Evidence of a reproduction-related function for pituitary adenylate cyclase-activating polypeptide-related peptide in an Anabantidae fish

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) is synthesized from a precursor, which includes the PACAP-related peptide (PRP; formerly known as GHRH-like peptide). PRP can act as a hypophysiotropic factor in teleosts by stimulating GH secretion. However, no information points to this peptide as a regulator of reproduction. Recently, the blue gourami *PRP-PACAP* cDNA was cloned and found to be expressed in the brain. Thus, the aims of the present study were to investigate the *PRP-PACAP* gene expression pattern during sexual behavior and oogenesis, and to learn its effect on pituitary hormonal transcription in pituitary cells. Examination of the *PRP-PACAP* expression profile during sexual behavior and oogenesis revealed that *PRP-PACAP* mRNA levels were higher in mature non-reproductively active males than in nest builders and juveniles; and higher in females with oocytes in the final maturation stage than in vitellogenic individuals. Stimulation of pituitary cells with blue gourami PRP (bgPRP) caused an increase in β LH subunit transcription levels only in females, whereas in males, it only brought about a rise in *GH* mRNA levels. These data were further supported by the presence of PRP receptor in the pituitary cells. Therefore, we propose that as a hypophysiotropic factor in the blue gourami, bgPRP may act differently on the gonadotropic axes in females and males, up-regulating gonadotropin mRNA in females and *GH* mRNA in males. This research provides a basis for the further understanding of the integrative network that regulates growth and reproduction, which may contribute to hormonal treatments and manipulations in aquaculture.

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Introduction

In teleosts, the somatotrophic axis is regulated by numerous hypothalamic peptides, such as the newly identified GHRH (Lee *et al.* 2007) and pituitary adenylate cyclase-activating polypeptide (PACAP). Both peptides have been implicated as GH-releasing factors. In all vertebrates, PACAP is synthesized from a longer precursor, the PACAP-related peptide (PRP; formerly known as GHRH or GHRH-like peptide)-PACAP. The physiological role of PACAP in reproduction and growth has been studied in detail, whereas information related to PRP function remains unknown. In non-mammalian vertebrates, a short alternatively spliced variant, lacking most of the PRP sequence, can be produced by an exon-skipping mechanism (Tam *et al.* 2007). Based on a sequence comparison, both peptides were classified as belonging to the secretin/glucagon superfamily (Sherwood *et al.* 2000). Sequence analysis revealed that PACAP is the most highly conserved member of this family, whereas

PRP is highly variable, from teleosts to mammals, both in sequence and length (Vaudry *et al.* 2009).

Although significant studies have been made of the structural and functional evolution of the PRP peptide, its physiological role as a regulator of growth and reproduction in teleosts has received little attention and is not fully understood.

Different immunoreactive (ir) neuronal populations of PRP in various brain regions of teleosts have been reported. Antiserum, directed against PRP, reacts with the pituitary in the pars distalis cell bodies and with neuronal cell bodies in the hypothalamus that project axonal pathways toward the median eminence and terminate primarily in the pars nervosa in *Gadus morhua* (Pan *et al.* 1985) and rainbow trout (Luo & McKeown 1989). In addition, in the zebrafish, ir-PRP recognized fibers that probably originated from the gustatory/visceral nucleus and innervated the ventral area of the telencephalon, hypothalamus, and the spinal cord (Castro *et al.* 2009). In Salmonidae (chum salmon and coho salmon), ir-PRP was identified at the

terminal nerve ganglion system, associated with the olfactory nerve and superficial basal regions of the olfactory bulbs (Parker & Sherwood 1990), and fibers containing ir-PRP have been found in the proximal pars distalis in the vicinity of the somatotrophs in several teleost species (Olivereau *et al.* 1990, Rao *et al.* 1996). The physiological relevance of PRP as a hypophysiotropic factor has been further confirmed by the identification of a specific high affinity G-protein-coupled receptor (GPCR) of PRP in the goldfish and zebrafish pituitary (Castro *et al.* 2009). Binding PRP to this receptor can elicit a cAMP increase in PRP receptor-transfected cells, indicating that PRP may exert biological activities through a specific receptor. However, such receptors were not found in fish belonging to the perciformes order. Moreover, two genes encoding for the PRP receptor were identified in the zebrafish, fugu, and chicken (Cardoso *et al.* 2003, Fradinger *et al.* 2005, Wang *et al.* 2010).

