General and Comparative Endocrinology 232 (2016) 174–184

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Sequence analysis, tissue distribution and molecular physiology of the GnRH preprogonadotrophin in the South American plains vizcacha (*Lagostomus maximus*)

Santiago Elías Charif [a,c,1], Pablo Ignacio Felipe Inserra [a,c,1], Noelia Paula Di Giorgio [b,c], Alejandro Raúl Schmidt [a,c], Victoria Lux-Lantos [b,c], Alfredo Daniel Vitullo [a,c,2], Verónica Berta Dorfman [a,c,*,2]

[a] Centro de Estudios Biomédicos, Biotecnológicos, Ambientales y Diagnóstico (CEBBAD), Universidad Maimónides, Ciudad Autónoma de Buenos Aires, Argentina
[b] Laboratorio de Neuroendocrinología, Instituto de Biología y Medicina Experimental (IByME)-CONICET, Ciudad Autónoma de Buenos Aires, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Ciudad Autónoma de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 23 July 2015
Revised 10 December 2015
Accepted 14 December 2015
Available online 15 December 2015

Keywords:
GnRH
GAP
Plains vizcacha
Reproductive axis

ABSTRACT

Gonadotropin-releasing hormone (GnRH) is the regulator of the hypothalamic-hypophyseal-gonadal (HHG) axis. GnRH and GAP (GnRH-associated protein) are both encoded by a single preprohormone. Different variants of GnRH have been described. In most mammals, GnRH is secreted in a pulsatile manner that stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The South-American plains vizcacha, *Lagostomus maximus*, is a rodent with peculiar reproductive features including natural poly-ovulation up to 800 oocytes per estrous cycle, pre-ovulatory follicle formation throughout pregnancy and an ovulatory process which takes place at mid-gestation and adds a considerable number of secondary corpora lutea. Such features should occur under a special modulation of the HHG axis, guided by GnRH. The aim of this study was to sequence hypothalamic GnRH preprogonadotrophin mRNA in the vizcacha, to compare it with evolutionarily related species and to identify its expression, distribution and pulsatile pattern of secretion. The GnRH1variant was detected and showed the highest homology with that of chinchilla, its closest evolutionarily related species. Two isoforms of transcripts were identified, carrying the same coding sequence, but different 5′ untranslated regions. This suggests a sensitive equilibrium between RNA stability and translational efficiency. A predominant hypothalamic localization and a pulsatile secretion pattern of one pulse of GnRH every hour were found. The lower homology found for GAP, also among evolutionarily related species, depicts a potentially different bioactivity.

1. Introduction

Gonadotropin-releasing hormone (GnRH), also known as luteinizing hormone-releasing hormone (LHRH), is the decapeptide involved in the modulation of fertility in mammals. According to its amino acid sequence composition, function, localization and embryonic origin, 30 GnRH peptides have been identified in nervous tissues from protochordates to vertebrates, with highly NH2– and COOH– terminal conserved sequences (Fernald and White, 1999; Lethimonier et al., 2004; Millar, 2005; Roch et al., 2014; Tsai, 2006; Tsai and Zhang, 2008). The first identified form of GnRH, named mammalian GnRH (mGnRH or GnRH1), was isolated from porcine and ovine brains (Burgus et al., 1972; Matsuo et al., 1971). This GnRH1 variant was also detected in chicken (King and Millar, 1982), and a second variant was later identified, namely cGnRH or GnRH2, by Miyamoto et al. (1984). Different biological functions for GnRH1 and GnRH2 were shown to exist (Cheng and Leung, 2005). A different variant of GnRH1 was firstly described in the guinea pig (*Cavia porcellus*), also reported in the capybara (*Hydrochoerus hydrochaeris*) (Montaner et al., 2002), and was originally termed gpGnRH (Grove-Strawser et al., 2002; Jimenez-Liñan et al., 1997). In addition, another form of GnRH

* Corresponding author at: Centro de Estudios Biomédicos, Biotecnológicos, Ambientales y Diagnóstico (CEBBAD), Universidad Maimónides, Hidalgo 775 6to piso, C1405BCK Ciudad Autónoma de Buenos Aires, Argentina.
E-mail address: dorfman.veronica@maimonides.edu (V.B. Dorfman).
[1] S.E. Charif and P.I.F. Inserra contributed equally to this work.
[2] A.D. Vitullo and V.B. Dorfman contributed equally to this work.

http://dx.doi.org/10.1016/j.ygcen.2015.12.012
0016-6480/

(sGnRH or GnRH3) firstly isolated from salmon, is now known to be present in all teleosts (Sherwood et al., 1986; Roch et al., 2014; Zohar et al., 2010). Most vertebrates possess at least two, usually three, variants of GnRH that differ in their amino acid sequence, localization and embryonic origin (Cheng and Leung, 2005). GnRH is synthesized as a preprogonadotrophin, including a signal peptide and an enzymatic cleavage site that separates GnRH from another sequence termed GnRH associated protein (GAP), originally proposed as prolactin inhibitory factor (PIF) (Adelman et al., 1986).

In the mammalian postnatal brain, GnRH is synthesized by a discrete specialized group of neurons scattered throughout the preoptic area of the hypothalamus in the basal forebrain, the ventromedial nucleus, and the arcuate nucleus (Silverman and Witkin, 1994; Urbanski et al., 1991, 1992). Most hypothalamic GnRH secreting neurons project towards the median eminence, releasing GnRH into the hypothalamic-hypophyseal portal circulation that transports the hormone to the anterior pituitary gland where it binds to its specific receptor and stimulates the synthesis/release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Krey and Silverman, 1978; Silverman et al., 1987; Silverman and Witkin, 1994; Witkin et al., 1995; Yin et al., 2009a,b). GnRH is secreted with a pulsatile pattern that varies across the reproductive cycle to differentially stimulate the release of FSH, when GnRH pulse frequency is low, or of LH, when the pulse frequency increases (Burger et al., 2008; Ciccone et al., 2010; Wildt et al., 1981).

In mammalian females, GnRH is delivered in a pulsatile manner throughout reproductive life except during pregnancy due to a negative feedback exerted by progesterone (which inhibits GnRH release) (Belchetz et al., 1978). In contrast, the South American plains vizcacha, *Lagostomus maximus*, seems to show variations of GnRH expression even during pregnancy (Dorfman et al., 2013). This mammal is a hystricognathe fossorial rodent that belongs to the family Chinchillidae, and inhabits the Pampean region of Argentina (Jackson et al., 1996; Wilson and Sanchez-Villagra, 2010). The ovary of the vizcacha shows the formation of pre-ovulatory follicles that is not interrupted throughout the 155-day long pregnancy. It also display a pseudo-ovulatory process around mid-gestation that adds a considerable number of oocyte-retaining secondary corpora lutea, with a consequent increase in circulating progesterone (P4) levels. Natural massive poly-ovulation, up to 800 oocytes per estrous cycle, the highest ovulatory rate recorded for a mammal, is so far described (Dorfman et al., 2013; Jensen et al., 2006, 2008; Weir, 1971a,b). These characters seem to arise from an unusual constitutive suppression of apoptosis that abolishes intra-ovarian oocyte dismissal caused by follicular atresia (Inserra et al., 2014; Jensen et al., 2006, 2008; Leopardo et al., 2011). These exceptional features should occur under a peculiar modulation of the hypothalamic-hypophyseal-gonadal (HHG) axis, guided by GnRH.

We have previously described that the distribution of GnRH in the hypothalamus of the vizcacha is mostly comparable to a variety of other mammalian species (Dorfman et al., 2011). Surprisingly, GnRH is also localized in the ventrolateral preoptic area of the hypothalamus of the vizcacha, where the supraoptic nucleus (SON) resides (Dorfman et al., 2011). This unusual localization of GnRH in SON has only been previously reported in the pig brain (Kineman et al., 1988), another well-known poly-ovulatory mammal. However, the specific variant of the vizcacha hypothalamic GnRH is not yet defined. In the present work, we aimed to determine the GnRH preprogonadotrophin mRNA sequence of the vizcacha to elucidate the variant/s of GnRH expressed in the hypothalamus, its distribution throughout different brain areas and other reproductive tissues, and to study its pulsatile hypothalamic pattern.

2. Materials and methods

2.1. Animals

Sixteen adult female plains vizcachas (2.5–3.0 kg body weight; 2–2.5 years old, age determined by the dry crystalline lens weight according to Jackson, 1986) were captured from a resident natural population at the Estación de Cría de Animales Silvestres (ECAS), Villa Elisa, Buenos Aires Province, Argentina. Animals were captured using live-traps located at the entrance of burrows. All experimental protocols concerning animals were conducted in accordance with the guidelines published in the NIH Guide for the care and use of laboratory animals (National Institutes of Health, 1985), and were reviewed and approved by the Institutional Committee on Use and Care of Experimental Animals (CICUAE) from Universidad Maimónides, Argentina. Handling and euthanasia of animals were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health, 1985). Appropriate procedures were performed to minimize the number of animals used. In order to obtain non-pregnant females, animals were captured in early-March according to the natural reproductive cycle, previously described by Llanos and Crespo (1952), and our own previous field expertise (Dorfman et al., 2011, 2013; Halperin et al., 2013; Inserra et al., 2014; Jensen et al., 2006, 2008; Leopardo et al., 2011).

