS (Pandolfi et al. 2006). All sections were incubated with PBS containing 5 % nonfat dry milk (blocking solution) at RT for 1 h. Next, they were incubated with rabbit anti-porcine NPY antisera

(1:2500 in PBS, Peninsula Laboratories Inc., CA, USA) at RT overnight. Then, sections were washed in PBS and incubated with biotinylated anti-rabbit IgG (1:500 in PBS, Sigma-Aldrich) at RT for 1 h. Afterward, they were incubated with Alexa Fluor 546 streptavidin (1:100 in PBS, Invitrogen, Carlsbad, CA, USA) (red) for 90 min. Subsequently, sections were incubated with rabbit anti-chum salmon GH antisera (1:1000 in PBS, lot 8502), anti-*Fundulus heteroclitus* β -LH antisera (1:500 in PBS), or anti-*Fundulus heteroclitus* β -FSH antisera (1:500 in PBS) at RT overnight. After washing slides in PBS, they were incubated with an fluorescein-conjugated anti-rabbit IgG (1:30 in PBS, Sigma-Aldrich, St. Louis, USA) (green) at RT for 90 min. Finally, they were mounted in PBS–glycerin (1:1) and analyzed by confocal laser microscopy (Olympus FV-30 attached to an Olympus Bx61 microscope).

This double-label immunofluorescence system with two primary antibodies raised in rabbit was performed since NPY immunoreactive (ir)-fibers and pituitary ir-cells are clearly identifiable from each other. Specificity controls were previously performed in our laboratory by preadsorption of primary antibody with an excess of its antigen (Pandolfi et al. 2001, 2006; Pérez Sirkin et al. 2013). In this work, additional controls were performed by omitting primary antibodies. Both tests showed no positive immunostaining which demonstrated the specificity of the antisera.

In vitro studies

Effect of NPY on hormone release

Adults of *C. dimerus* were euthanized by decapitation under anesthesia (benzocaine 0.1 %), and pituitaries were removed from males and females. Sampling procedures were similar to those described previously by Cánepa et al. (2008). Briefly, four glands for each sex were removed and placed individually in a 96-multiwell plates containing 80 % (v/v) Leibovitz L15 medium (Gibco, Invitrogen, Carlsbad, CA, USA), pH 7.4, 10 % fetal bovine serum, 10 mM Hepes, 100 IU/ml penicillin, and 100 μ g/ml of streptomycin, and maintained in a dark incubator at 27 °C. After 3 h preincubation, the medium was replaced by fresh medium, and pituitaries were incubated for 24 h in the same conditions (day 1). Later, the medium was recovered and stored at –20 °C, with 1 μ l of protease

inhibitor cocktail (Sigma-Aldrich), in order to establish basal conditions of hormone release. Pituitaries were then randomly incubated in the absence or presence of porcine NPY at 2, 20, or 200 nM (Bachem, Bubendorf, Switzerland, Cat No. H-4430). After 24 h (day 2), each medium was removed and stored frozen, with 1 μ l of protease inhibitor cocktail (Sigma-Aldrich), in order to establish stimulatory conditions of hormone release. Sexes were analyzed separately. Each experiment was repeated four times ($n = 4$) for each sex.

Effect of NPY on hormone mRNA expression

Pituitaries were removed from males and females of *C. dimerus* and individually placed in wells with cultured medium in the same conditions as described above. After 3 h preincubation, medium was replaced by fresh medium as an internal control, or medium containing porcine NPY (Bachem; 2, 20, 200 nM). Then, the plates were incubated in a dark incubator at 27 °C for 4 h. Later, pituitaries were individually collected and were immediately homogenized in 500 μ l of TRI Reagent (MRC, Inc., Cincinnati, USA) for further analysis. Sexes were analyzed separately. Each experiment was repeated four times ($n = 4$) for each sex.

Western blot

Western blot analysis with heterologous antisera mentioned above was used to determine hormone release into the culture medium as it was described in detail in Cánepa et al. (2008). Briefly, samples (15 μ l) from each pituitary culture medium diluted in 5 \times sample buffer (120 mM Tris Base, pH: 6.8, 3 % dodecylsulfate, 10 % glycerol, and 1 % β -mercaptoethanol) were subjected to 15 % sodium dodecyl-sulfatepolyacrylamide gels electrophoresis (SDS-PAGE). The proteins and molecular markers (Genbiotech, Buenos Aires, Argentina) were then transferred onto a nitrocellulose membrane (Amersham Biosciences, UK) at 90 V for 1 h. Membranes were washed in Tris-buffered saline with Tween (TBST) at pH 7.5 (100 mM Tris–HCl, 0.9 % NaCl, 0.1 % Tween-20) and blocked with TBST containing 5 % nonfat dry milk at RT for 1 h. Then, they were incubated with GH antiserum (anti-chum salmon GH, 1:2000 in TBST), β -LH antiserum (*Fundulus*

heteroclitus, 1:1000 in TBST), or β -FSH antiserum (*F. heteroclitus*, 1:1000 in TBST) at 4 °C overnight. After three washes in TBST, membranes were incubated with a biotinylated anti-rabbit IgG (1:2000 in TBST) at RT for 1 h, washed again, and then incubated with peroxidase-conjugated streptavidin (1:1500 in TBST) at RT for 1 h. Ir-bands were visualized using chemiluminescence detection reagents (Sigma-Aldrich) and luminescent image analyzer LAS-1000 plus (Fuji Photo Film). In all cases, blotting and developing conditions were repeated twice.