Most studies in fish have focused on the involvement of PRP (formerly known as GHGH-LP or GRF) and PACAP in growth control. A recent study in the African catfish fry has shown that recombinant PRP enhanced growth promotion, as well as increasing the total protein concentration (Carpio *et al.* 2008). This ability may be related to the effect of GHRH on pituitary GH, inducing protein synthesis and growth, which has been widely studied in teleosts. PACAP has a more potent effect on GH release than does PRP; however, such effects are varied among species. PRP can stimulate GH release from pituitary cells in several teleosts: goldfish (Vaughan *et al.* 1992), rainbow trout (Luo *et al.* 1990), and sockeye salmon (Parker *et al.* 1997), and a synthetic hexapeptide of PRP increased the cell number of rat GH-secreting cells (Goth *et al.* 1992). In contrast, in turbot (*Psetta maxima*) and zfGHRH-R-transfected CHO cells, PRP did not stimulate GH release (Montero *et al.* 2000, Rousseau *et al.* 2001, Lee *et al.* 2007).

Differential regulation during development and reproduction stages, as a result of the ratio between PACAP short and long variants, has been shown to occur in the turkey, suggesting that PRP may play an important role during hypothalamic development and reproduction (Yoo *et al.* 2000). The key regulators of reproduction at the pituitary levels are the gonadotropins. The effect of PACAP on gonadotropin release has been extensively studied in several teleosts, among them, the blue gourami (Levy *et al.* 2010), tilapia (Vaudry *et al.* 2009), and goldfish (Wong *et al.* 2000). However, the possible effect of PRP on gonadotropin regulation in fish remains obscure.

The blue gourami (*Trichogaster trichopterus*) belongs to the suborder Labyrinthici (characterized by the presence of an air-filled breathing cavity (the labyrinth), located above the gills under the operculum) and

to the family Anabantidae. Among them, the blue gourami, which belongs to this genus, serves as a useful model in studying the role of endocrine regulation on reproduction, since it is multi-spawning and male dependent, with asynchronous ovary development (Jackson *et al.* 1994). Thus, each stage of its gonadal development can be controlled and examined separately in the laboratory (Degani 1993a,b, Jackson *et al.* 1999). The secretion and gene expression pattern of β FSH, β LH (Degani *et al.* 1997, 2003a, Mananos *et al.* 1997, Jackson *et al.* 1999), and GH (Goldberg *et al.* 2004, Degani *et al.* 2006), as well as sex steroid secretion during gonadal development in male and female blue gourami, have been previously reported (Degani & Boker 1992a,b). Recently, our laboratory cloned the full length of the PRP-PACAP cDNA sequence, measured the mRNA expression profiles of PACAP in the blue gourami during different states of reproduction, and examined the role of PACAP in regulating pituitary hormone transcription (Levy *et al.* 2010).

Towards understanding the physiological role of PRP in growth and reproduction of teleosts, this study aimed to discover the changes in PRP-PACAP gene expression during oogenesis and sexual behavior in both males and females, as well as to investigate the potential hypophysiotropic effect of PRP on GH and gonadotropin gene expression in the blue gourami fish, *in vitro*. Our *in vivo* and *in vitro* results imply that PRP may be involved in the regulation of reproduction in teleosts, and provide an insight into its potential physiological roles.

Materials and methods

Fish and sampling procedure

Blue gourami fish (*T. trichopterus*), which were maintained and bred at MIGAL Laboratories in northern Israel, were used in this study. The fish were grown in containers (2×2×0.5 m) at a temperature of 27 °C under a light regime of 12 h light:12 h darkness cycle (Jackson *et al.* 1994) and fed an artificial diet (45% protein and 7% fat), supplemented by live food (*Artemia salina*). Brains were collected from females and males at various stages of gonadal development and the somatic and gonadal weights were recorded for the gonadosomatic index (GSI) calculations. The gonadal samples were processed for histological determination of the reproductive stage. The stages in females were previtellogenesis (PV; GSI=0.911±0.269), low vitellogenesis (LV; GSI=3.93±1.351), high vitellogenesis (HV; GSI=7.893±1.241), and maturation (MS; GSI=10.901±2.142; Jackson *et al.* 1999). The stages in males were juvenile (GSI=0.046±0.014), mature non-reproductively active (GSI=0.28±0.03), and mature

reproductively active (nest builders) ($GSI = 0.33 \pm 0.03$) (Degani *et al.* 2003a).

Histological analysis

Gonadal samples were fixed in Bouin, and subsequently processed for light microscopy. Paraffin sections of 6 µm were stained with hematoxylin and eosin, as previously described (Jackson *et al.* 1994).