2.2. Tissue collection

Animals were anaesthetized by intramuscular injection of 13.5 mg/kg body weight ketamine chlorhydrate (Holliday Scott S.A., Buenos Aires, Argentina) and 0.6 mg/kg body weight xylazine chlorhydrate (Richmond Laboratories, Veterinary Division, Buenos Aires, Argentina). Animals were sacrificed by trained technical staff, by an intracardiac injection of 0.5 ml/kg body weight Euthanyl™ (Sodic Pentobarbital, Sodic Diphenilhidanthoine, Brouwer S.A., Buenos Aires, Argentina). After rapid removal of the whole brain, the hypothalamus was dissected out to a depth of approximately 4 mm with the following borders: the anterior edge of the optic chiasm, the anterior edge of the mammillary bodies, and the two hypothalamic sulci on either lateral side, as previously reported (Dorfman et al., 2013). Other brain regions like striatum, brain cortex, hippocampus, midbrain, olfactory bulb, pineal gland, white matter, cerebellum, and spinal cord were also dissected out and frozen together with non-nervous tissues like anterior hypophysis, ovary, and breast. Tissues were stored at −80 °C until used.

2.3. Total RNA isolation

Tissues were homogenized with TRIzol™ (Invitrogen, California, USA.) according to the manufacturer's instructions to extract total RNA. Concentration was quantified by absorption at 260 nm (Genequant, Amersham Biosciences, England) and integrity confirmed in a 1% agarose gel electrophoresis (Genbiotech, Argentina) in Tris (0.09 M) – boric acid (0.045 M) – EDTA (0.05 M) (TBE) buffer (pH 8.3). RNA integrity was confirmed when the presence of S28 and S18 rRNA subunits were observed. Tissues from four different animals were tested.

2.4. RNA ligase mediated rapid amplification (RLM-RACE) of 5′ and 3′ cDNA ends

Hypothalamic GnRH preprogonadotrophin mRNA was amplified using the 5′ RLM-RACE method. For each end 3 μg of total isolated RNA was reverse-transcribed using the Gene-Racer™ kit

(L1500, Invitrogen Life Technologies, USA). For 5′ end, RNA was dephosphorylated by incubation with 1 μl RNase Out™ (40U/μl), 1 μl calf intestinal phosphatase (CIP, 10U/μl), 1 μl CIP Buffer 10× and DEPC water to 10 μl for 1 h at 50 °C. The obtained RNA was precipitated with phenol–chloroform and washed with 70% ethanol. To remove 5′ end Cap structure from the mRNA, dephosphorylated RNA was incubated with 1 μl RNase Out™ (40U/μl), 1 μl tobacco acid pyrophosphatase (TAP, 0.5U/μl) and 1 μl TAP Buffer for 1 h at 37 °C. The obtained RNA was precipitated with phenol–chloroform and washed with 70% ethanol. To ligate the GeneRacer™ oligo RNA to the decapped 5′ mRNA, 7 μl of decapped mRNA and 0.25 μg of the GeneRacer oligo RNA were incubated with 1 μl Buffer Ligase (10×), 1 μl ATP (10 mM), 1 μl RNase Out™ (40U/μl) and 1 μl T4 RNA ligase (5U/μl) for 1 h at 37 °C. The obtained ligated mRNA was precipitated with phenol–chloroform and washed with 70% ethanol. Ten microliter of the ligated mRNA were retrotranscribed by incubation with 1 μl GeneRacer™ Oligo dT primer (50 μM), 1 μl dNTPs (10 mM), 4 μl DTT (0.1 M), 1 μl RNaseOut™ (40U/μl) and 1 μl Superscript III™ retrotranscriptase (200U/μl) for 1 h at 50 °C. Then, 1 μl of RNase H (2U/μl) was added and incubated for 20 min at 37 °C. RNA from HeLa cells was used as control. After each step, RNA integrity was checked using 1% agarose gel electrophoresis. Amplification of the GnRH preprogonadotrophin was developed by incubation of 2 μl of each obtained cDNA with 3 μl 5′ GeneRacer™ primer (10 μM) and 1 μl specific reverse primer (10 μM) for 5′ end, or 3 μl 3′ GeneRacer™ primer (10 μM) and 1 μl specific forward primer (10 μM) for 3′ end, with the addition of 5 μl of PCR Buffer Taq Polymerase (10×), 1 μl dNTP mix (10 mM), 1.25 μl Taq Polymerase (1U/μl, Fermentas, Thermo Scientific, MA, USA), 4 μl $MgCl_2$ (25 mM) and DEPC water up to a final volume of 50 μl. Specific forward and reverse primers were designed by aligning published sequences of the preprogonadotrophin mRNA of chinchilla (*Chinchilla lanigera*, NCBI Reference Sequence XM_005373712.1), guinea pig (*C. porcellus*, NCBI Reference Sequence NM_001172956.1), degu (*Octodon degus*, NCBI Reference Sequence XM_004630672.1), mouse (*Mus musculus*, NCBI Reference Sequence NM_008145.2), rat (*Rattus norvegicus*, NCBI Reference Sequence NM_012767.2) and two sequence isoforms of human that differ in the 5′ UTR (*Homo sapiens*, NCBI Reference Sequences NM_000825.3 and NM_001083111.1). Sequences were aligned using Bioedit Software (version 7.2.5, Ibis Biosciences, Carlsbad, CA, USA), the homologous conserved fragments were determined and the primers designed over them. Specific primers and length of the obtained amplified products are listed in Table 1. Cycling parameters were: Two minutes at 94 °C followed by a first round PCR of 5 cycles: 30 s at 94 °C and 1 min at 72 °C; followed by a second round of 5 cycles: 30 s at 94 °C and 1 min at 70 °C; followed by a third round of 25 cycles: 30 s at 94 °C, 30 s at 68 °C and 1 min at 72 °C; followed by 10 min at 72 °C. Negative controls were performed in parallel by omission of cDNA or primers. Amplified products were run in a 1.5% agarose gel and their presence detected with an UV transilluminator (Labnet DyNALight TM-26, USA). Gel bands were excised, purified with the MinElute™ Gel Extraction kit (Qiagen, Hilden, Germany), and sequenced with a 3130xl Genetic Analyzer (Applied Biosystems, California, USA) by the Genomic Unit of the Biotechnology Institute from the Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina, using the Bioedit software (Ibis Biosciences, California, USA).

2.5. GnRH preprogonadotrophin sequence analysis

The different regions of the vizcacha preprogonadotrophin mRNA sequence were determined by comparison with published sequences of close evolutionarily related rodents like chinchilla (NCBI Reference Sequence XM_005373712.1), guinea pig (NCBI Reference Sequence NM_001172956.1) and degu (NCBI Reference Sequence XM_004630672.1), far evolutionarily related rodents like mouse (NCBI Reference Sequence NM_008145.2) and rat (NCBI Reference Sequence NM_012767.2) and two sequence isoforms of human (NCBI Reference Sequences NM_000825.3 and NM_001083111.1). All the sequences were aligned using Bioedit Software (version 7.2.5, Ibis Biosciences, Carlsbad, CA, USA) and the homology percentage was calculated using the DnaSP software (version 5.10.1, Universidad de Barcelona, Spain) and BLAST (NCBI, NIH, Maryland, USA).

Table 1
Primers employed for 5′ and 3′ RACE-PCR and for quantitative PCR (qPCR).

Target	Primers	Product[a]
5′ RACE	F: GeneRacer™ 5′ Primer (GeneRacer™ Kit) R: 5′ CGAGGCTGGTGGAGGGTGCATTCC 3′	345 bp
3′ RACE	F: 5′ GGAATGCACCCTCCACCAGCCTCG 3′ R: GeneRacer™ 3′ Primer (GeneRacer™ Kit)	251 bp
GnRH-GAP	F: 5′ CAGCACTGGTCCTATGGGTTGCG 3′ R: 5′ TTCCTCTTCAATCAGACGTTCC 3′	148 bp
Beta-actin	F: 5′ GGAGACGGGTCACCCACAC 3′ R: 5′ CTACTCAGGCCGGGGAGGTAG 3′	80 bp

[a] Product = Amplified product length. GeneRacer™ Kit (L1500, Invitrogen Life Technologies, California, U.S.A.).

2.6. RNA isolation and quantitative polymerase chain reaction (qPCR)

Three microgram of total RNA was treated with 1 μl DNaseI (Invitrogen, California, USA) in 1 μl DNase Reaction Buffer (10×, Invitrogen, California, USA) and DEPC water to 10 μl for 30 min at 37 °C. The reaction was stopped by the addition of 1 μl EDTA (50 mM, Invitrogen, California, USA) and incubated for 10 min at 65 °C. The RNA was reverse-transcribed into first-strand cDNA using 1.5 μl random hexamer primers (50 μM, Applied Biosystems, California, USA), 1 μl reverse transcriptase (200U/μl, RevertAid™ M-MuLV, Fermentas, Massachusetts, USA), 4 μl First Strand Buffer (5×, Fermentas, Massachusetts, USA), 1 μl dNTP mix (10 mM, Invitrogen, California, USA), 1 μl RNase inhibitor (40U/μl, Ribolock™, Fermentas, Massachusetts, USA) and 2 μl DTT (0.1 M, Invitrogen, California, USA) at 20 μl final volume reaction. The reverse transcriptase was omitted in control reactions, where the absence of PCR-amplified DNA indicated the isolation of RNA free of genomic DNA. The reaction was carried out at 72 °C for 10 min, 42 °C for 60 min and stopped by heating at 70 °C for 10 min. cDNA was stored at −20 °C until used. For qPCR, reverse transcripted cDNA was mixed with 6 μl SYBR™ Green PCR Master Mix (Applied Biosystems, UK) and 2 μl forward and reverse oligonucleotide primer mix (0.3 μM). Specific primers and length of the obtained amplified products are listed in Table 1. Cycling parameters for GnRH-GAP are: Ten minutes at 95 °C followed by 40 cycles of: 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C; whereas, specific cycling conditions for beta-actin are: Ten minutes at 95 °C followed by 40 cycles of: 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Forward and reverse oligonucleotide primers for GnRH-GAP were designed based on the sequenced vizcacha preprogonadotrophin cDNA. Beta-actin forward and reverse primers were designed by comparison of published sequences of beta-actin mRNA of rat (NCBI Reference Sequence NM 031144.3), mouse (NCBI Reference Sequence NM 007393.3), chinchilla (NCBI Reference Sequence XM 005397842.1), guinea pig (NCBI Reference Sequence NM 001172909.1) and human (NCBI Reference Sequence NM 001101.3). Bioedit Software (version 7.2.5, Ibis Biosciences, Carlsbad, CA, USA) was used to align the sequences and the primers were designed over the homologous conserved fragments. Oligonucleotides were obtained from Invitrogen (Life Technologies, California, USA). The oligonucleotide primers used are listed in Table 1. Quantitative measures were performed using a Stratagene MPX500 cycler (Stratagene, California,