The GH, β -LH, and β -FSH ir-bands from different treatments were semi-quantified by densitometric analysis with ImageJ Software and normalized against a 148-kDa protein only present in culture medium, in order to correct possible variations in the SDS-PAGE loading. The 148-kDa band was visualized by Ponceau-S in the nitrocellulose membrane and digitalized, and its optical density was quantified with ImageJ Software.

Hormone release from each pituitary was evaluated as follows: 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, and thus the optical density values of each ir-band from day 2 were normalized to those from day 1. This normalization allowed us to compare values obtained from different pituitaries.

Quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was obtained by using TRI Reagent (MRC) following manufacturer's instructions. Then, 0.5 μ g of total RNA was treated with DNase I (Sigma-Aldrich), and cDNA was synthesized using random primers (Genbiotech) and MMLV enzyme (Promega, Madison, WI, USA) as described previously by Pérez Sirkin et al. (2013).

GH, β -FSH, β -LH, and 18S ribosomal RNA transcripts levels were quantified by fluorescent-based RT-qPCR using cDNA products diluted 1:10 in autoclaved distilled water. RT-qPCR was performed by using FastStart Universal SyBR green Master (5 μ l, ROCHE) with a mixture of forward- and reverse-specific primers designed from sequences previously obtained (1.5 μ l; primers concentrations: 0.6 μ M for GH and β -LH; 1 μ M for FSH, 18S; Table 1), 2.5 μ l of cDNA template and 1 μ l of autoclaved distilled water

per tube (Pérez Sirkin et al. 2013). The RT-qPCR protocol was as follows: initial denaturation at 95 °C for 10 min and 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 20 s. Each sample was run in duplicate. Negative controls were performed by template omission. For each gene amplification, a single melting peak was obtained and their efficiencies were close to 100 %. The expression of 18S was stable among the replication measures, and it was used as reference gene because of its minimum variation between treatments (data not shown). The starting concentration (N0) per sample was calculated by LinRegPCR as initial fluorescence in arbitrary units which takes into account PCR efficiencies per sample, amplicon, and tissue. N0 was used as the response variable (Ramakers et al. 2003; Ruijter et al. 2009).

Statistic analysis

For hormone release in the culture medium and RT-qPCR data (N0), comparisons between group means were made by using one-way ANOVAs followed by Dunnett's multiple comparison test. Some variable were transformed to $\ln(x)$ in order to meet normality and homoscedasticity assumptions. Statistical significance was established at the $p < 0.05$ level. Data are presented as mean \pm SEM (standard error of the mean).

Results

Double-label immunofluorescence

As a first approach, double-label immunofluorescences were performed to elucidate possible contacts between NPY fibers and LH, FSH, and GH cells. We observed NPY ir-fibers at proximal *pars distalis* level, particularly in the region near the somatotrophic cells. Specifically, a great number of contacts were detected between NPY ir-fibers and GH ir-cells (Fig. 1a–d). Few contacts were observed between NPY ir-fibers and FSH ir-cells (Fig. 1e–h), and no contacts with LH ir-cells were found (data not shown).

In vitro pituitary cultures

To investigate a possible effect of NPY over the expression and release of GH, LH, and FSH, cultures

Table 1 Primers used for mRNA expression by real-time PCR

Name	Forward primer (5' → 3')	Reverse primer (5' → 3')	GenBank accession No.
FSH	GTGAAGGACAGTGCTACCAG	GGACATCGCTCTGTGTACTTC	EU315918
GH	GTCAGTCGTGTGTTGGGTGTC	CGAGCAGGTGGAGGTGTTGG	HQ660075
LH	ACACTGCATCACCAAGGAC	ACAGTCGGGAAGCTCAAATG	EU315919
18S	GGAGTATGGTTGCAAAGCTG	TCTGTCAATCCTTTCCGTGTC	Unpublished