RNA extraction and cDNA synthesis

Total RNA was extracted from freshly excised whole brains of males ($n=21$) and females ($n=33$), using Trizol reagent (Invitrogen), according to the manufacturer's recommendations. First-strand cDNA was synthesized by the Verso-Reverse-IT 1st Strand Synthesis Kit (ABgene, Epsom, UK) from 0.5 to 2 µg total RNA, with an incubation of 1 h at 57 °C, followed by 2 min at 94 °C.

Partial cloning of the PRP receptor of the blue gourami pituitary

Based on the full-length sequence of *Danio rerio* (accession number NM_001131052), *Fugu rubripes* (accession number AJ296145), and *Carassius auratus* (accession number AF048819), gene-specific primers were designed for the cloning of the partial cDNA sequence of the PRP receptor of the blue gourami brain (Table 1). The 5' end PRP receptor was amplified by the SMART-RACE cDNA Amplification Kit, using the

primer, PRPrecR4, according to the manufacturer's recommendations (Clontech). Following 5' end amplification, nested PCR was performed using specific primers, PRPrecR4 and PRPrecf2. The amplified PCR product was cloned into the pGEM-T vector (Promega), which was propagated in *Escherichia coli* cells. The recombinant plasmid was then extracted using the SV miniprep (Promega), and the sequence of the amplified product was determined (HyLabs, Rehovot, Israel). Only one PRP receptor transcript was identified. The PCR was carried out in a total volume of 50 µl, consisting of 10× PCR buffer, 10 mM each deoxynucleotide triphosphate, 1 µM each primer, and 0.5 U of Taq advantage 2 polymerase (Clontech), using the Thermal Cycler (Bio-Rad Laboratories, Inc.). The PCR products were visualized on a u.v. transilluminator after electrophoresis on a 2% agarose gel containing ethidium bromide. To confirm the specificity of the PCR, the identity of each PCR product was verified by sequencing.

Amino acid sequence comparison between the blue gourami PRP and PRP receptor and the GHRH and GHRH receptor

Multiple sequence alignment (Lasergene 6 software, DNASTAR, Madison, WI, USA) of the partial sequence of the blue gourami PRP (bgPRP) receptor with fish PRP receptor amino acid revealed that the blue gourami partial PRP receptor sequence shares an 84–91% identity with the amino acid sequence of the fish PRP receptor, and only 45% with the fish GHRH receptor. A comparison, between the amino acid sequences of mature bgPRP and the first 27 amino acids of GHRH of fish and mammals, demonstrated that PRP shares a 37% identity with GHRH of fish and mammals. Sequence comparisons were employed using the ClustalW method.

Table 1 Nucleotide sequences of primers for pituitary adenylate cyclase-activating polypeptide-related peptide (PRP) and PRP receptor expression by reverse-transcribed PCR (A) and for real-time PCR (B)

Primer name	Synthesis direction		Sequence
(A)			
GHRHr183	Reverse		5'-TACACAGGAGGGCAGTTTGG-3'
PRP31f	Forward		5'-AGGGAGATCCTGGGTCAGTT-3'
PRPrecf2	Forward		5'-TTCCAGTTCAGCATCCT-3'
PRPrecR4	Reverse		5'-GTGTAGTGCATCCCAACA-3'
PRPrecR6	Reverse		5'-GAGTAGTGCATCCCAACAGAGGG-3'
Primer name	Genes	Synthesis direction	Sequence
(B)			
GHRHr183	PRP	Reverse	5'-TACACAGGAGGGCAGTTTGG-3'
PRP31f	PRP	Forward	5'-AGGGAGATCCTGGGTCAGTT-3'
GLHexfor1	LH	Forward	5'-CCTGACTGTCTCCTGGTGT-3'
GLHexrev1	LH	Reverse	5'-TTTGCTTTTGGTTTGCTGTG-3'
GFSHexpfor1	FSH	Forward	5'-GTTGTGTCATGGCAGCAGTGT-3'
GFSHexrev1	FSH	Reverse	5'-CCTCGTGGTAGCAATGCTCT-3'
GGHExpfor	GH	Forward	5'-TTCACAACCGCTATGGACAA-3'
GGHExprev	GH	Reverse	5'-TGACGCTGCTCTTCAATCTG-3'
expG18Sf	r18s	Forward	5'-CCGTCGTAGTTCCGACCATA-3'
expG18Sr	r18s	Reverse	5'-CCCTTCGTCAATTCCTTTA-3'

