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Identification of a cDNA encoding for *Ghrelin* in the testis of the frog *Pelophylax esculentus* and its involvement in spermatogenesis

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ABSTRACT

GHRELIN (GHRL) is an acylated peptide that contains 28-amino acids prevalently expressed in the stomach of several species. Specifically, it contributes to energy balance, but some new evidence highlights its role in the regulation of reproductive functions. In fact, this protein has been detected at testicular level in the tubular and interstitial compartments of several vertebrate species, and previous research has demonstrated that GHRL affects various aspects of spermatogenesis and steroidogenesis. GHRL clearly plays an inhibitory role in mammalian reproduction, in contrast GHRL stimulates reproductive functions in non mammalian vertebrate. We have focused our attention on the comparative aspect of GHRL, thus studying its expression in an amphibian seasonal breeder, *Pelophylax esculentus*, to verify the presence and localization, of *Ghrl* transcript variations during the frog reproductive cycle, in order to demonstrate that *Pelophylax esculentus* may represent a useful animal model to assess the role of GHRL in male fertility.

1. Introduction

GHRELIN (GHRL) is a peptidyl hormone that was recently identified as an endogenous ligand for the growth hormone (GH) secretagogue-receptor (GHS-R) (Kojima et al., 1999). GHRL is an acylated peptide that contains 28-amino acids and is prevalently expressed in the stomach (Kojima et al., 1999; Kojima et al., 2001). Additionally, it is expressed at low levels in other organs, such as the hypothalamus, pituitary, kidney, placenta, heart, ovaries and testes (Parhar et al., 2003).

Several papers have reported the potential involvement of GHRL in the regulation of a large assay of endocrine and non-endocrine functions, including the control of GH secretion; in addition, its orexant and adipogenic activity contributes to energy balance (Casanueva and Dieguez, 2002). Recently, growing evidence indicated that GHRL may exert a role in the regulation of several aspects of reproductive function (for review see Tena-Sempere, 2005; Lorenzi et al., 2009). It has been suggested that GHRL participates in the hypothalamus-pituitary-gonads regulatory network by suppressing luteinizing hormone (LH) secretion in rats (Martini et al., 2006). Notably, GHRL expression in rat and human testes has been characterized and has been strongly indicated as a cause for biological action in the male gonads (Tena-Sempere et al., 2002; Barreiro et al., 2002; Gaytan et al., 2004). Additionaly, although the *Ghrl* gene has been documented as hormonally regulated, the mechanisms of its action are not fully understood (for review see Lorenzi et al., 2009; Unniappan, 2010).

Testicular *Ghrl* gene expression has been demonstrated throughout postnatal development, and the GHRL protein was detected in Leydig cells (LC) from adult rat testes (Tena-Sempere et al., 2002). Moreover, GHRL injections induce a decrease in the number of spermatogonia, Sertoli and LC in rats (Kheradmand et al., 2009; Sirotkin et al., 2008). In addition, Barreiro et al., 2002, demonstrated that the *Ghrl* mRNA signal became undetectable in rat testes following selective LC elimination, after treatment with 1,2-ethane dimethane sulphonate (EDS), which is an alkylating agent that exerts specific cytotoxic action on LC of a majority of mammalian and non mammalian vertebrates (for review Minucci et al., 1992; Palmiero et al., 2003).

Specifically for non mammalian vertebrates, the structure of GHRL and some biological activities have been characterized for some teleost fishes (Parhar et al., 2003; Unniappan et al., 2002; Kaiya et al., 2003a,b,c; Murashita et al., 2009), amphibians (Kaiya et al., 2001) birds (Kaiya et al., 2002) and reptiles (Kaiya et al., 2004). However, no information has been reported to date concerning the temporal and spatial expression pattern of GHRL in the testes of a non mammalian vertebrate. Galas et al., 2002 provided the immunohistochemical evidence of a GHRL-related peptide in the brain and stomach of *Pelophylax esculentus* (previously: *Rana esculenta*). The aim of this study is to identify the *Ghrl* cDNA in this seasonal breeder, in order to detect *Ghrl* mRNA expression in frog testes during the annual

Abbreviations: GHRL, Grelin; GHS-R, Growth hormone secretagogue-receptor; LH, Luteinizing hormone; LC, Leydig cells; EDS, 1,2-ethane dimethane sulphonate; E_2, 17β-estradiol; T, Testosterone; KRB, Krebs Ringer Buffer; CPA, Cyproterone acetate.

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spermatogenic cycle and its variations after treatments with the two major hormones involved in spermatogenesis, i.e. 17β-estradiol (E_2) and testosterone (T).

2. Material and methods

2.1. RNA and first strand cDNA synthesis

Total RNA was isolated from testis, brains, livers, ovaries, hearts, stomachs and Harderian gland of *P. esculentus* according to Chomczynski and Sacchi (1987). Each tissue from different animals was pooled and homogenized in 10 mL lysis buffer (guanidine thiocyanate 4 mol L^{-1}, sodium citrate 25 mmol L^{-1} pH 7.0, 0.5% w/v sarcosyl and 2-β-mercaptoethanol 0.1 mol L^{-1}) followed by extraction with phenol acid:chloroform:isoamyl alcohol (50:49:1) and precipitation with isopropanol. The resulting RNA pellet was dissolved in water.

Five μg of total RNA were reverse-transcribed into cDNA using 40 ng random hexameric primer and 100 U Superscript III RT enzyme (Invitrogen, Paisley, UK) according to manufacturer's instruction.

