Chromatographic and immunological identification of GnRH (Gonadotropin-releasing hormone) variants. Occurrence of mammalian and a salmon-like GnRH in the forebrain of an eutherian mammal : Hydrochaeris hydrochaeris (Mammalia, Rodentia

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Chromatographic and immunological identification of GnRH (Gonadotropin-releasing hormone) variants. Occurrence of mammalian and a salmon-like GnRH in the forebrain of an eutherian mammal: *Hydrochaeris hydrochaeris* (Mammalia, Rodentia).

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Abstract

The molecular variants of Gonadotropin releasing hormone (GnRH) in brain extracts of the eutherian mammal *Hydrochaeris hydrochaeris* (Mammalia, Rodentia) were characterized. An indirect method combining reverse-phase high-performance liquid chromatography (RP-HPLC) and radioimmunoassay (RIA) with different antisera was used. Two different forebrain regions (olfactory bulbs and preoptic–hypothalamic region) were analyzed. Characterization of RP-HPLC fractions from preoptic–hypothalamic extracts with three different RIA systems revealed two immunoreactive GnRH (ir-GnRH) peaks coeluting with mammalian GnRH (mGnRH) and salmon GnRH (sGnRH) synthetic standards. These results were additionally supported by serial dilution studies with specific antisera. Similar results were obtained from olfactory bulb extracts with the same methodology. However, a third ir-GnRH peak in a similar position to that of chicken GnRH II (cIIGnRH) synthetic standard was revealed. As far as we know, this is the first report showing chromatographic and immunological evidences for the presence of a second GnRH variant in the forebrain of an eutherian mammal.

Keywords: Gonadotropin-releasing hormone; GnRH; HPLC; Radioimmunoassay; Mammalian; Capybara

1. Introduction

GnRH, originally isolated from ovine and porcine brains, is the hypothalamic hormone that regulates the reproductive system by stimulation of gonadotropins from the anterior pituitary gland [1]. This hormone was considered to be a unique molecular form, until GnRH molecular variants were demonstrated. To date, nine molecular variants have been characterized in vertebrates [2,3] and two other forms were recently characterized form a protochordate [4]. They all are decapeptides, with a very conserved structure: amino-terminus (pGlu) and carboxy-terminus (NH_2) are modified and conserved, all of them having common positions in 1, 2, 4, 9 and 10.

cIIGnRH is expressed together with mGnRH in the brain of most of metatherian species studied [5–7]. Most of the eutherian species studied as yet have only mGnRH,

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Table 1
Radioimmunoassay characteristics

				Percentage of cross-reactivity				
Antiserum	^{125}I-GnRH	STD	Titer	mGnRH	cIGnRH	cIIGnRH	sGnRH	IIGnRH
m1076	mGnRH	mGnRH	1:50 000	100	59	0.4	0.3	< 0.01
EL-15	mGnRH	mGnRH	1:175 000	100	< 0.01	< 0.01	< 0.01	0.05
cII678	mGnRH	mGnRH	1:40 000	100	416	81	473	3.1
cII675	cIIGnRH	cIIGnRH	1:40 000	< 0.01	< 0.01	100	< 0.01	< 0.01
s1668	sGnRH	sGnRH	1:10 000	< 0.01	< 0.01	0.7	100	0.4
sGnRH≠2	sGnRH	sGnRH	1:300 000	< 0.01	< 0.01	1.58	100	0.08
PBL≠45	sGnRH	sGnRH	1:250 000	100	133	25.9	100	24
PBL≠49	sGnRH	sGnRH	1:150 000	93.3	333	32.6	100	2.0

PBL≠45, PBL≠49 and CII678 antisera were used in the screening assays. The following antisera were used in homologous assays: m1076, EL-15, CII675, S1668 and anti sGnRH≠2. ^{125}I-GnRH: radioiodinated hormone; STD: synthetic standard; IIGnRH: lamprey GnRH I. The percentages of crossreactivities of the different RIA systems used were reported by Dr. King, Dr. Okuzawa [24] and Dr. Yu [19], and retested in our conditions. The percentages of crossreactivities of the RIA system with EL-15 were calculated in our laboratory.

with the order Insectivora excepted [3]. mGnRH is coexpressed with cIIGnRH [7,9,10] in the brain of different species of this primitive order [8]. However, there are few reports concerning identification of GnRH variants in eutherian mammal species other than Insectivora [11–13].

The aim of this study was to determine the nature of GnRH variants in the brain of the amphibian mammal *Hydrochaeris hydrochaeris*. It is commonly named 'capybara', 'carpincho' or water hog. This is the largest living rodent and can not be confused with any other form [14]. It may be 1.25 m (50 in.) long and weight 50 kg or more. It is shy and associates in groups along the banks of lakes and rivers of South America. It swims and dives readily and commonly enters water to elude predators. It is strictly vegetarian. The female bears a single litter of three to eight young each year and gestation takes about 100 to 120 days [15].