Expression of PRP–PACAP and its receptor in the brain and pituitary

Reverse transcription (RT) was performed at 42 °C for 2 h in a total volume of 10 µl, which consisted of 2 µg total RNA from brain and pituitary tissues ($n=5-10$) (Verso cDNA Kit, ABgene). The PCR was performed according to the manufacturer's instructions in a final volume of 50 µl, which contained 1 unit of *Thermus aquaticus* advantage 2 polymerase (Clontech). The primers used for PRP–PACAP and PRP receptor identification are summarized in Table 1, and the PRP–PACAP primer locations are listed in Fig. 1A. The amplification programmes were for PRP–PACAP – 50 cycles of 94 °C for 30 s; 50 °C for 60 s; 72 °C for 60 s, followed by a 5 min final extension at 72 °C; and for the PRP receptor – 50 cycles of 94 °C for 30 s; 58 °C for 30 s;

Figure 1 Expression of blue gourami *PRP-PACAP* and PRP receptor. Total RNA from gourami brains and pituitaries ($n=5$) was extracted and reverse transcribed, and the resulting cDNA was amplified by PCR. (A) The location of the primer set that is specific for PRP peptide in the *PRP-PACAP* long transcript. (B) The lower panel shows representative results of five to ten replicates. PCR products were separated on a 2.5% agarose gel containing ethidium bromide. The predicted size of the *PRP-PACAP* partial fragment derived from the brain precursor was 282 bp, and the predicted size of the PRP receptor partial fragment is 405 bp. r18s – the internal PCR product control, a fragment of 150 bp, derived from gourami 18S rRNA cDNA. B, brain; P, pituitary; V, control without cDNA.

72 °C for 30 s. In order to validate that there were no false positive products, PCRs containing water instead of cDNA were examined. Ten microliter volumes of PCR products were analyzed by electrophoresis on a 2% agarose gel, containing ethidium bromide for visualization of DNA bands.

Primary culture of dispersed pituitary cells

Pooled pituitaries of females ($n=58$) or males ($n=60$) were minced with a surgical blade under a dissecting microscope with fine forceps and immediately transferred to ice-cold M-199 medium, containing 10 mM HEPES, 0.3% BSA, 0.003 U/ml penicillin, 0.003 mg/ml streptomycin, and 0.0013 U/ml nystatin (basal medium). After incubation at 25 °C for 1 h, cells were dissociated by trypsin and several aspirations through a needle, followed by centrifugation at 2000 g for 5 min and suspension in M-199 medium, containing 10% FCS and 10 mM HEPES, 0.003 U/ml penicillin, 0.003 mg/ml streptomycin, and 0.0013 U/ml nystatin (growth medium). The cells were immediately plated in 24-well plates at a density of 3×10^5 cells/well for 72 h at 28 °C, 5% CO₂, and 90% humidity. Growth medium was changed to M-199 medium, containing 0.1% BSA, 10 mM HEPES, 0.003 U/ml penicillin, 0.003 mg/ml streptomycin, and 0.0013 U/ml nystatin (stimulating medium). Following 15 min of incubation

at 28 °C, the cells were stimulated with bgPRP or with fish fGHRH, diluted in stimulating medium. The bgPRP was synthesized, based on the first 45 amino acids of the sequence of the blue gourami mRNA (Levy *et al.* 2010; Genescript, Piscataway, NJ, USA), and the fGHRH was synthesized according to the first 27 amino acids of the sequence of the non-mammalian vertebrate (Lee *et al.* 2007; Genescript). After incubation, the medium was removed, and the cells were harvested by scraping, for RNA extraction and cDNA using verso cDNA kit (ABgene) as described above.