USA). Data were collected from the threshold value, taken at the 72 °C extension phase, continuously stored during reaction and analyzed by the complementary computer software (MxPro3005P v4.10 Build 389, Schema 85, Stratagene, California, USA). To confirm specificity of the signal, the results were validated based on the quality of dissociation curves generated at the end of the qPCR runs. Relative quantitation of gene expression was normalized to that of beta-actin as housekeeping gene. For the assessment of quantitative differences in the cDNA target between samples, the mathematical model of Pfaffl was applied (Pfaffl, 2001). An expression ratio was determined for each sample by calculating $(E_{\text{target}})^{\Delta Ct(\text{target})}/(E_{\text{b-actin}})^{\Delta Ct(\text{b-actin})}$, where E is the efficiency of the primer set and ΔCT is the difference in the threshold cycle with $\Delta Ct = \text{Ct}_{\text{(normalization cDNA)}} - \text{Ct}_{\text{(experimental cDNA)}}$. The amplification efficiency of the primer set was calculated from the slope of a standard amplification curve of log (ng cDNA) per reaction vs. CT value ($E = 10^{-(1/\text{slope})}$). An efficiency of 2.0 ± 0.1 was considered optimal. Each sample was analyzed in triplicate along with non-template controls to monitor contaminating DNA. Purity of the amplified products was confirmed by 2% agarose gel electrophoresis (Biodynamics, Buenos Aires, Argentina). The presence of the amplified sequence was detected with an UV transilluminator (Labnet DyNALight TM-26, USA). To confirm the identity of the amplified product, gel bands were excised, purified with the MinElute™ Gel Extraction kit (Qiagen, Hilden, Germany) and sequenced with a 3130xl Genetic Analyzer (Applied Biosystems, California, USA) by the Genomic Unit of the Biotechnology Institute, Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina, using the Bioedit Software (version 7.2.5, Ibis Biosciences, Carlsbad, CA, USA). Tissues from four different animals were tested.

2.7. Radioimmunoassay (RIA) for GnRH detection

Fifty microgram of hypothalamus, striatum, brain cortex, hippocampus, midbrain, olfactory bulb, pineal gland, white matter, cerebellum, spinal cord, anterior hypophysis, ovary and breast were homogenized in 100 µl HCl (0.1 N), centrifuged for 30 min at 13,000*g* and supernatants recovered. All procedures were carried out at 4 °C. GnRH concentration was determined as previously described in mouse (Di Giorgio et al., 2013) and validated for vizcacha (Dorfman et al., 2013). Briefly, samples were analyzed in duplicate using anti-GnRH antiserum (rabbit polyclonal HU-60 that recognizes GnRH1 with higher affinity than GnRH2 (Mongiat et al., 2006), final dilution 1:50,000) kindly provided by Dr. Urbanski (Division of Neuroscience, Oregon National Primate Research Center). GnRH was iodinated with ^{125}I (NEZ 033H Iodine 125, Perkin Elmer, Life and Analytical Science, Waltham, Massachusetts, USA) by the chloramine-T method (Greenwood et al., 1963). Intra- and inter-assay coefficient of variation was 7.2% and 11.6%, respectively. The detection limit was 1.5 pg. Protein content of each sample was determined by the Bradford method (Bradford, 1976). Results were expressed as the ratio between GnRH value obtained by RIA and the protein content. Tissues from four different animals were tested.

2.8. GnRH pulsatility measured by radioimmunoassay (RIA)

To synchronize the estrous cycle, non-pregnant females captured in early-March were induced to ovulate through intramuscular injection of 250 IU/day of pregnant mare's serum gonadotropin (PMSG, Novormon 5000, Syntex, Argentina) during three consecutive days, followed by one intramuscular injection of 1000 IU of human chorionic gonadotropin (hCG, Ovusyn, Syntex, Argentina) at the fourth day, according to Willis (2011). Animals were sacrificed five days after the last injection. After rapid brain removal, whole hypothalamus was dissected out as detailed above and weighted. GnRH pulsatility was measured *in vitro* as previously described by Catalano et al. (2010). Once removed, hypothalami were placed in 500 µl Krebs–Ringer buffer (115 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.56 mM $CaCl_2$, and 20 mM $NaHCO_3$; pH 7.4) supplemented with 0.1% bovine serum albumin, 25 mM glucose and 16 mM HEPES buffer and refrigerated for at least 1 h. Then, hypothalami were preincubated in gelatin pre-coated eppendorf tubes with 500 µl of supplemented Krebs–Ringer buffer for 30 min at 37 °C, followed by incubation in fresh supplemented Krebs–Ringer buffer for 6 h at 37 °C. During incubation, the medium from each tube was collected at 7.5-min intervals, replaced with fresh medium and stored at −20 °C. GnRH concentration was determined by RIA as described above. GnRH pulsatile parameters were determined using the computer algorithm Cluster8 developed by Veldhuis and Johnson (1986) (Pulse_XP software, http://mljohnson.pharm.virginia.edu/home.html). A 2 × 2 cluster configuration and a *t*-statistic of 2 for the upstroke and downstroke, to maintain false-positive and false-negative error rates < 10%, were used as suggested by Martinez de la Escalera et al. (1992). Tissues from five different animals were tested.

2.9. Statistical analysis

Values were expressed as mean ± standard deviation (SD). All the experiments were performed in duplicate. Results were evaluated using one-way analysis of variance (ANOVA) and comparisons among groups were made by Fisher, Scheffe and Bonferroni's Multiple Comparison tests. Statistical analysis was performed using Prism 4.0 (GraphPad Software Inc., San Diego, California, USA). Differences were considered significant when $p < 0.05$.

3. Results

3.1. Sequencing of the hypothalamic GnRH preprogonadotrophin

To amplify the vizcacha hypothalamic GnRH preprogonadotrophin, sequences of chinchilla, guinea pig, degu, mouse, rat and two variants of human were aligned and the region with the highest homology used to design forward and reverse primers for 3′ and 5′ RACEs respectively. Amplified 3′ and 5′ RACE products are shown in Fig. 1. The 3′ RACE showed an amplified product of around 300 base pair (bp) whereas the 5′ RACE showed two bands of 300 bp and 350 bp approximately (Fig. 1). To obtain the full length GnRH preprogonadotrophin sequence, the three bands were dissected out, every product was sequenced independently using the specific forward and reverse primers detailed in Table 1, and the obtained forward and reverse sequences were compared to fill the single sequence resulting gaps by comparison among paired sequences. Lastly, 5′ and 3′ sequences were assembled. This resulted in two cDNA sequences: a longer sequence of about 526 bp and a shorter one of 492 bp. Both sequences differed in 34 nucleotides (nt) localized at the 3′ end of the 5′ untranslated region (UTR), yielding two alternatives 5′ UTR fragments of 94 nt or 60 nt respectively, where the shorter lacks 34 nt (Fig. 2). The open reading frame (ORF) of 279 bp, encoding a deduced aminoacid sequence of 92 residues, was identical for both isoforms of the mRNA and exhibited the typical GnRH preprogonadotrophin structure including a signal peptide of 69 nt length, followed by 30 nt that correspond to the sequence of mammalian GnRH and the 168 nt length GAP sequence, both separated by 9 nt coding a 3 amino acid enzymatic cleavage site (Fig. 2). The signal peptide starts with the initial codon ATG and the sequence for GAP ends with the termination codon TAA. The 3′ UTR was 154 nt length including the poly-adenylation signal AGTAAA (a close variant of

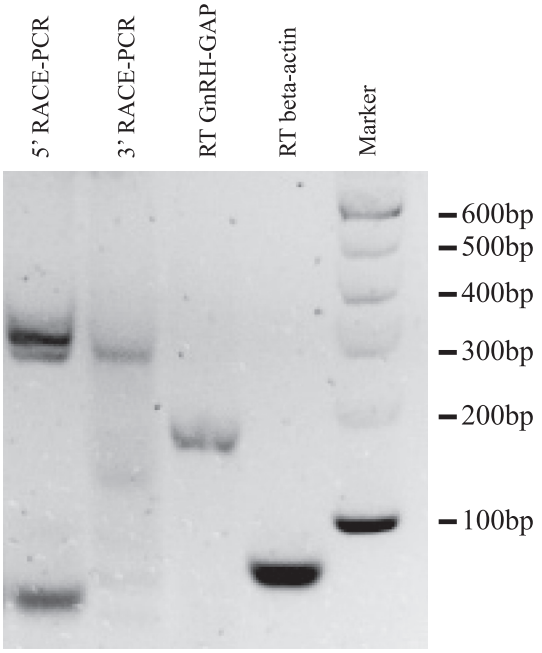


Fig. 1. GnRH amplification. Representative image of electrophoresis analysis in a 2% agarose gel of the obtained amplified products. Both RACE-PCR 5′ and 3′ tails, and specific GnRH-GAP amplified products were detected. Line 1: 5′ RACE-PCR product, Line 2: 3′ RACE-PCR product, Line 3: Real time PCR (RT) GnRH-GAP product, Line 4: RT beta-actin product, Line 5: Marker (100 Kb DNA ladder).

the classical consensus sequence AAUAAA previously described by Zhao et al., 1999) located 3 nt to 5′ side of the Poly A tail, and one ATTTA sequence, which is frequently present in the 3′ non-coding regions of transcripts that exhibit short half-lives and is involved in the targeting of mRNAs for rapid turnover (Gao et al., 2009) (Fig. 2). The nucleotide sequence data of both isoforms of GnRH1 of *L. maximus* have been submitted to the Gen-Bank (Accession Number KT003537 and KT336553).