2.2. PCR cloning and sequencing

Based on the published sequence for *Ghrl* mRNA of *Rana catesbeiana* (EMBL data bank accession No AB058510), were designed a pair of primers (ghrelin up: 5'-ttgtcttgttctgcctgctg-3'; ghrelin rev: 5'-ctgacccgagagagaggagg-3') to amplify *Ghrl* cDNA of the *P. esculentus* stomachs and testis. PCR reactions (25 μL volume) were performed in the presence of 0.4 μg of cDNA, 0.3 U Taq polymerase and its buffer 1x, $MgCl_2$ 1.5 mM, dNTP 0.2 mmol L^{-1} and 5 pmol of each primer. PCR amplification was carried out for 40 cycles with denaturing at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min. Amplification product was electrophoresed on 1.5% w/v agarose gel and visualized with ethidium bromide staining at UV light. The DNA fragment (275 bp) was purified by QIAGEN gel extraction kit (QIAGEN, Hilden, Germany) and cloned into the pGemT Easy vector (Promega Corporation, Madison, WI) according to the manufacturer's instruction. Sequence determination was performed on both strands with the dideoxynucleotide chain termination method of Sanger (Sanger and Coulson, 1975).

2.3. PCR and quantification

A pair of specific primer (*fg*hrelin up: 5'-cctgctgtggacagaggag-3'; *fg*hrelin down: 5'-ctcctgcgtcagcttcatact-3') were designed on the cDNA sequences for *P. esculentus Ghrl* to amplify *Ghrl* cDNA (196 bp) from different tissues of the frog and in the testis during the annual cycle using a semiquantitative PCR system.

A region of 356 bp mRNA fragment coding for a frog ribosomal protein fP1 (EMBL data bank accession No AJ298875), was amplified with specific oligonucleotide primers (fP1 for: 5'-tacgagcgtccatcacacac-3', fP1 rev: 5'-agaccaaagcccatgtcatc-3') as control. PCR amplification was carried out for 30 cycles under the conditions previously described. The expected RT-PCR products were separated by agarose gel (1.5% w/v) electrophoresis and the quantification was performed using Gel Doc 1000 Instrument (Biorad Laboratories, Milan, Italy). The relative amount of the *Ghrl* mRNA was calculated by the *fGhrl/fP1* ratio values and graphed as relative densitometry. Three independent assays were carried out to assess the statistic significance.

2.4. Phylogenetic analysis

Full-length protein sequences of pre-progrelin from different animal species were analyzed by MEGA4 with TT (Jones, Taylor & Thornton) matrix in the Neighbor-Joining method, to generate a phylogenetic tree. The reliability of each tree node was assessed by the bootstrap method with 500 replications. GenBank Accession nos. of the species used are as follow: *P. esculentus*, CAJ20255.1; *Rana catesbeiana*, BAB71718.1; *Oreochromis niloticus*, BAC65152.1; *Anguilla japonica*, BAB96565.1; *Carassius auratus*, AAN16216.1; *Danio rerio*, ACJ76436.1; *Gallus gallus*, AAP56234.1; *Homo sapiens*, BAA89371.1; *Rattus norvegicus*, NP_067701.1; *Mus musculus*, BAB19046.1; *Sus scrofa*, AAK19243.1; *Meriones unguiculatus*, AAO06965.1.

2.5. Histology

The testes fixed by immersion in Bouin fluid, were dehydrated in ethanol, cleared in xilene and embedded in paraffin. Tissue sections (5 μm) were stained with haematoxylin-eosin for a monthly check of the testicular activity or used for the *in situ* hybridization.

2.6. In situ hybridization

Frog *Ghrl* cDNA was cloned into the pGemT Easy vector (Promega Corporation, Madison, WI, USA). A clone was linearized with either *ApaI* (antisense probe) or *PstI* (sense probe) to produce template for *in vitro* transcription using digoxigenin-UTP (Roche Diagnostics) exactly as recommended by the manufacturer. The *in situ* hybridization was carried out as reported previously (de Rienzo et al., 2001) using sense (control) or antisense DIG-labelled cRNA on testis sections of frogs collected during the annual cycle.

2.7. Statistical analysis

Densitometric data were expressed as the mean ± SD of three independent RT-PCR determinations. Relative amount of *fghre/fP1* densitometric analysis was obtained using a Gel Doc 1000 Instrument (Biorad Laboratories, Milan, Italy). One-way ANOVA followed by Duncan's test for multigroup comparison assesses the significance of differences at $P<0.05$ and $P<0.01$.

2.8. Animals

Adult male frogs, *P. esculentus*, were collected monthly ($n=10$/month) in the surrounding of Naples by a local dealer. Animals were maintained in plastic tanks (23×16×11cm) with food and water available *ad libitum*. Frogs were killed by decapitation under anaesthesia (MS-222, Sigma, St. Louis, MO, USA), and the testis were dissected, quickly frozen by immersion in liquid nitrogen and stored at −80 °C until RNA extraction. In addition, male and female frogs ($n=10$/gender) were used to dissect ovary, testis, stomach, heart, brain, liver and Harderian gland to study sexual dimorphism.

This project was approved by the Italian Ministry of Education and Research (MIUR).

2.9. In vitro experiments

2.9.1. Experiment A

The frogs collected in the months of February and October (46 frogs/month) were divided as follows: (a) 10 animals were killed and the testes were immediately excised and used as initial control; (b) 36 animals were sacrificed and the testes (6 testes/time/groups) were incubated in Krebs Ringer Buffer (KRB) plus 17β-estradiol (E_2; 10^{-6} mol L^{-1}) or KRB plus E_2 and its antagonist ICI 182-780 (10^{-4} mol L^{-1}) for 2, 6 and 24 h respectively.

2.9.2. Experiment B

The frogs ($n=46$) collected in the months of February and October were divided as follows: (a) 10 animals were killed and the testes were immediately excised and used as initial control; (b) 36 animals were sacrificed and the testes (6 testes/time/groups) were incubated in KRB