Real-time PCR

In order to compare the *PRP-PACAP* mRNA levels in the brain of the gourami individuals, or mRNA levels of the *GH*, *β LH*, and *β FSH* in the primary culture of dispersed pituitary cells, the relative abundance of mRNA was normalized with the mRNA of the endogenous reference gene, 18S subunit of rRNA (*18S rRNA*), by the comparative threshold cycle (C_T) method, according to Pfaffl (2001). The relative amount of each gene was calculated by the formula $2^{-\Delta C_T}$, where ΔC_T corresponds to the difference between the C_T measured for each target gene, and that determined for *18S rRNA*. To validate this method, serial dilutions were prepared from a brain cDNA sample, and the efficiencies of gene amplifications were compared by plotting ΔC_T versus log (template), according to the method of Muller *et al.* (2002). Linear regressions of the plots showed R^2 values of 0.99. Gene-specific primers for the real-time PCR (Table 1) were designed using Primer3 Software. To each of the above PCR mixtures, SYBR Green Master Mix (ABgene) was added, and amplification was carried out in a RotorGene 3000 Sequence Detection System (Corbett Research, Mortlake, Australia) under the following conditions: for *PRP-PACAP* – initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s; for *β LH* – initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, annealing at 60 °C for 15 s, and extension at 72 °C for 20 s; for *β FSH* – initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, annealing at 57 °C for 20 s, and extension at 72 °C for 20 s; for *GH* – initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 64 °C for 20 s, 72 °C for 20 s, and 83 °C for 15 s; and for *18S rRNA* – initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 64 °C for 20 s, and extension at 72 °C for 20 s. Amplifications of each target gene and reference gene (*18S rRNA*) cDNAs were performed simultaneously in separate tubes in duplicates, and the results were analyzed with the Q-Gen software (Simon 2003). Dissociation curve analysis was run after each real-time experiment to ensure that there was only one product. To control for false

positives, a non-template negative control was run for each primer pair.

Statistical analysis

Data are presented as the mean $\pm$ S.E.M. The significance of the differences between group means of hormone mRNA levels was determined by one-way ANOVA, followed by a posteriori Bonferroni or LSD *post hoc* tests using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

Results

PRP-PACAP and PRP receptor expression in the brain and pituitary

In order to determine whether *PRP-PACAP* is expressed in brains and pituitaries, total RNA from these organs of gourami fish was reverse transcribed. Reactions, using cDNA derived from brain RNA and

a set of primers specific to a segment of the gourami *PRP-PACAP* gene (Fig. 1A), produced a 282 bp fragment (Fig. 1B). No product was obtained from the pituitary cDNA. The PRP receptor fragment was identified in both brains and pituitaries. The expression of r18S was also measured in order to provide an internal control for the quantity of RNA template in each RT-PCR aliquot. Amplification of r18S in brain and pituitary samples gave a single product of 150 bp (Fig. 2B), indicating that there was no degradation of RNA in the preparation of the pituitary RNA samples.

The expression pattern of *PRP-PACAP* throughout the reproductive cycle and during sexual behavior in blue gourami females and males

Relative mRNA levels of *PRP-PACAP* were determined in brains excised from females and males. The females examined were in the previtellogenic (maintained alone, without males; PV), low vitellogenic (LV), and high vitellogenic (HV) stages, or were paired with

Figure 2 Histological determination of various reproductive stages in females (A–D) and males (E–G) of the blue gourami. (A) Previtellogenic stage (PV). Perinuclear oocyte with an intracellular spherical structure (a). Oocyte at cortical alveolar stage (b). Note the irregular outgrowth of the nuclear periphery. (B) Low vitellogenic stage. Note the low percentage of oocytes in the advanced vitellogenic stage (c). Lipid droplets (L). (C) High vitellogenic stage. Note the high percentage of oocytes at the advance vitellogenic stage (c). Yolk vesicle accumulation (black arrow). (D) Maturation stage (MS). Note that the appearance of oocytes at the maturation stage (d) is characterized by the fusion of lipid vesicles into one vesicle (L). (E) Juvenile male. Note the absence of spermatozoa (SZ) in the middle of the lobule. (F) Mature non-reproductive male. A high concentration of SZ is observed in the middle of the lobule (black arrow). (G) Mature fish during sexual behavior. Note the decrease in the number of SZ in the center of the lobule. Cellular spermatogenesis stages are shown in E and F at the periphery of the lobule: SG, spermatogonia; SC, spermatocyte; ST, spermatids. Sections were stained with hematoxylin and eosin.

Figure 3 Relative mRNA levels of *PRP-PACAP* in brains from blue gourami females at different stages of oogenesis: pre-vitellogenesis (PV), low vitellogenesis (LV), high vitellogenesis (HV), and maturation (MS). Total brain RNA was reverse transcribed, and the resulting cDNA was used in quantitative real-time PCR. The relative amount of the *PRP-PACAP* mRNA was normalized to that of 18S rRNA by the C_T cycle method, where $2^{-\Delta C_T}$ reflects the relative amount of *PRP-PACAP* transcription. Each histogram represents the average of independent measurements (mean $\pm$ S.E.M.; $n=5-7$). Different letters above the histograms denote significant differences among the mRNA levels ($P < 0.05$, by ANOVA, Bonferroni *post hoc* test).