The obtained sequence was aligned with the previously described GnRH preprogonadotrophin sequences of other rodents and showed a high degree of homology. A 92% identity was determined with the complete GnRH preprogonadotrophin of *C. lanigera*, 87% with *C. porcellus*, 88% with *O. degus*, 81% with *R. norvegicus*, 81% with *M. musculus* and 81% with both isoforms of *H. sapiens* (Fig. 3A and Table 2). The nucleotide sequence corresponding to the GnRH peptide of vizcacha showed a high 93.33% homology with the mammalian GnRH variant whereas GAP sequence showed the highest homology (93.57%) with the GAP sequence of *C. lanigera.* On the other hand, the localization of the polyadenylation sequence of *L. maximus* coincides with that described for *C. porcellus* (Fig. 3). The amino acids sequence of GnRH deduced for vizcacha seems to be identical to that for *C. lanigera*, *O. degus*, *M. musculus*, *R. norvegicus* and *H. sapiens.* However, when comparing with *C. porcellus*, a T × C substitution in the first nucleotide of the second codon and a G × T substitution in the first nucleotide of the seventh codon were detected. In addition, when comparing with *H. sapiens*, three nucleotide substitutions were also detected (Fig. 3B).

3.2. Distribution of GnRH in brain subregions and reproductive organs

Specific primers were designed to amplify vizcacha GnRH based on the above sequenced preprogonadotrophin including the whole predicted GnRH sequence and a 5′ portion of GAP. The amplified fragment yielded an expected single band of 189 bp (Fig. 1). The obtained product was sequenced to confirm identity. Contents of both nucleotide and protein GnRH were studied in the hypothalamus and compared with other regions of the nervous system like hippocampus, brain cortex, striatum, midbrain, corpus callosum, spinal cord, cerebellum, pineal gland and olfactory bulb, and with reproductive organs such as anterior hypophysis, ovary and breast. mRNA as well as protein expression were significantly increased in the hypothalamus with respect to the other analyzed areas of the nervous system, and in relation to anterior hypophysis, ovary and breast (Fig. 4). In addition, anterior hypophysis and ovary showed significantly higher GnRH mRNA and protein levels than breast (Fig. 4).

3.3. GnRH pulsatility

GnRH release was measured from whole *ex vivo* hypothalamic explants. Analyzed GnRH in media samples every 7.5 min during 6 h, revealed a pulsatile delivery pattern of 6 pulses, approximately one pulse/hour. Strikingly, pulses of GnRH with higher amplitude were observed around the 4th and 5th pulses. Representative pulsatile profile and individual values are depicted in Fig. 5.

4. Discussion

The present study is the first to describe the complete nucleotide sequence of the GnRH preprogonadotrophin in the hypothalamus of the South American plains vizcacha, *L. maximus*, which corresponds to the GnRH1 variant. In addition, predominant hypothalamic localization among brain areas and reproductive tissues and a pulsatile secretion pattern of one pulse every hour were observed.

The deduced hypothalamic sequence showed the three classical components of the GnRH preprogonadotrophin: signal peptide, GnRH decapeptide and GAP (Adelman et al., 1986). Comparing the obtained sequence of the GnRH preprogonadotrophin in the vizcacha with that among species, 4 exons could be predicted (Tsai and Zhang, 2008). It is likely that the first exon would contain the 5′ UTR, the second exon would encode for GnRH and 5′ end of GAP, the entire third exon would encode for GAP and the fourth exon would encode for the resting sequence of GAP followed by the 3′ UTR (Fig. 2). However, this assumption cannot be confirmed until the entire GnRH gene is characterized. The presence of two mRNA isoforms of the GnRH preprogonadotrophin carrying different 5′ UTR fragments was unexpected. The agarose gel electrophoresis revealed that in the vizcacha hypothalamus the shortest transcript was less abundant than the longest isoform. This analysis does not determine if the two isoforms of 5′ UTR may have different functions but they might reflect a post-transcriptional regulation of GnRH rendering a sensitive equilibrium between RNA stability and translational efficiency. It cannot be ascertained whether the different transcripts obtained were produced by alternative promoters from a single GnRH preprogonadotrophin gene. Similar observations were reported for the neuronal nitric oxide synthase (nNOS) which shows transcripts with diverse 5′ UTRs that did not affect the encoded protein sequence and are generated by alternative multiple promoters (Wang et al., 1999). Diverse 5′ UTRs may be the substrate for different cell specific tissue factors that may modulate translational efficiency in a specific endocrine situation. This may be the case during different reproductive stages as pregnancy, ovulation or lactation in this mammal with such peculiar reproductive features. On the other hand, differences on the 3′ UTR length have been previously described in gpGnRH. In that case, the 3′ UTR fragment showed two isoforms resulting from the presence of two different polyadenylation sites (Jimenez-Liñan et al., 1997).

GnRH shows a high degree of conservation among close related caviomorphs like chinchilla, degu and guinea pig, and a lesser

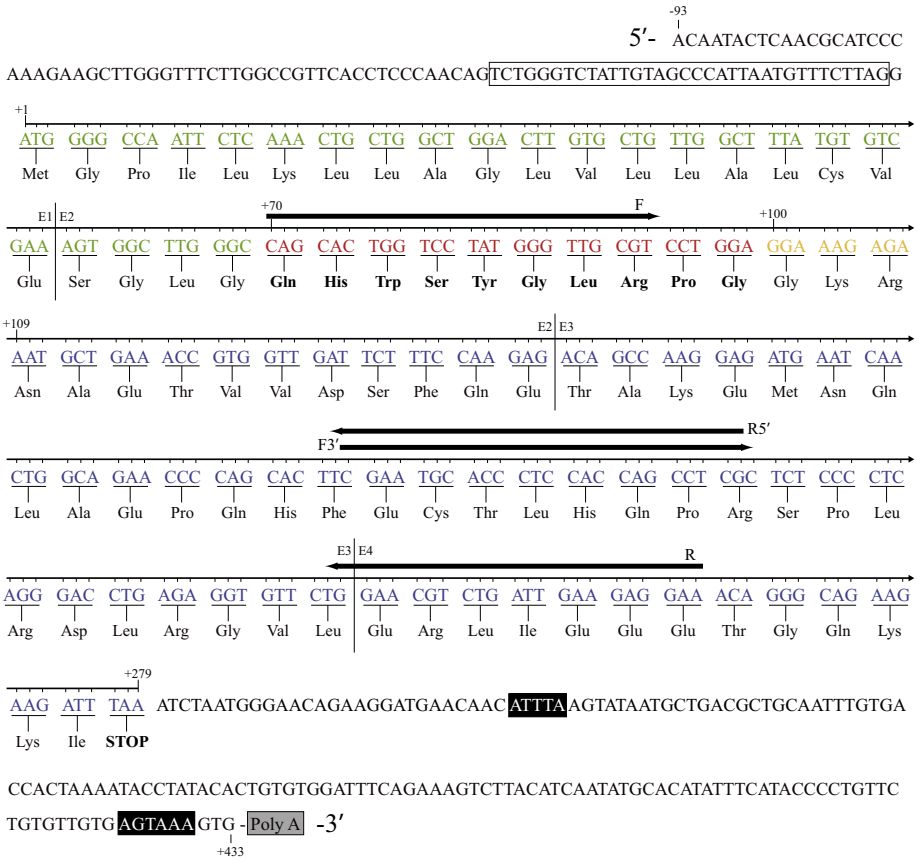


Fig. 2. Full-length cDNA sequence and deduced amino acid sequence coding the hypothalamic GnRH preprogonadotrophin of vizcacha. Green: nucleotides for signal peptide. Red: nucleotides for GnRH. Orange: nucleotides for the enzymatic cleavage site. Blue: nucleotides for GAP. Black: nucleotides for 5′ and 3′ untranslated regions (UTR) respectively. 5′ UTR is indicated with negative numbers and represents the larger 5′ UTR isoform (nucleotides not found in the shorter isoform are boxed). Nucleotide numbers are indicted above the sequence. The start codon of the open reading frame sequence is indicated as +1. The last part of the GnRH preprogonadotrophin, corresponding to GAP, ends at nucleotide 279 with the TAA codon. The polyadenylation signal (Poly A) and the ATTTA sequence are black shaded in the 3′ tail. Arrows over the sequence indicate location of forward (F) and reverse (R) primers used to amplify the specific vizcacha GnRH-GAP fragment, and specific primers used in the RACE-PCR, R5′ (reverse primer) and F3′ (forward primer). Deduced amino acid sequence is indicated under the nucleotides sequence. GnRH predicted amino acids are in bold. Deduced exon-exon boundaries are indicated (E*n*). We have submitted both isoforms of GnRH1 of *Lagostomus maximus* nucleotide sequence to the Gen-Bank (Accession Number KT003537 and KT336553).