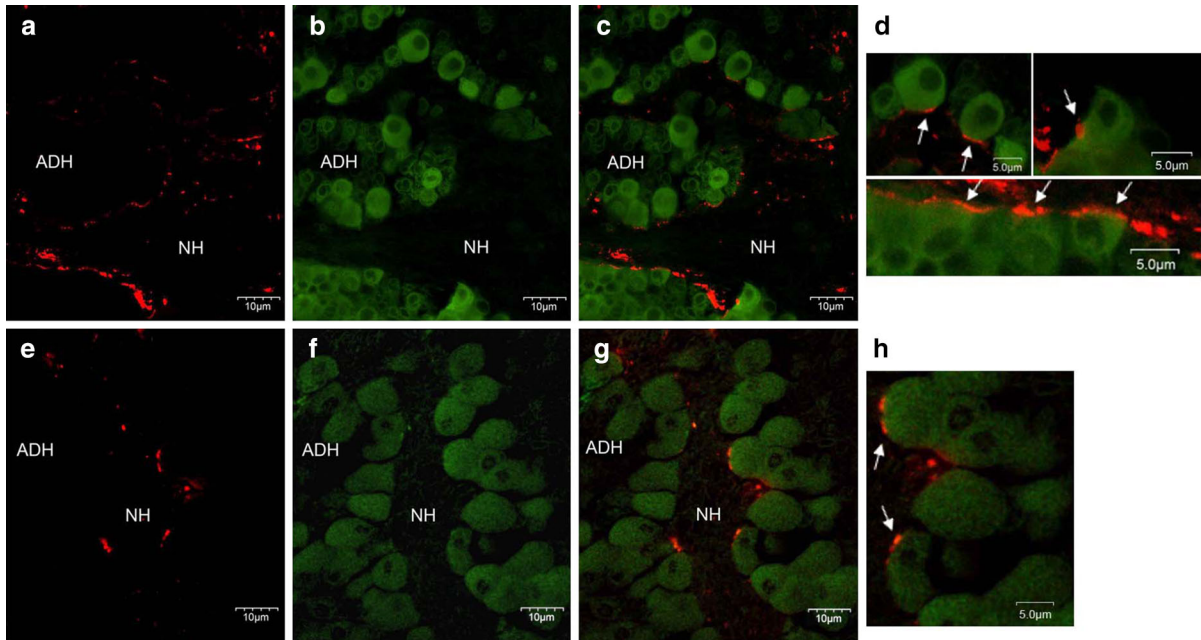


Fig. 1 Contacts between NPY fibers and GH or β -FSH cells detected by double-label immunofluorescence in parasagittal sections of *C. dimerus* pituitaries. **a** NPY fibers (red) in the neurohypophysis. Scale bar 10.0 μ m. **b** GH ir-cells (green) in the proximal *pars distalis* of the ADH. Scale bar 10.0 μ m. **c** Overlay of **a** and **b** showing numerous NPY ir-fibers in close association with GH ir-cells. Scale bar 10.0 μ m. **d** Detail of NPY fibers and GH cells contact (white arrows). Scale bar

5.0 μ m. **e** NPY fibers (red) in the neurohypophysis. Scale bar 10.0 μ m. **f** β -FSH ir-cells (green) in the proximal *pars distalis* of the ADH. Scale bar 10.0 μ m. **g** Overlay of **a** and **b**, showing few NPY ir-fibers in close association with β -FSH ir-cells. Scale bar 10.0 μ m. **h** Detail of NPY fibers and FSH cells contact (white arrows). Scale bar 5.0 μ m. NH: neurohypophysis, ADH: adenohypophysis. (Color figure online)

of intact pituitary glands from females and males were challenged against different NPY concentrations. Although no change in GH mRNA expression was observed in the presence of NPY (Fig. 2a, b), there was an increase in GH release to the culture medium at 2 nM for males ($p = 0.043$ vs. control, Fig. 2c) and 20 nM for females ($p = 0.028$ vs. control, Fig. 2d). Pituitary β -FSH release showed an increase at 20 nM for males ($p = 0.026$ vs. control, Fig. 3c) and 200 nM for females ($p = 0.033$ vs. control, Fig. 3d), without changes in β -FSH mRNA expression at any concentration assayed (Fig. 3a, b). Finally, NPY increased

the expression of β -LH mRNA at 20 nM only in females ($p = 0.028$; Fig. 4a, b) and its release at 2 nM ($p = 0.049$) and 200 nM ($p = 0.005$) for males and 200 nM for females ($p = 0.018$) compared with each control (Fig. 4c, d, respectively).

Discussion

From the first reports of Breton et al. (1989, 1991) and Peng et al. (1990, 1993a, b, c) to more recent works by Mazumdar et al. (2006) and Wu et al. (2012), NPY has