males to induce the final maturation stage (MS). The reproductive stage was assessed by the histology of the ovaries, presented in Fig. 2A–D. The males tested were those maintained without females, i.e. juveniles and non-reproductive adult males and males, which were reproductively active and built bubble nests. The histology of their testes is shown in Fig. 2E–G respectively. *PRP-PACAP* mRNA levels were significantly higher in the paired, reproductively active females with oocytes in the MS, than in unpaired females (PV, LH, and HV; 2.6-, 4.08-, and 2.81-fold respectively) ($P < 0.05$, by ANOVA and Bonferroni *post hoc* test; Fig. 3). *PRP-PACAP* mRNA levels were significantly augmented in mature non-reproductive males, as compared to in mature reproductive (16.269-fold) and juvenile males (7.534-fold; Fig. 4). No significant difference was found between the *PRP-PACAP* mRNA levels of the mature reproductive fish and the juveniles.

Effect of bgPRP and fGHRH on βLH , βFSH , and GH mRNA levels in a culture of dispersed gourami pituitary cells

In order to study the direct effect of GHRH-like peptides on the expression of pituitary genes, pituitary cells from females or males were treated with synthetic bgPRP or fGHRH, and the expression levels of βLH , βFSH , and GH were determined. Figures 5 and 6 summarize the effects of bgPRP and fGHRH on pituitary hormone gene expression levels in a culture of dispersed pituitary cells of gourami females and males respectively. In females, bgPRP treatment of cells significantly increased βLH and βFSH mRNA levels (2.8- and 2.5-fold respectively), with a maximal

response at 24 h. No effect on GH mRNA levels was observed (Fig. 5). In male-derived cells, bgPRP brought about a significant rise in GH mRNA levels (Fig. 6), with a maximal response at 48 h following treatment (3.7-fold). In contrast, fGHRH caused an increase in the βLH mRNA levels, in female cells 24 h after treatment and in male cells 48 h after treatment (10.2- and 14.8-fold respectively); fGHRH treatment increased GH mRNA levels in male-derived (97.4-fold) and in female-derived cells 24 and 48 h (2.23- and 2.27-fold respectively) after treatment, whereas βFSH mRNA levels rose 48 h following fGHRH treatment only in female-derived cells (5.93-fold).

Discussion

In the present study, we show that *PRP-PACAP* gene expression in the brain changes during different stages of oogenesis and sexual behavior of the blue gourami male and female. In addition, the identification of the PRP receptor in the pituitary was supported by the *in vitro* bgPRP hypophysiotropic effect on pituitary hormone gene expression. The results showed that *PRP-PACAP* expression varies significantly throughout the reproductive cycle in the blue gourami males and females, and that it can up-regulate GH and gonadotropin gene expression respectively through its receptor in dispersed pituitary cell cultures.

As a preliminary step, *PRP-PACAP* mRNA expression was examined in the brain and pituitary of the blue gourami fish. The exclusive high expression level of *PRP-PACAP* in the brain is in agreement with previous reports on catfish (McRory *et al.* 1995), zebrafish (Fradinger & Sherwood 2000), and lungfish

Figure 4 Relative mRNA levels of *PRP-PACAP* in brains from blue gourami males at different stages of reproduction: juvenile, mature non-reproductive, and mature reproductive. Total RNA from brains was reverse transcribed for quantitative real-time PCR. The relative amount of *PRP-PACAP* mRNA was normalized to that of 18S rRNA by the C_T cycle method, where $2^{-\Delta C_T}$ reflects the relative amount of the specific gene precursor transcripts. Each histogram represents the average of independent measurements (mean $\pm$ S.E.M.; $n=4-9$). A different letter above the histogram denotes its significant difference from the mRNA levels of the other histograms ($P < 0.05$ ANOVA and Bonferroni *post hoc* test).

Figure 5 The effect of blue gourami PRP (bgPRP) and fish GHRH (fGHRH) on β LH, β FSH, and GH mRNA levels in a culture of dispersed pituitary cells from gourami females. A gourami pituitary cell culture of high vitellogenic non-reproductive females was treated with the following peptides: 10 nM of synthetic blue gourami PRP (bgPRP) or 10 nM fish GHRH for 24 and 48 h. Total RNA was extracted and reverse transcribed to cDNA, which was used as a template for the real-time PCR. β LH, β FSH, and GH mRNA levels were normalized to that of 18S rRNA by the C_T cycle method, where $2^{-\Delta C_T}$ reflects the relative amount of specific gene precursor transcripts. Each histogram represents the mRNA level of the hormone relative to that of the non-treated cells, which was set at 1. A different letter above the histogram denotes its significant difference from the mRNA levels of the non-treated cells ($P < 0.05$ ANOVA and Bonferroni *post hoc* test).