extent of homology with more distantly related rodents like mouse and rat (Kochman, 2012); however, the peptide sequence remains mostly unchanged except for the guinea pig (Jimenez-Liñan et al., 1997). This reinforces the importance of the conserved role of GnRH in the regulation of the HHG axis, specifically on hypophyseal gonadotropin synthesis and release, during the 500 million years of vertebrate evolution (Adams et al., 2002; Millar et al., 2004). Whereas vizcacha GnRH showed a complete identity with the mammalian variant, GAP nucleotide sequence showed the highest homology with that of chinchilla, its closest evolutionarily related species. When comparing the vizcacha GnRH nucleotide sequence with that of guinea pig, the nucleotide substitutions predict two amino acids changes as expected (histidine instead of tyrosine in the second position, and leucine instead of valine in the seventh place), as previously described by Jimenez-Liñan et al. (1997); however, when compared with human, the three nucleotide changes detected (at codons 6, 7 and 8) resulted in silent substitutions. The high conservation of GnRH among species confirms once again the relevance of this hormone, among other factors as kisspeptin, dopamine, etc., to guarantee reproduction. On the other hand, the range of homology determined for GAP, even among closely related species, suggests that the bioactivity of this peptide may be different among species (Tsai, 2006). Differences in GAP length have been previously reported between the preprogonadotrophins for GnRH1 and GnRH2 with a shorter GAP for the former precursor (Cheng and Leung, 2005). GAP is co-released with GnRH from the median eminence and is able to inhibit prolactin secretion and stimulate LH and FSH release albeit less effectively than GnRH (Wetsel and Srinivasan, 2002). GnRH expression has been described from protochordates to vertebrates (Guilgur et al., 2006; Millar, 2005; Somoza et al., 2002; Tsai, 2006). GnRH1 nucleotide and protein expression is much more abundant in the hypothalamus when compared with other tissues; however, it is also expressed in extra-hypothalamic reproductive tissues of vizcacha like hypophysis and ovary. Similar distribution and expression pattern have been previously described among mammals (Krsmanovic et al., 2000; Miller et al., 1996; Peng et al., 1994; Ramakrishnappa et al., 2005; Schirman-Hildesheim et al., 2005). According to Krsmanovic et al. (2000), the presence of GnRH1 in the adenohypophysis, the major site of expression of GnRH receptors, suggests local regulatory effects of the decapeptide over the control of LH and FSH secretion, with special incidence in the vizcacha mid-pregnancy where an increase of serum LH is reported. Similar autocrine regulation of cell function may occur in the ovary where GnRH modulates steroidogenesis by having effect on the production of steroid hormones and apoptosis in ovarian follicle and corpus luteum (Ramakrishnappa et al., 2005).

High abundance of GnRH2 has been identified in the midbrain of a variety of vertebrates like rat, frog, fish, and stingray, and it is involved in sensory and behavioral regulation (Forlano et al., 2000;

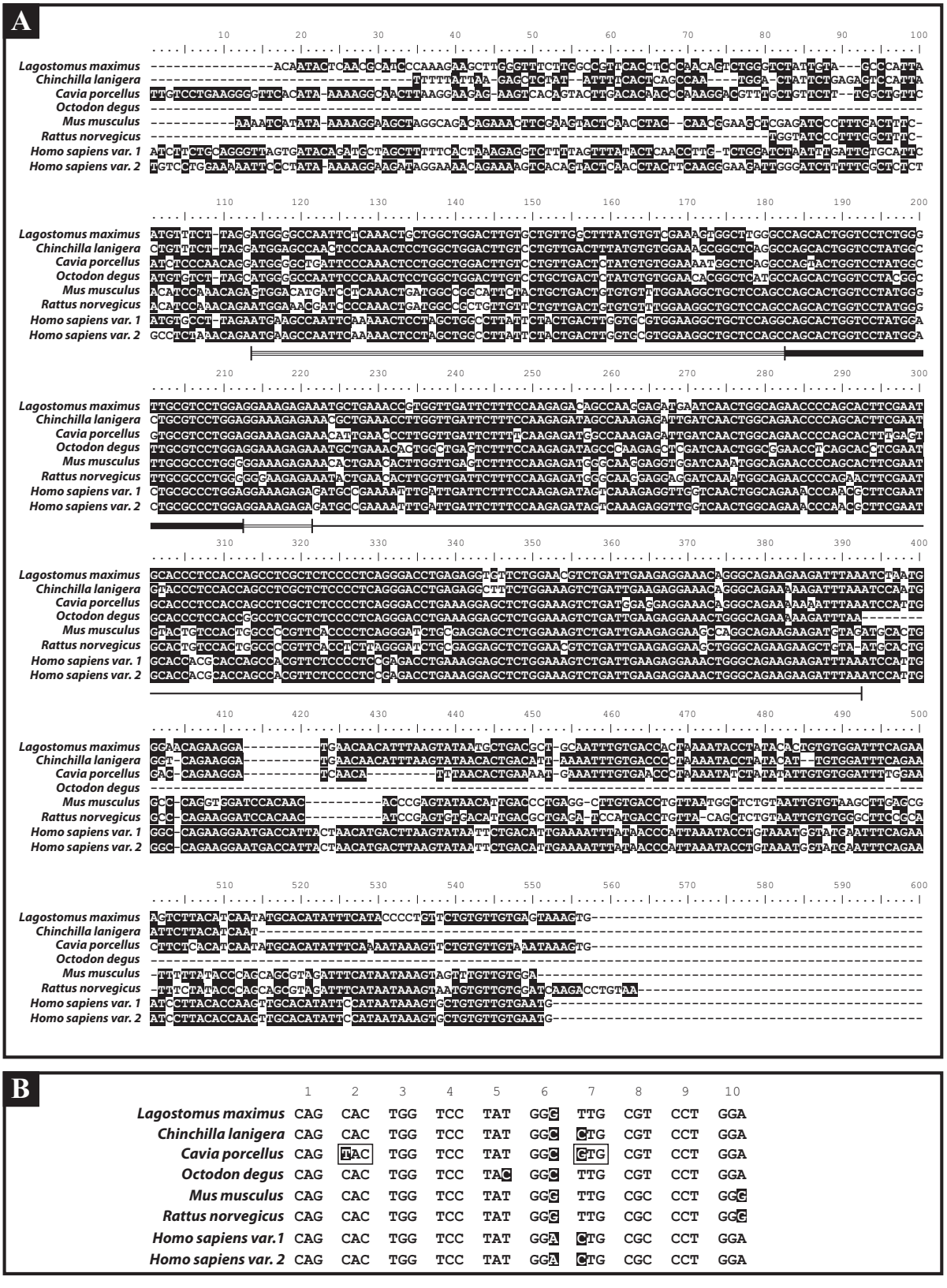


Fig. 3. Alignment of the vizcacha nucleotide GnRH preprogonadotrophin sequence with those previously reported in other species. (A) The obtained GnRH preprogonadotrophin nucleotide sequence of South American plains vizcacha (*L. maximus*) was aligned with that of chinchilla (*C. lanigera*), guinea pig (*C. porcellus*), degu (*O. degus*), mouse (*M. musculus*), rat (*R. norvegicus*) and human (*H. sapiens*, GnRH1 isoforms 1 and 2). To maximize homology, sequences were aligned according to the GnRH sequences. Nucleotides were numbered according to the first nucleotide of the guinea pig sequence and numbers are indicated above the sequences. Human 5′ UTRs corresponding to both isoforms show only the 3′ side of each one. Homologue nucleotides are black shaded. The triple line indicates the signal peptide, the thick line indicates GnRH sequence, the double line indicates the enzymatic cleavage site sequence and single line indicates GAP sequence. (B) Alignment of GnRH nucleotide sequences of *L. maximus*, *C. lanigera*, *C. porcellus*, *O. degus*, *M. musculus*, *R. norvegicus* and *H. sapiens*. Non-homologous nucleotides are black shaded and codons coding for different amino acids are boxed. The numbers of codons are indicated above the sequences.

Table 2
Percentage of homology of GnRH of *L. maximus* with other species.

Species	*Lagostomus maximus*		
	PreProGnRH (%)	GnRH (%)	GAP (%)
Mus musculus	81.00	93.33	82.46
Rattus norvegicus	81.00	93.33	83.63
Cavia porcellus	87.00	90.00	87.72
Octodon degus	88.00	93.33	88.30
Chinchilla lanigera	92.00	93.33	93.57
Homo sapiens variant 1	81.00	93.33	83.63
Homo sapiens variant 2	81.00	93.33	83.63

Palmieri et al., 2008; Parhar et al., 2005; Sukhan et al., 2013; Wang et al., 2001). Considering that the anti-GnRH antiserum used for RIA is capable of detecting both GnRH1 and GnRH2 variants, GnRH expression in the midbrain was expected to be higher than in the other extrahypothalamic areas. However, the previously proved low affinity of the antiserum used here against GnRH2 (Mongiat et al., 2006) may justify the low level of GnRH detected in the midbrain of the vizcacha when compared with hypothalamus.