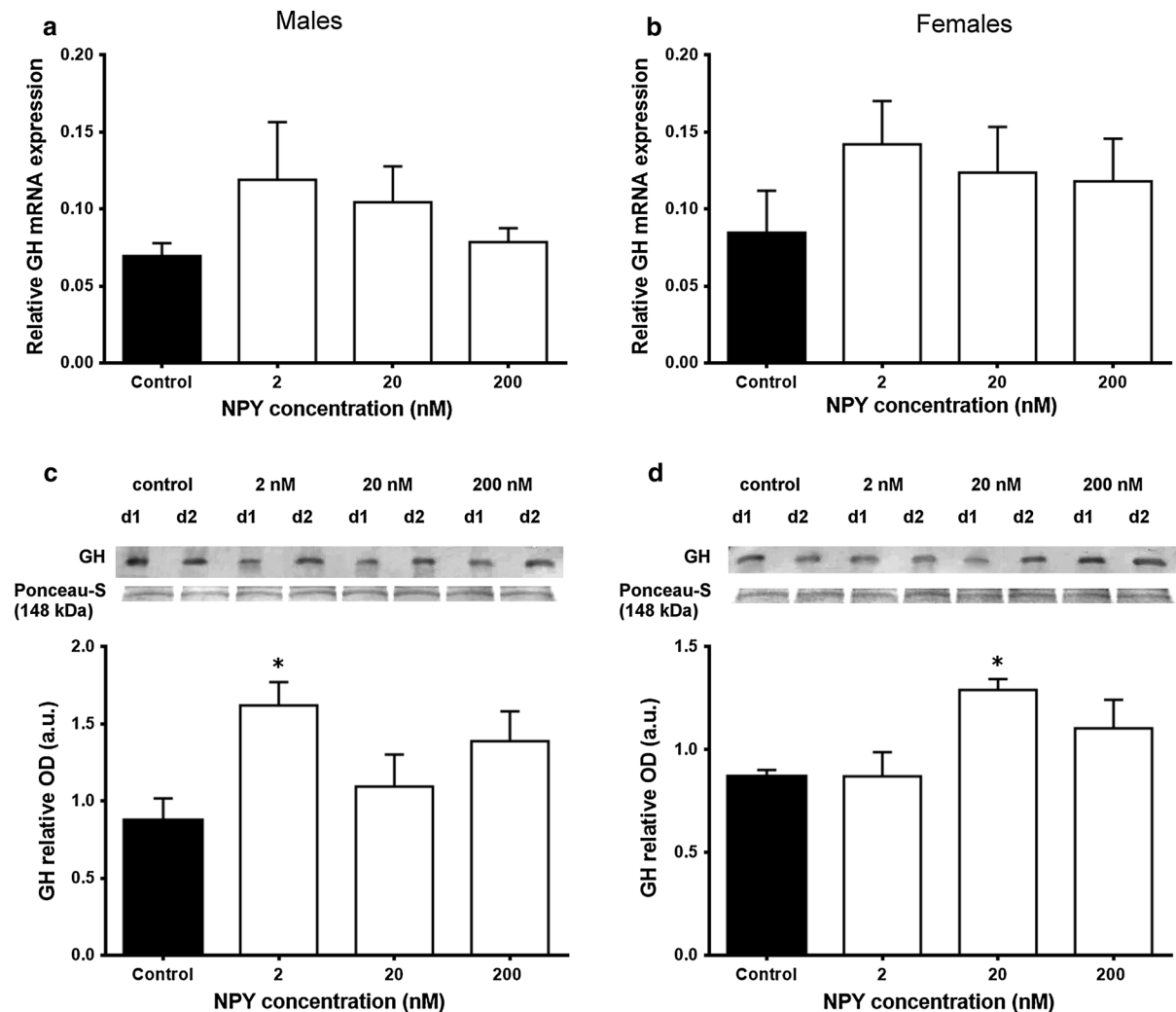


Fig. 2 In vitro effect of NPY on GH mRNA expression and protein release from pituitaries of *C. dimerus* males (left) and females (right). **a, b** Analysis of GH mRNA expression after 4 h incubation of pituitaries from males (**a**) and females (**b**) in either the absence (control) or the presence of increasing concentrations of NPY (2–200 nM). GH mRNA was normalized against 18S gene expression. **c, d** Representative immunoblots and

semiquantitative analysis of GH release to the culture media from males (**c**) and females (**d**) pituitaries from day 1 (d1) and following treatment with NPY (2–200 nM), day 2 (d2). Values are expressed in arbitrary units (a.u.) as mean \pm SEM of optical density of GH released on day 2/optical density of GH released on day 1 (relative optical density, OD). Results are presented as mean \pm SEM ($n = 4$). * $p < 0.05$

been considered as a GH- and LH-releasing factor only in females and on a given stage of the reproductive cycle. Moreover, limited information is available on NPY modulation of FSH in fish. The response of these pituitary hormones to NPY stimulation has not been analyzed taking into account possible differences between sexes. In the present work, we evaluated whether NPY modulates the expression and release of LH, FSH, and GH in both sexes of *C. dimerus*. Two experimental approaches were conducted to

accomplish this objective. First, we performed double-label immunofluorescences between NPY fibers and each pituitary cell type. A great number of contacts were seen between NPY fibers and GH cells, whereas few contacts between NPY fibers and FSH cells and none with LH cells were observed. These results suggested that NPY could act directly upon somatotrophs and indirectly upon gonatotrophs, or if NPY acts directly over both cell populations, the effect on GH could be greater than that on gonatotrophs. On