(Lee *et al.* 2009). In this study, a partial sequence of the PRP receptor transcript was identified in the brain and pituitary of the blue gourami fish as a GPCR subfamily B-I member. This partial transcript encodes for the PRP receptor and includes the following domains: TM2, TM3, TM4, and TM5, part of the ICL1 and the full sequences of ICL2, ECL1, and ECL2. They provide critical information necessary for specific interactions with ligands and intracellular signaling (Harmar 2001). This receptor might be able to bind PRP and transmit intracellular signals, which are important to the physiological relevance of bgPRP, *in vivo*. To date, the PRP receptor has been found to be expressed only in non-mammalian species. Among fish, the PRP receptor has been detected in goldfish, zebrafish, and fugu (Cardoso *et al.* 2003, Fradinger *et al.* 2005, Wang *et al.* 2010).

The presence of the PRP-PACAP transcript in the brain alone, and its receptor in the pituitary, as well, is in agreement with findings in goldfish (Chan *et al.* 1998) and chicken (Wang *et al.* 2010). They imply that PRP exerts conserved tissue-specific functions in the central nervous system in non-mammalian vertebrates and that its function might be mediated by a specific receptor.

As of yet, there have been no reports on a possible relationship between brain PRP-PACAP mRNA and reproduction in fish. In this study, PRP-PACAP expression was detected in the brain of the blue gourami females during different reproductive stages. The higher mRNA levels that were obtained in the brain of females, with oocytes at the final MS, as compared to vitellogenic and non-vitellogenic females, imply that PRP as well as PACAP peptides may be involved in the regulation of the final MS of oocytes

which occurs as a result of an interaction with males. This expression pattern is in correlation with the GnRH3 expression pattern in the brain of the gourami females (Levy *et al.* 2009), suggesting that PRP-PACAP may be associated with the regulation of the gonadotropic axis on the hypothalamic pituitary level. These findings are not in agreement with a previous study, in which PRP-PACAP expression levels did not change during the reproductive stages in the turkey (Yoo *et al.* 2000). Such differences can be explained by the fact that the role of PRP has been lost through evolution, as supported by recent findings in birds, demonstrating that the PRP receptor lacks the ligand-binding domain (Wang *et al.* 2010).

On the other hand, in males, high expression levels of PRP-PACAP were detected only in non-reproductively active adults, which were kept separated from females. These fish were characterized by a high concentration of spermatozoa in the middle of the testes lobes and did not build a nest of bubbles. Although PRP is encoded by a single gene that also encodes PACAP, its mRNA expression pattern in males does not correlate with PACAP expression in the brain of the blue gourami male. This is because the latter can be transcribed in a short transcript, independently of the PRP-PACAP long transcript, due to the exon-skipping phenomenon (Levy *et al.* 2010). As opposed to PRP-PACAP expression in females, in the male brain, PRP-PACAP expression does not correlate with GnRH3 expression, hinting that in males PRP-PACAP may not be involved in the regulation of the gonadotropic axis.

The expression of PRP-PACAP in the brain and of its receptor in the pituitary in the blue gourami raised the hypothesis that PRP may act as a hypophysiotropic

Figure 6 The effect of blue gourami PRP (bgPRP) and fish GHRH (fGHRH) on β LH, β FSH, and GH mRNA levels in a culture of dispersed pituitary cells from gourami males. A gourami pituitary cell culture of mature non-reproductive females was treated with the following peptides: 10 nM of synthetic blue gourami PRP (bgPRP) or 10 nM fish GHRH for 24 and 48 h. Total RNA was extracted and reverse transcribed to cDNA, which was used as a template for the real-time PCR. β LH, β FSH, and GH mRNA levels were normalized to that of 18S rRNA by the C_T cycle method, where $2^{-\Delta C_T}$ reflects the relative amount of specific gene precursor transcripts. Each histogram represents the mRNA level of the hormone relative to that of the non-treated cells, which was set at 1. A different letter above the histogram denotes its significant difference from the mRNA levels of the non-treated cells ($P < 0.05$ ANOVA and Bonferroni *post hoc* test).

regulator in the blue gourami. The newly identified *GHRH* gene in fish (Lee *et al.* 2007) raised a question regarding the biological role of PRP. Because of the structural similarity between PRP and GHRH, which was noted in an earlier paper describing the gene that encodes *PRP-PACAP* in humans, it was suggested that PRP may act as a GH-releasing factor (Ohkubo *et al.* 1992).