The normal reproductive development and fertility depends in part on the frequency of GnRH pulses determining LH or FSH delivery. Variations in GnRH expression have been also reported related to sex, developmental stage and estrous cycle (Schirman-Hildesheim et al., 2005; Watanabe et al., 2009). Pulsatility of GnRH has been previously studied in hypothalamic explants of mice and rats (Di Giorgio et al., 2013; Fernández et al., 2010). Here, we detected that hypothalamic explants of vizcacha studied nine days after induction of ovulation (similar to early luteal phase) show one pulse of GnRH every hour which may represent a high pulsatile frequency when compared with previous descriptions for other mammals in which pulse frequency in luteal phase is nearer to one pulse every two hours (Williams et al., 1990; Wilson et al., 1984). While a fast pulse frequency of GnRH favors LH delivery, a slow pulse frequency favors FSH secretion (Burger et al., 2008; Marshall et al., 1991). In the present case, one pulse every hour frequency may be favoring LH synthesis and delivery, which correlates with the high hypophyseal LH contents and serum LH observed in these studied animals (unpublished results). This is in accordance with the descriptions that during LH surge, GnRH levels remain elevated (Moenter et al., 1990). On the other hand, the amplitude is another important factor involved in the GnRH message transmission. In this work, it was shown that high amplitude of GnRH pulse was observed around 4th and 5th hour of assay. This could be the result of the isolation of the hypothalamus from the whole animal where the explants are relieved from the ovarian negative feedback. In addition, GnRH promoter activity also seems to operate in a pulsatile manner, where the secretory pulse of GnRH and its episodic expression are closely associated (Nunez et al., 1998; Vazquez-Martinez et al., 2002). Further analysis of the GnRH promoter and its enhancers would contribute to elucidate this issue.

In conclusion, the knowledge of GnRH mRNA sequence, distribution and molecular physiology in *L. maximus* represents an important contribution to employ this animal in the research of

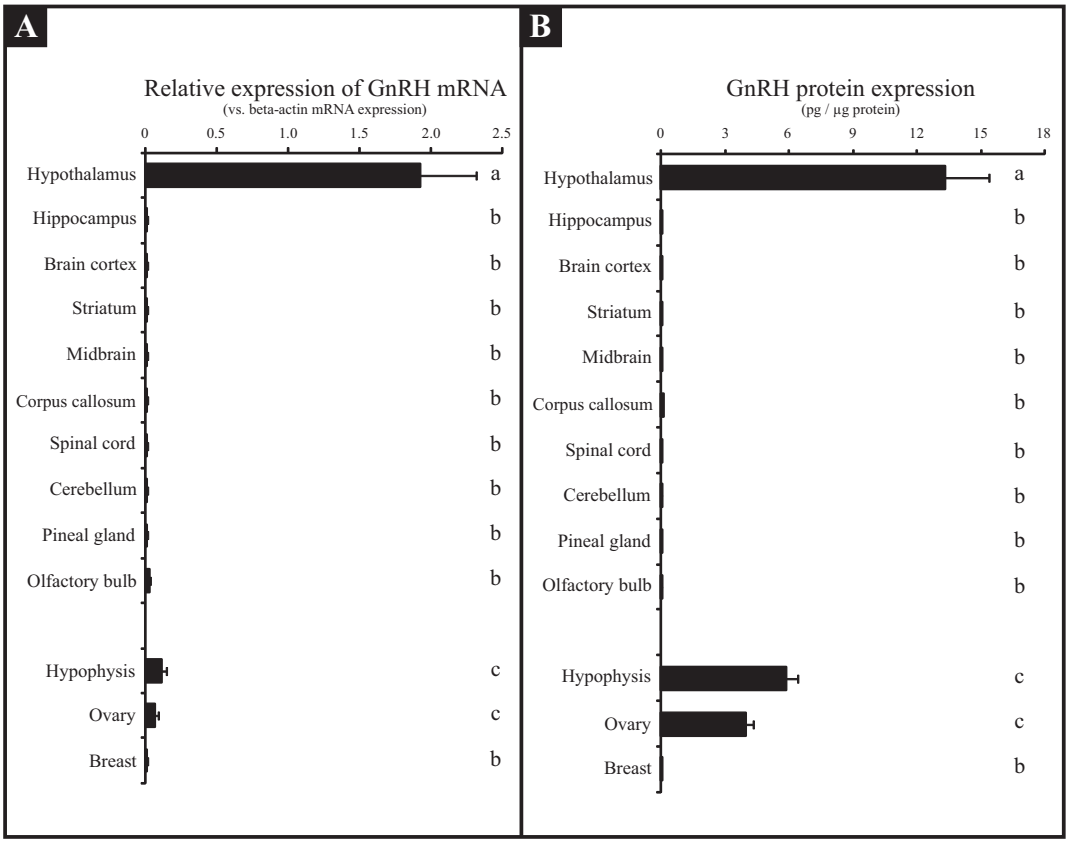
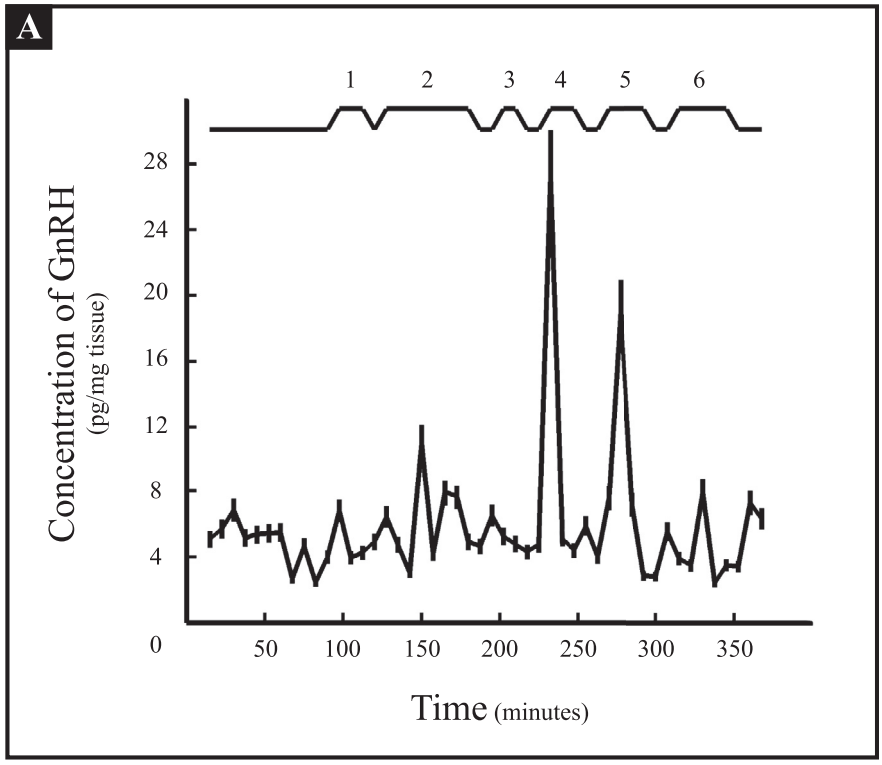


Fig. 4. Distribution of GnRH in the brain regions and reproductive organs of vizcacha. mRNA (A) and peptide (B) GnRH expression was significantly higher in the hypothalamus in relation with the other studied brain areas, whereas extra-brain areas like hypophysis and ovary showed significantly increased expression with respect to breast. The expression of GnRH mRNA was related to beta-actin mRNA content whereas the expression of protein GnRH was related to the total protein content. Different letters show significant differences with $p < 0.05$ as determined by ANOVA.



B

Animal number	Number of pulses during 6 hours	Mean intervals between pulses (minutes)	Mean mass secreted by pulse (mean area per pulse)	Total mass secreted (total area of pulses)
1	6	46.5	185.6	2123
2	5	58.1	336.5	2559
3	6	54.0	214.5	3986
4	5	56.2	531.9	5819
5	5	48.7	183.3	3064
Average	**5.4**	**52.7**	**290.4**	**3510**
S.E.	**0.2**	**2.2**	**66.6**	**655.2**

Fig. 5. Analysis of GnRH pulsatility. (A) Representative GnRH pulsatile profile from female hypothalamic explants determined by radioimmunoassay (RIA). Six pulses were detected during 6 h and the number of each pulse is indicated above the graph, whereas the bottom line indicates the time lapse of the GnRH release study. (B) Summary of the analyzed pulsatile parameters with individual values for each analyzed animal. Average and S.E. (Standard Error) are shown.

reproductive abnormalities including hypothalamic amenorrhea, hyperprolactinaemia, hyperandrogenemia and polycystic ovary syndrome that involve disruption of the normal pulsatile GnRH secretion.

Acknowledgments

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (PIP N° 0225) granted to VBD and by Fundación Científica Felipe Fiorellino, Universidad Maimónides, Argentina. Authors are especially grateful to the Ministerio de Asuntos Agrarios, Dirección de Flora y Fauna, Buenos Aires Province for enabling animal capture, to the personnel of ECAS for their invaluable help in trapping and handling the animals, to Dr. Sergio Ferraris and his veterinarian staff for their essential help on vizcachas handling and anesthetizing, and to Lic. María Constanza Gariboldi for her contribution on submitting the nucleotide sequences to the Gen-Bank.

References

Adams, B.A., Vickers, E.D., Warby, C., Park, M., Fischer, W.H., Craig, A.G., Rivier, J.E., Sherwood, N.M., 2002. Three forms of gonadotropin-releasing hormone, including a novel form, in a basal salmonid, *Coregonus clupeaformis*. Biol. Reprod. 67, 232–239.

Adelman, J., Mason, A.J., Hayflick, J.S., Seeburg, P.H., 1986. Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat. Proc. Natl. Acad. Sci. 83, 179–183.

Belchetz, P.E., Plant, T.M., Nakai, Y., Keogh, E.J., Knobil, E., 1978. Hypophyseal responses to continuous and intermittent delivery of hypopthalamic gonadotropin-releasing hormone. Science 202, 631–633.