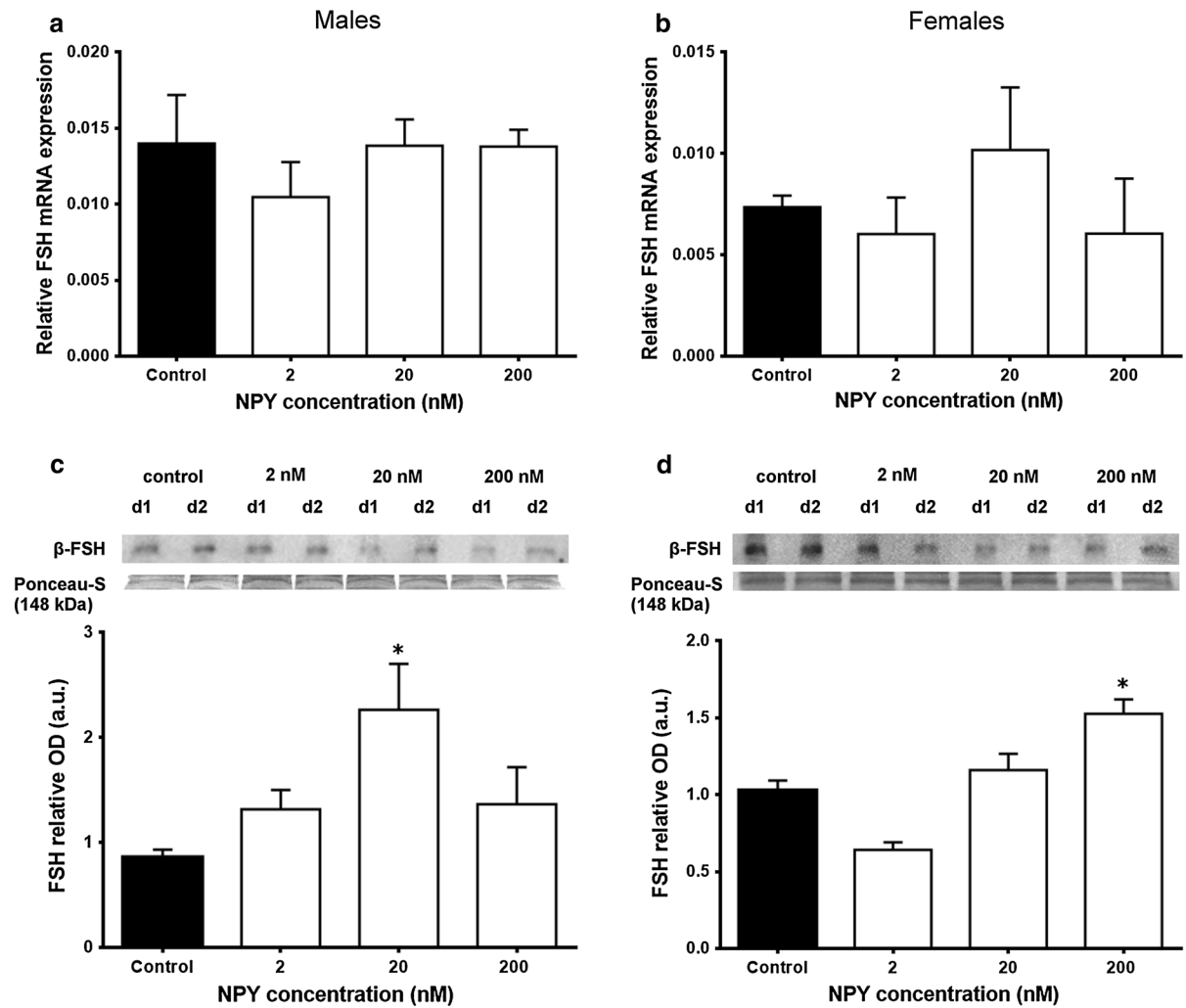


Fig. 3 In vitro effect of NPY on β -FSH mRNA expression and protein release from pituitaries of *C. dimerus* males (left) and females (right). **a, b** Analysis of β -FSH mRNA expression after 4 h incubation of pituitaries from males (**a**) and females (**b**) in either the absence (control) or the presence of increasing concentrations of NPY (2–200 nM). β -FSH mRNA was normalized against 18S gene expression. **c, d** Representative immunoblots and semiquantitative analysis of β -FSH release to

the culture media from males (**c**) and females (**d**) pituitaries from day 1 (d1) and following treatment with NPY (2–200 nM), day 2 (d2). Values are expressed in arbitrary units (a.u.) as mean \pm SEM of optical density of β -FSH released on day 2/optical density of β -FSH released on day 1 (relative optical density, OD). Results are presented as mean \pm SEM ($n = 4$). * $p < 0.05$

the contrary, in other studies conducted in different species, it has been shown that NPY fibers contact LH (gonadotropin II)-producing cells (goldfish, Kah et al. 1989; catfish (*Clarias batrachus*), Gaikwad et al. 2003; Subhedra et al. 2005). Possible species-specific or technical differences, including antibodies used, could exist. In this work, morphological studies were performed with specific antibodies for each gonadotropin, and no cross-reaction between them was

observed (Pandolfi et al. 2006). As far as we know, this is the first report that shows NPY fibers in close contact mainly with GH and to a lesser extent with FSH-producing cells by double-label immunofluorescence.

A possible effect of NPY on GH, FSH, and LH synthesis and release was analyzed by performing in vitro culture studies of females and males pituitary glands separately. These assays showed an NPY-releasing effect on GH and LH in *C. dimerus* females,