In the chicken, a close evolutionary relationship between the GHRH receptor and the PRP receptor has been shown, however, as in the blue gourami, bgPRP, and in zebrafish, PRP-R (the zebrafish PRP receptor) shares a 40% identity with the zebrafish and the human GHRH receptor (Wang *et al.* 2010).

In a subsequent step, the effect of PRP on pituitary hormone gene expression was examined in a pituitary culture of dispersed cells, obtained from males or females *in vitro*. In addition, the effects of the non-mammalian (fish) GHRH and bgPRP were compared. A gender-related hypophysiotropic effect of bgPRP and fGHRH on pituitary hormone gene expression was demonstrated in pituitary cultures obtained from females and males. For the first time, it is shown that PRP can up-regulate both β FSH and β LH mRNA levels after different incubation times in pituitary cultures of dispersed cells obtained from females. In males, PRP can up-regulate only *GH* mRNA levels. These data support previous findings that demonstrate a stimulatory effect of PRP on GH release. In the carp, PRP could stimulate GH release from cultured goldfish pituitary glands, and in goldfish, a PRP injection elevated GH serum levels (Vaughan *et al.* 1992). Synthetic carp PRP was active in releasing GH from enzymatically dispersed rainbow trout pituitary fragments in a concentration-dependent manner (Luo *et al.* 1990, Parker *et al.* 1997). These results, in addition to the PRP expression pattern in the brain of the blue gourami at different reproductive stages, imply that in females, PRP is involved in the regulation of reproduction at the transcription level of pituitary hormones.

Similar to bgPRP in *in vitro* assays, a sex-dependent effect of fGHRH on β FSH and *GH* gene expression was demonstrated in this study, probably because the receptors are structurally related (Cardoso *et al.* 2005). Functional assays revealed that the PRP receptors in both zebrafish and goldfish are most sensitive to one of their PRP, but both also bind GHRH (Lee *et al.* 2007). The increase in *GH* mRNA levels upon fGHRH stimulation in pituitary cells derived from males in this study is in agreement with a previous report, which showed that an i.p. injection of a human pancreatic GHRH fragment provoked an increase in serum GH levels in goldfish (Peter *et al.* 1984). The results of the present study also show that, as opposed to bgPRP, fGHRH may also be involved in the regulation of reproduction in males, via up-regulation of *LH* mRNA

levels. In ruminants, exogenous administration of GHRH increased GH and LH plasma levels (Mondal *et al.* 2006). The comparison, between the effects of bgPRP and fGHRH on pituitary hormone gene expression, implies that both GHRH and PRP systems, which exist in this fish, have different cellular receptor distribution and signal transduction pathways in males and females.

Furthermore, molecular cloning of gourami cDNA, encoding for the GHRH peptide, is clearly required to determine whether it shares a high percent of sequence similarity and the same biological activity, on the receptor, as well as on the cellular levels.

In conclusion, in this study, we report for the first time 1) a sex-related association between *PRP-PACAP* and growth and reproduction, 2) identification of PRP receptors in the brain and pituitary, and 3) a direct hypophysiotropic effect of PRP on hormonal gene transcription in the blue gourami fish. In females, *PRP-PACAP* gene expression is associated with the final MS of oocytes, as supported by *in vitro* up-regulation of gene expression of pituitary hormones on the gonadotropic axis. These results are in agreement with data from a previous study in our laboratory (Goldberg *et al.* 2004). The highest levels of *GH* mRNA were found in immature females during HV and maturation. Goldberg *et al.* (2004) suggested that GH may play a role in the gonadal cycle of the female blue gourami. Here, high levels of *GH* mRNA, obtained during the late stages of the gonadal cycle, validate the concept that GH participates in reproduction. On the other hand, in males, a higher *PRP-PACAP* gene expression was detected in adult non-reproductively active males, which correlates with results obtained in an *in vitro* up-regulation of *GH* mRNA levels by PRP. Degani *et al.* (2003b) determined the mRNA level of GH in blue gourami, showing that GH levels remained high in mature males, and therefore hypothesized that GH may be involved in spermatogenesis. Taken together, the results of this study imply that in males, PRP may be involved in spermatogenesis, which occurs before sexual behavior (indicated by nest building) and in the release of spermatozoa during sexual behavior, but is not involved in egg fertilization.

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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