Bradford, M.M., 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Burger, L.L., Haisenleder, D.J., Aylor, K.W., Marshall, J.C., 2008. Regulation of intracellular signaling cascades by GnRH pulse frequency in the rat pituitary: roles for CaMK II, ERK, and JNK activation. Biol. Reprod. 79, 947–953.

Burgus, R., Butcher, M., Amoss, M., Ling, N., Monahan, M., Rivier, J., Fellows, R., Blackwell, R., Vale, W., Guillemin, R., 1972. Primary structure of the ovine hypothalamic luteinizing hormone-releasing factor (LRF). Proc. Natl. Acad. Sci. 69, 278–282.

Catalano, P.N., Di Giorgio, N.P., Bonaventura, M.M., Bettler, B., Libertun, C., Lux-Lantos, V., 2010. Lack of functional $GABA_B$ receptors alters GnRH physiology and sexual dimorphic expression of GnRH and GAD-67 in the brain. Am. J. Physiol. 298, E683–E696.

Cheng, C.K., Leung, P.C.K., 2005. Molecular biology of gonadotropin-releasing hormone (GnRH)-I, GnRH-II, and their receptors in humans. Endocr. Rev. 26, 283–306.

Ciccone, N.A., Xu, S., Lacza, C.T., Carroll, R.S., Kaiser, U.B., 2010. Frequency-dependent regulation of follicle-stimulating hormone beta by pulsatile gonadotropin-releasing hormone is mediated by functional antagonism of bZIP transcription factors. Mol. Cell. Biol. 30, 1028–1040.

Di Giorgio, N.P., Catalano, P.N., López, P.V., González, B., Semaan, S.J., López, G.C., Kauffman, A.S., Rulli, S.B., Somoza, G.M., Bettler, B., Libertun, C., Lux-Lantos, V.A., 2013. Lack of functional GABAB receptors alters Kiss1, GnRH1 and Gad1 mRNA expression in the medial basal hypothalamus at postnatal day 4. Neuroendocrinology 98, 212–223.

Dorfman, V.B., Fraunhoffer, N., Inserra, P.I.F., Loidl, C.F., Vitullo, A.D., 2011. Histological characterization of gonadotropin-releasing hormone (GnRH) in the hypothalamus of the South American plains vizcacha (*Lagostomus maximus*). J. Mol. Histol. 42, 311–321.

Dorfman, V.B., Saucedo, L., Di Giorgio, N.P., Inserra, P.I.F., Fraunhoffer, N., Leopardo, N.P., Halperín, J., Lux-Lantos, V., Vitullo, A.D., 2013. Variation in progesterone receptors and GnRH expression in the hypothalamus of the pregnant South American plains vizcacha, *Lagostomus maximus* (Mammalia, Rodentia). Biol. Reprod. 89, 115.

Fernald, R.D., White, R.B., 1999. Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. Front. Neuroendocrinol. 20, 224–240.

Fernández, M., Bourguignon, N., Lux-Lantos, V., Libertun, C., 2010. Neonatal exposure to bisphenol A and reproductive and endocrine alterations resembling the polycystic ovarian syndrome in adult rats. Environ. Health Perspect. 118, 1217–1222.

Forlano, P.M., Maruska, K.P., Sower, S.A., King, J.A., Tricas, T.C., 2000. Differential distribution of gonadotropin-releasing hormone-immunoreactive neurons in the stingray brain: functional and evolutionary considerations. Gen. Comp. Endocrinol. 118, 226–248.

Gao, Pf, Cao, Gq, Zhao, Ht, Zhang, Gx, Jiang, Ys, Qd, Wang, 2009. Molecular cloning and characterization of pigeon (*Columba liva*) ubiquitin and ubiquitin-conjugating enzyme genes from pituitary gland library. Int. J. Biol. Sci. 5, 34–43.

Greenwood, F.C., Hunter, W.M., Glover, J.S., 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. 89, 114–123.

Grove-Strawser, D., Sower, S.A., Ronsheim, P.M., Connoly, J.B., Bourn, C.G., Rubin, B. S., 2002. Guinea pig GnRH: localization and physiological activity reveal that it, not mammalian GnRH, is the major neuroendocrine form in guinea pigs. Endocrinology 143, 1602–1612.

Guilgur, L.G., Moncaut, N.P., Canário, A.V.M., Somoza, G.M., 2006. Evolution of GnRH ligands and receptors in gnathostomata. Comp. Biochem. Physiol. A 144, 272–283.

Halperin, J., Dorfman, V.B., Fraunhoffer, N., Vitullo, A.D., 2013. Estradiol, progesterone and prolactin modulate mammary gland morphogenesis in adult female plains vizcacha (*Lagostomus maximus*). J. Mol. Histol. 44, 299–310.

Inserra, P.I.F., Leopardo, N.P., Willis, M.A., Freysselinard, A.L., Vitullo, A.D., 2014. Quantification of healthy and atretic germ cells and follicles in the developing and post-natal ovary of the South American plains vizcacha, *Lagostomus maximus*: evidence of continuous rise of the germinal reserve. Reproduction 147, 199–209.

Jackson, J.E., 1986. Determinación de edad en la vizcacha (*Lagostomus maximus*) en base al peso del cristalino. Vida Silvestre 1, 41–44.

Jackson, J.E., Branch, L.C., Villarreal, D., 1996. *Lagostomus maximus*. Mammalian Species 543, 1–6.

Jensen, F., Willis, M.A., Albamonte, M.S., Espinosa, M.B., Vitullo, A.D., 2006. Naturally suppressed apoptosis prevents follicular atresia and oocyte reserve decline in the adult ovary of *Lagostomus maximus* (Rodentia, Caviomorpha). Reproduction 132, 301–308.

Jensen, F., Willis, M.A., Leopardo, N.P., Espinosa, M.B., Vitullo, A.D., 2008. The ovary of the gestating South American plains vizcacha (*Lagostomus maximus*): suppressed apoptosis and corpora lutea persistence. Biol. Reprod. 79, 240–246.

Jimenez-Liñan, M., Rubin, B.S., King, J.C., 1997. Examination of guinea pig luteinizing hormone-releasing hormone gene reveals a unique decapeptide and existence of two transcripts in the brain. Endocrinology 138, 4123–4130.

Kineman, R.D., Leshin, L.S., Crim, J.W., Rampacek, G.B., Kraeling, R.R., 1988. Localization of luteinizing hormone-releasing hormone in the forebrain of the pig. Biol. Reprod. 39, 665–672.

King, J.A., Millar, R.P., 1982. Structure of chicken hypothalamic luteinizing hormone-releasing hormone. II. Isolation and characterization. J. Biol. Chem. 257, 10729–10732.

Kochman, K., 2012. Evolution of gonadotropin-releasing hormone (GnRH) structure and its receptor. J. Anim. Feed Sci. 21, 3–30.

Krey, L.C., Silverman, A.J., 1978. The luteinizing hormone-releasing hormone (LH-RH) neuronal networks of the guinea pig brain. II. The regulation of gonadotropin secretion and the origin of terminals in the median eminence. Brain Res. 157, 247–255.

Krsmanovic, L.Z., Martinez-Fuente, A.J., Arora, K.K., Mores, N., Tomić, M., Stojilkovic, S.S., Catt, K.J., 2000. Local regulation of gonadotroph function by pituitary gonadotropin-releasing hormone. Endocrinology 141, 1187–1195.

Leopardo, N.P., Jensen, F., Willis, M.A., Espinosa, M.B., Vitullo, A.D., 2011. The developing ovary of the South American plains vizcacha, *Lagostomus maximus* (Mammalia, Rodentia): massive proliferation with no sign of apoptosis-mediated germ cell attrition. Reproduction 141, 633–641.

Lethimonier, C., Madigou, T., Muñoz-Cueto, J.A., Lareyre, J.J., Kah, O., 2004. Evolutionarily aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. Gen. Comp. Endocrinol. 135, 1–16.

Llanos, A.C., Crespo, J.A., 1952. Ecología de la vizcacha (*Lagostomus maximus maximus* Blainv.) en el noreste de la Provincia de Entre Ríos. Revista de Investigaciones Agrícolas 6, 289–578.

Marshall, J.C., Dalkin, A.C., Haisenleder, D.J., Paul, S.J., Ortolano, G.A., Kelch, R.P., 1991. Gonadotropin-releasing hormone pulses: regulators of gonadotropin synthesis and ovulatory cycles. Recent Prog. Horm. Res. 47, 155–187.

Martinez de la Escalera, G., Choi, A.L., Weiner, R.I., 1992. Generation and synchronization of gonadotropin-releasing hormone (GnRH) pulses: intrinsic properties of the GT1-1 GnRH neuronal cell line. Proc. Natl. Acad. Sci. U.S.A. 89, 1852–1855.

Matsuo, H., Baba, Y., Nair, R.M., Arimura, A., Schally, A.V., 1971. Structure of the porcine LH- and FSH-releasing hormone I. The proposed amino acid sequence. Biochem. Biophys. Res. Commun. 43, 1334–1339.

Millar, R.P., 2005. GnRHs and GnRH receptors. Anim. Reprod. Sci. 88, 5–28.

Millar, R.P., Lu, Z., Pawson, A.J., Flanagan, C.A., Morgan, K., Maudsley, S.R., Troskie, B., Ott, T., Millar, M., Lincoln, G., Sellar, R., Faurholm, B., Scobie, G., Kuestner, R., Terasawa, E., Katz, A., 2004. Gonadotropin-releasing hormone receptors. Endocr. Rev. 25, 235–275.