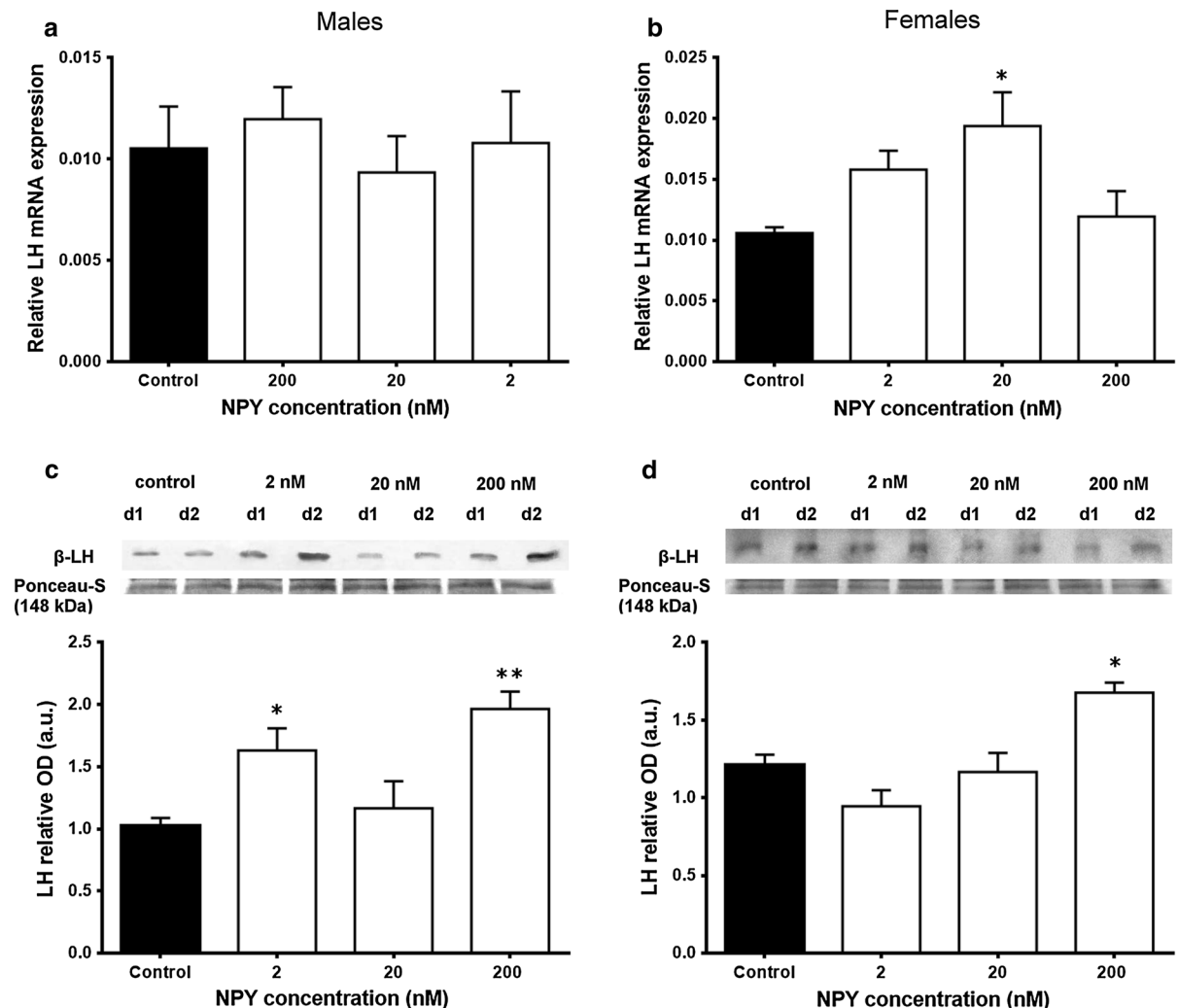


Fig. 4 In vitro effect of NPY on β -LH mRNA expression and protein release from pituitaries of *C. dimerus* males (left) and females (right). **a, b** Analysis of β -LH mRNA expression after 4 h incubation of pituitaries from males (**a**) and females (**b**) in either the absence (control) or the presence of increasing concentrations of NPY (2–200 nM). β -LH mRNA was normalized against 18S gene expression. **c, d** Representative immunoblots and semiquantitative analysis of β -LH release to the

culture media from males (**c**) and females (**d**) pituitaries from day 1 (d1) and following treatment with NPY (2–200 nM), day 2 (d2). Values are expressed in arbitrary units (a.u.) as mean \pm SEM of optical density of β -LH released on day 2/optical density of β -LH released on day 1 (relative optical density, OD). Results are presented as mean \pm SEM ($n = 4$). * $p < 0.05$; ** $p < 0.01$

confirming in vivo (Breton et al. 1991; Peng et al. 1993b; Mazumdar et al. 2006) and in vitro (Kah et al. 1989; Breton et al. 1989; Danger et al. 1991; Peng et al. 1990, 1993a, c; Cerdá-Reverter et al. 1999; Peyon et al. 2001) studies conducted in other species. In turn, in this work, a lower concentration of NPY was needed to stimulate GH release in females. These results were similar to those obtained by Peng et al. (1990, 1993a) in goldfish. In addition, NPY increased

β -LH mRNA expression in females as reported by Gur et al. (2002), who also observed that NPY effect was mediated via protein kinase C (PKC), which in turn activated MAPK kinase (MEK)-extracellular signal-regulated kinase (ERK) cascade. Furthermore, in the present work, we observed a stimulatory effect of NPY on GH and LH release in males, without changing its mRNA expression. We observed that LH increased at the lowest and highest concentrations assayed for

males. Although this observation is difficult to explain, one possibility is that at least two types of NPY receptors, with different sensitivity, are implicated in the direct and/or indirect action of NPY over LH secretion, as it was suggested by Peng et al. (1993a) and Mazumdar et al. (2006).

The effect of NPY on FSH release was unexplored in teleost fish until now. In the present study, NPY stimulated FSH release in males and females without changes in its expression. These last results agree with those obtained by Gur et al. (2002) where NPY did not stimulate β -FSH mRNA. The expression of β -FSH seems to be dependent on gonadotropin-releasing hormone (GnRH), activin, inhibin, and steroids levels rather than neuropeptides in both sexes (Yaron et al. 2003). To the best of our knowledge, this is the first report that shows that NPY could act as a FSH-releasing factor in both males and females fish.

Considering the fact that GH responded at lower NPY concentrations assayed than both gonadotropins, specially in females, along with the results obtained in morphological studies, NPY seems to exert a direct effect on GH release. However, we cannot rule out possible indirect actions of NPY through GnRH or somatostatin neurons as it has been seen by other authors (Peng et al. 1993a, b; Gaikwad et al. 2003, 2005; Subhedar et al. 2005).

