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

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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

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(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