Miller, G.M., Alexander, J.M., Klibanski, A., 1996. Gonadotropin-releasing hormone messenger RNA expression in gonadotroph tumors and normal human pituitary. J. Clin. Endocrinol. Metab. 81, 80–83.

Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K., Matsuo, H., 1984. Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. Proc. Natl. Acad. Sci. U.S.A. 81, 3874–3878.

Moenter, S.M., Caraty, A., Karsch, F.J., 1990. The estradiol induced surge of GnRH in the ewe. Endocrinology 127, 1375–1384.

Mongiat, L.A., Fernández, M.O., Lux-Lantos, V.A.R., Guilgur, L.H., Somoza, G.M., Libertun, C., 2006. Regul. Pept. 136, 50–57.

Montaner, A.D., Mongiat, L., Lux-Lantos, V.A., Warby, C., Chewpoy, B., Bianchi, M.S., Libertun, C., Rivier, J.E., Sherwood, N.M., Somoza, G.M., 2002. Guinea pig gonadotropin-releasing hormone: expression pattern, characterization and biological activity in rodents. Neuroendocrinology 75, 326–338.

National Institutes of Health, 1985. Health Research Extension Act of 1985, Public Law 99-158. Animals in Research. Office of Laboratory Animal Welfare, NIH.

Nunez, L., Faught, W.J., Frawley, L.S., 1998. Episodic gonadotropin-releasing hormone gene expression revealed by dynamic monitoring of luciferase reporter activity in single, living neurons. Proc. Natl. Acad. Sci. U.S.A. 95, 9648–9653.

Palmieri, G., Acone, F., Desantis, S., Corriero, A., Ventriglia, G., Addis, P., Genovese, S., Aprea, A., Spedicato, D., Losurdo, M., Deflorio, M., Di Summa, A., De Metrio, G., 2008. Brain morphology and immunohistochemical localization of the gonadotropin-releasing hormone in the blue tune, *Thunnus thynnus*. Eur. J. Histochem. 52, 19–28.

Parhar, I.S., Soga, T., Ogawa, S., Ogawa, S., Pfaff, D.W., Sakuma, Y., 2005. Nonmammalian gonadotropin-releasing hormone molecules in the brain of promoter transgenic rats. Proc. Natl. Soc. Sci. U.S.A. 102, 5880–5885.

Peng, C., Fan, N.C., Ligier, M., Väänänen, J., Leung, P.C., 1994. Expression and regulation of gonadotropin-releasing hormone (GnRH) and GnRH receptor messenger ribonucleic acids in human granulosa-luteal cells. Endocrinology 135, 1740–1746.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real time RT-PCR. Nucleic Acids Res. 29, e45.

Ramakrishnappa, N., Rajamahendran, R., Lin, Y.M., Leung, P.C., 2005. GnRH in non-hypothalamic reproductive tissues. Anim. Reprod. Sci. 88, 95–113.

Roch, G.J., Busby, E.R., Sherwood, N.M., 2014. GnRH receptors and peptides: skating backward. Gen. Comp. Endocrinol. 209, 118–134.

Schirman-Hildesheim, T.D., Bar, T., Ben-Aroya, N., Koch, Y., 2005. Differential gonadotropin-releasing hormone (GnRH) and GnRH receptor messenger ribonucleic acid expression patterns in different tissues of the female rat across the estrous cycle. Endocrinology 146, 3401–3408.

Sherwood, N.M., Sower, S.A., Marshak, D.R., Fraser, B.A., Brownstein, M.J., 1986. Primary structure of gonadotropin-releasing hormone from lamprey brain. J. Biol. Chem. 261, 4812–4819.

Silverman, A.J., Witkin, J.W., 1994. Biosynthesis of gonadotropin-releasing hormone during the rat estrous cycle: a cellular analysis. Neuroendocrinology 59, 545–551.

Silverman, A.J., Jhamandas, J., Renaud, L.P., 1987. Localization of luteinizing hormone-releasing hormone (LHRH) neurons that project to the median eminence. J. Neurosci. 7, 2312–2319.

Somoza, G.M., Miranda, L.A., Strobl-Mazzulla, P., Guilgur, L.G., 2002. Gonadotropin-releasing hormone (GnRH): from fish to mammalian brains. Cell. Mol. Neurobiol. 22, 589–609.

Sukhan, Z.P., Kitano, H., Salvaraj, S., Yoneda, M., Yamaguchi, A., Matsuyama, M., 2013. Identification and distribution of three gonadotropin-releasing hormoe (GnRH) isoforms in the brain of a clupeiform fish, *Engraulis japonicus*. Zoolog. Sci. 30, 1081–1091.

Tsai, P.S., 2006. Gonadotropin-releasing hormone in invertebrates: structure, function, and evolution. Gen. Comp. Endocrinol. 148, 48–53.

Tsai, P.S., Zhang, L., 2008. The emergence and loss of gonadotropin-releasing hormone in protostomes: orthology, phylogeny, structure, and function. Biol. Reprod. 79, 798–805.
Urbanski, H.F., Doan, A., Pierce, M., 1991. Immunocytochemical investigation of luteinizing hormone-releasing hormone neurons in Syrian hamsters maintained under long or short days. Biol. Reprod. 44, 687–692.
Urbanski, H.F., Doan, A., Pierce, M., Fahrenbach, W.H., Collins, P.M., 1992. Maturation of the hypothalamo-pituitary-gonadal axis of male Syrian hamsters. Biol. Reprod. 46, 991–996.
Vazquez-Martinez, R., Leclerc, G.M., Wierman, M.E., Boockfor, F.R., 2002. Episodic activation of the rat GnRH promoter: role of the homeoprotein oct-1. Mol. Endocrinol. 16, 2093–2100.
Veldhuis, J.D., Johnson, M.L., 1986. Cluster analysis: a simple, versatile, and robust algorithm for endocrine pulse detection. Am. J. Physiol. 250, E486–E493.
Wang, Y., Newton, D.C., Robb, G.B., Kau, C.L., Miller, T.L., Cheung, A.H., Hall, A.V., VanDamme, S., Wilcox, J.N., Marsden, P.A., 1999. RNA diversity has profound effects on the translation of neuronal nitric oxide synthase. Proc. Natl. Acad. Sci. U.S.A. 96, 12150–12155.
Wang, L., Yoo, M.S., Kang, H.M., Im, W.B., Choi, H.S., Bogerd, J., Kwon, H.B., 2001. Cloning and characterization of cDNAs encoding the GnRH1 and GnRH2 precursors from bullfrog (*Rana catesbeiana*). J. Exp. Zool. 289, 190–201.
Watanabe, M., Sakuma, Y., Kato, M., 2009. $GABA_A$ receptors mediate excitation in adult rat GnRH neurons. Biol. Reprod. 81, 327–332.
Weir, B.J., 1971a. The reproductive organs of the female plains viscacha, *Lagostomus maximus*. J. Reprod. Fertil. 25, 365–373.
Weir, B.J., 1971b. The reproductive physiology of the plains viscacha, *Lagostomus maximus*. J. Reprod. Fertil. 25, 355–363.
Wetsel, W.C., Srinivasan, S., 2002. Pro-GnRH processing. Prog. Brain Res. 141, 221–241.
Wildt, L., Häusler, A., Marshall, G., Hutchison, J.S., Plant, T.M., Belchetz, P.E., Knobil, E., 1981. Frequency and amplitude of gonadotropin-releasing hormone stimulation and gonadotropin secretion in the rhesus monkey. Endocrinology 109, 376–385.
Williams, C.L., Thalabard, J.C., O'Byrne, K.T., Grosser, P.M., Nishihara, M., Hotchkiss, J., Knobil, E., 1990. Duration of phasic electrical activity of the hypothalamic gonadotropin-releasing hormone pulse generator and dynamics of luteinizing hormone pulses in the rhesus monkey. Proc. Natl. Acad. Sci. U.S.A. 87, 8580–8582.
Willis, M.A., 2011. Calidad y funcionalidad de las gametas femeninas en un modelo mamífero con poliovulación masiva y supresión de apoptosis folicular. Buenos Aires, Argentina: Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica. Thesis.
Wilson, L.A.B., Sanchez-Villagra, M.R., 2010. Diversity trends and their ontogenetic basis: an exploration of allometric disparity in rodents. Proc. R. Soc. London B 207, 1227–1234.
Wilson, R.C., Kesner, J.S., Kaufman, J.M., Uemura, T., Akema, T., Knobil, E., 1984. Central electrophysiologic correlates of pulsatile luteinizing hormone secretion in the rhesus monkey. Neuroendocrinology 39, 256–260.
Witkin, J.W., O'Sullivan, H., Silverman, A.J., 1995. Novel associations among gonadotropin-releasing hormone neurons. Endocrinology 136, 4323–4330.
Yin, W., Mendenhall, J.M., Monita, M., Gore, A.C., 2009a. Three-dimensional properties of GnRH neuroterminals in the median eminence of young and old rats. J. Comp. Neurol. 517, 284–295.
Yin, W., Wu, D., Noel, M., Gore, A.C., 2009b. Gonadotropin-releasing hormone neuroterminals and their microenvironment in the median eminence: effects of aging and estradiol treatment. Endocrinology 150, 5498–5508.
Zhao, J., Hyman, L., Moore, C., 1999. Formation of mRNA 3′ ends in eukaryotes: mechanism, regulation, and interrelationships with other steps in mRNA synthesis. Microbiol. Mol. Biol. Rev. 63, 405–445.
Zohar, Y., Muñoz-Cueto, J.A., Elizur, A., Kah, O., 2010. Neuroendocrinology of reproduction in teleost fish. Gen. Comp. Endocrinol. 165, 438–455.