Interestingly, although NPY effect is stimulatory in males and females, there is a difference between NPY concentrations that proved to be effective in both sexes. Specifically, male pituitaries were more sensitive since it responded at lower NPY concentrations, in terms of GH and gonadotropins release. Considering that testosterone is more effective than estradiol in enhancing NPY effects on pituitary hormone release (Peng et al. 1993c), differences between testosterone levels in both sexes could be responsible for NPY sensitivity. Furthermore, it has been demonstrated that NPY effects on hormone secretion vary with seasonal changes (Breton et al. 1989; Danger et al. 1991; Peng et al. 1990, 1993c; Peyon et al. 2001, 2003; Senthilkumar et al. 2001) and NPY gene expression in brain areas depends on sex steroids, particularly testosterone (Peng et al. 1994; Sakharkar et al. 2005). In addition, in mammals, it is known that Y1 receptor expression could be dependent on 17β -estradiol as it varies during estrous cycle in rat (Xu et al. 2000). In our study, we failed to measure sex steroids levels due to the low volume of plasma available. For this

reason, we took special attention in selecting animals with similar GSI. Other possibilities that we cannot rule out are the differences in the number and/or abundance of NPY receptor types between sexes. NPY receptor types have been described only in few fish species (Matsuda et al. 2012), and no information about possible sex-dependent differences in receptor type abundance is available in the literature, even in mammals. This could be an interesting issue to elucidate in future studies.

In conclusion, NPY acts as a GH-, LH-, and FSH-releasing factor, in a dose- and sex-dependent way in *C. dimerus*. Compared to gonadotropins, NPY seems to exert a direct effect on GH release. Taking into account these results and previous reports, NPY could be a link among feeding status, reproduction, and growth.

Acknowledgments We thank Dr. Kawauchi for the GH antisera, Dr. Akio Shimizu through Dr. M. Pandolfi for the LH and FSH antisera, and Dr. Gustavo M. Somoza, Dr. Alicia G. Faletti, Mr. Martín R. Ramallo, and Dr. Dante Paz for their valuable contributions. This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (Grant Number: PIP: 0276. P.V.), Agencia Nacional de Promoción Científica y Tecnológica (Grant Number: PICT 2008–2005. P.V.), and Universidad de Buenos Aires (Grant Number: 20020120100280 P.V.).

Conflict of interest The authors declare no conflict of interest.

References

- Breton B, Mikolajczyk T, Danger JM, Gonnet F, Saint-Pierre S, Vaudry H (1989) Neuropeptide Y (NPY) modulates in vitro gonadotropin release from rainbow trout pituitary glands. *Fish Physiol Biochem* 7(1–6):77–83
- Breton B, Mikolajczyk T, Popek W, Bieniarz K, Epler P (1991) Neuropeptide Y stimulates in vivo gonadotropin secretion in teleost fish. *Gen Comp Endocrinol* 84(2):277–283
- Cánepa MM, Pandolfi M, Maggese MC, Vissio PG (2006) Involvement of somatolactin in background adaptation of the cichlid fish *Cichlasoma dimerus*. *J Exp Zool A Comp Exp Biol* 305(5):410–419
- Cánepa M, Pozzi A, Astola A, Maggese MC, Vissio P (2008) Effect of salmon melanin-concentrating hormone and mammalian gonadotrophin-releasing hormone on somatolactin release in pituitary culture of *Cichlasoma dimerus*. *Cell Tissue Res* 333(1):49–59
- Cerdá-Reverter JM, Sorbera LA, Carrillo M, Zanuy S (1999) Energetic dependence of NPY-induced LH secretion in a teleost fish (*Dicentrarchus labrax*). *Am J Physiol* 277(6 Pt 2):R1627–R1634
- Chang JP, Habibi HR (2002) Intracellular integration of multifactorial neuroendocrine regulation of goldfish

- somatotrope functions. In: Small B, MacKinlay D (eds) Developments in understanding fish growth. Symposium proceedings of the international congress on the biology of fish. University of British Columbia, Vancouver, pp 5–14
- Danger JM, Breton B, Vallarino M, Fournier A, Pelletier G, Vaudry H (1991) Neuropeptide-Y in the trout brain and pituitary: localization, characterization, and action on gonadotropin release. *Endocrinology* 128(5):2360–2368
- Gaikwad A, Biju KC, Subhedar N (2003) GnRH-LH secreting cells axis in the pituitary of the teleost *Clarias batrachus* responds to neuropeptide Y treatment: an immunocytochemical study. *Gen Comp Endocrinol* 131(2):126–133
- Gaikwad A, Biju KC, Muthal PL, Saha S, Subhedar N (2005) Role of neuropeptide Y in the regulation of gonadotropin releasing hormone system in the forebrain of *Clarias batrachus* (Linn.): immunocytochemistry and high performance liquid chromatography-electrospray ionization-mass spectrometric analysis. *Neuroscience* 133(1):267–279
- Gur G, Bonfil D, Safarian H, Naor Z, Yaron Z (2002) Pituitary adenylate cyclase activating polypeptide and neuropeptide Y regulation of gonadotropin subunit gene expression in tilapia: role of PKC, PKA and ERK. *Neuroendocrinology* 75(3):164–174
- Kah O, Pontet A, Danger JM, Dubourg P, Pelletier G, Vaudry H, Calas A (1989) Characterization, cerebral distribution and gonadotropin release activity of neuropeptide Y (NPY) in the goldfish. *Fish Physiol Biochem* 7(1–6):69–76
- Kalra SP, Dube MG, Pu S, Xu B, Horvath TL, Kalra PS (1999) Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocr Rev* 20(1):68–100
- Larhammar D (1996) Evolution of neuropeptide Y, peptide YY and pancreatic polypeptide. *Regul Pept* 62(1):1–11
- Matsuda K, Sakashita A, Yokobori E, Azuma M (2012) Neuroendocrine control of feeding behavior and psychomotor activity by neuropeptide Y in fish. *Neuropeptides* 46(6):275–283
- Mazumdar M, Lal B, Sakharkar AJ, Deshmukh M, Singru PS, Subhedar N (2006) Involvement of neuropeptide Y Y1 receptors in the regulation of LH and GH cells in the pituitary of the catfish, *Clarias batrachus*: an immunocytochemical study. *Gen Comp Endocrinol* 149(2):190–196
- Pandolfi M, Paz DA, Maggese C, Meijide FJ, Vissio PG (2001) Immunocytochemical localization of different cell types in the adenohypophysis of the cichlid fish *Cichlasoma dimerus* (Heckel, 1840). *Biocell* 25(1):35–42
- Pandolfi M, Lo Nostro FL, Shimizu A, Pozzi AG, Meijide FJ, Vazquez GR, Maggese MC (2006) Identification of immunoreactive FSH and LH cells in the cichlid fish *Cichlasoma dimerus* during the ontogeny and sexual differentiation. *Anat Embryol (Berl)* 211(5):355–365
- Peng C, Huang YP, Peter RE (1990) Neuropeptide Y stimulates growth hormone and gonadotropin release from the goldfish pituitary in vitro. *Neuroendocrinology* 52(1):28–34
- Peng C, Chang JP, Yu KL, Wong AO, Van Goor F, Peter RE, Rivier JE (1993a) Neuropeptide-Y stimulates growth hormone and gonadotropin-II secretion in the goldfish pituitary: involvement of both presynaptic and pituitary cell actions. *Endocrinology* 132(4):1820–1829
- Peng C, Humphries S, Peter RE, Rivier JE, Blomqvist AG, Larhammar D (1993b) Actions of goldfish neuropeptide Y on the secretion of growth hormone and gonadotropin-II in female goldfish. *Gen Comp Endocrinol* 90(3):306–317
- Peng C, Trudeau VL, Peter RE (1993c) Seasonal variation of neuropeptide Y actions on growth hormone and gonadotropin-II secretion in the goldfish: effects of sex steroids. *J Neuroendocrinol* 5(3):273–280
- Peng C, Gallin W, Peter RE, Blomqvist AG, Larhammar D (1994) Neuropeptide-Y gene expression in the goldfish brain: distribution and regulation by ovarian steroids. *Endocrinology* 134(3):1095–1103
- Pérez Sirkin DI, Suzuki H, Cánepa MM, Vissio PG (2013) Orexin and neuropeptide Y: tissue specific expression and immunoreactivity in the hypothalamus and preoptic area of the cichlid fish *Cichlasoma dimerus*. *Tissue Cell* 45(6):452–459
- Peyon P, Zanuy S, Carrillo M (2001) Action of leptin on in vitro luteinizing hormone release in the European sea bass (*Dicentrarchus labrax*). *Biol Reprod* 65(5):1573–1578
- Peyon P, Vega-Rubín de Celis S, Gómez-Requeni P, Zanuy S, Pérez-Sánchez J, Carrillo M (2003) In vitro effect of leptin on somatolactin release in the European sea bass (*Dicentrarchus labrax*): dependence on the reproductive status and interaction with NPY and GnRH. *Gen Comp Endocrinol* 132(2):284–292
- Ramakers C, Ruijter JM, Deprez RH, Moorman AF (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* 339(1):62–66
- Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, Moorman AF (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res* 37(6):e45
- Sakharkar AJ, Singru PS, Sarkar K, Subhedar NK (2005) Neuropeptide Y in the forebrain of the adult male cichlid fish *Oreochromis mossambicus*: distribution, effects of castration and testosterone replacement. *J Comp Neurol* 489(2):148–165
- Senthilkumaran B, Okuzawa K, Gen K, Kagawa H (2001) Effects of serotonin, GABA and neuropeptide Y on seabream gonadotropin releasing hormone release in vitro from preoptic-anterior hypothalamus and pituitary of red seabream, *Pagrus major*. *J Neuroendocrinol* 13(5):395–400
- Subhedar N, Gaikwad A, Biju KC, Saha S (2005) Role of neuropeptide Y (NPY) in the regulation of reproduction: study based on catfish model. *Fish Physiol Biochem* 31(2–3):167–172
- Valassi E, Scacchi M, Cavagnini F (2008) Neuroendocrine control of food intake. *Nutr Metab Cardiovasc Dis* 18(2):158–168
- Volkoff H, Hoskins LJ, Tuziak SM (2010) Influence of intrinsic signals and environmental cues on the endocrine control of feeding in fish: potential application in aquaculture. *Gen Comp Endocrinol* 167(3):352–359
- Wu S, Li B, Lin H, Li W (2012) Stimulatory effects of neuropeptide Y on the growth of orange-spotted grouper (*Epinephelus coioides*). *Gen Comp Endocrinol* 179(2):159–166
- Xu M, Urban JH, Hill JW, Levine JE (2000) Regulation of hypothalamic neuropeptide Y Y1 receptor gene expression during the estrous cycle: role of progesterone receptors. *Endocrinology* 141(9):3319–3327
- Yaron Z, Gur G, Melamed P, Rosenfeld H, Elizur A, Levaviv-Sivan B (2003) Regulation of fish gonadotropins. *Int Rev Cytol* 225:131–185