This model bears special interest because of the following reasons: this animal belongs to the suborder Hystricomorpha considered to be one of the oldest groups of rodents [16]. It is the only living species belonging to the family Hydrochaeridae. This group underwent great radiation in America during Pliocene and Pleistocene periods [16]. Its anatomical features are similar to those of the oldest extinguished rodents [16,17]. Some authors consider this group the mother group of all remaining rodents [18]. Its origin is found in fauna from African Eocene, members from which, migrated across the South Atlantic to South America by rafts [19]. However there are no data about brain physiology except the paper of Affanni et al. [20].

2. Materials and methods

2.1. Animals

Two adult males and one adult female, weighing among 20–50 kg, of *Hydrochaeris hydrochaeris* were used. They were captured by licensed animal officers in the surrounding areas of Diamante (Province of Entre Ríos, Argentina). They were immediately taken to the laboratory. They were anesthetized by a combination of ketamine hydrochloride (40 mg/kg, i.m.) and sodium thiopental (60 mg/kg, i.m.). The brains were removed, and brain regions were obtained by dissection. The pieces were immediately frozen in dry ice, pooled and stored at −70°C until extraction of peptides.

2.2. Tissue extraction

Preoptic–hypothalamic region (4.5 g) and olfactory bulbs (1.5 g) were independently homogenized in acetone: 1 M HCl (100:3, v/v) at 4°C with an Ultraturrax homogenizer. The extraction mixtures were stirred at 4°C and filtered through Whatman N°.1 filter paper. The insoluble material was reextracted in acetone: 0.01 M HCl in 40% of the original volume, stirred for 5 min and refiltered. The combined filtrates were treated with petroleum ether (b.p. 30–60°C) for five successive times as previously described [21,22]. The final aqueous phases were then concentrated to less than 1 ml using a vacuum concentrator.

2.3. Reverse phase-high performance liquid chromatography

The concentrated extracts were filtered through a 0.45 μm Millipore HA filter. They were injected through 1 ml injection loop onto a Lichrospher 100 RP18 column (4 × 250 mm; 5 μm particle size, Merck) with a C18 guard column.

A Konic liquid chromatograph was used. It was programmed according Sherwood et al. [21]. The flow rate was kept at ml/min. The filtrate was injected at the beginning of a 10 min period of 17% acetonitrile in TEAF (0.25 M formic acid adjusted with triethylamine to pH 6.5). The acetonitrile concentration was then increased linearly over a 7 min period to 24%. The elution continued under isocratic conditions for a further 53 min.

Blank chromatographs injecting 1 ml of TEAF were performed before each RP-HPLC of tissue extracts to

check possible contamination of the system. The fractions were radioimmunoassayed under the same conditions as tissue extracts. Fresh standards of GnRH (200 ng of each one) were pooled and diluted in TEAF. They were injected together and chromatographed after the tissue extracts chromatography. Mammalian GnRH, chicken GnRH I (cIGnRH), cIIGnRH and sGnRH were obtained from Peninsula Laboratories Inc. (San Carlos, CA, USA).

RP-HPLC collected fractions were lyophilized and resuspended in RIA buffer. Ir-GnRH was tested using different GnRH RIA systems.

2.4. Radioimmunoassays

Radioimmunoassays were made using synthetic mGnRH, cIIGnRH and sGnRH as some of us previously described [23–25]. The different GnRH standards were radiolabeled following established protocols [26] with minor modifications [23]. Extracts of preoptic–hypothalamic region and olfactory bulbs were analyzed for ir-GnRH in a first screening. PBL≠45, PBL≠49 and cII678 assays were used. See Table 1 for RIA systems and antisera characteristics.

Fractions with ir-GnRH suspected to be caused by an endogenous determined variant were pooled and then analyzed with specific RIAs. The slopes of the displacement curves were compared with those of synthetic standards by ANOVA.

3. Results

The elution positions of the different GnRH synthetic standards were: mGnRH, between 20–22 min; cIGnRH, 22–24 min; cIIGnRH, 27–29 min. The more hydrophobic sGnRH eluted between 50–60 min depending upon the chromatography.

Three different RIA systems were used as a first screening (see Table 1).

3.1. Preoptic-hypothalamic region

RP-HPLC fractions of extracts from this region revealed two main ir-GnRH peaks when analyzed with the three RIA systems (Fig. 1a, b and c). The first ir-GnRH eluting peak (fractions 22–24) coeluted with mGnRH synthetic standard. The second one (fractions 60–63) coeluted with synthetic sGnRH.

Fractions showing ir-mGnRH and ir-sGnRH were pooled independently and serial dilution displacements were performed. Fractions 22–24, which coeluted with synthetic mGnRH, were then tested with two homologous mGnRH RIA systems using m1076 and E-15 antisera (Fig. 2a and b). The slopes of serial dilutions of pooled fractions were not significantly different from those of synthetic standards. The same analysis was performed in fractions

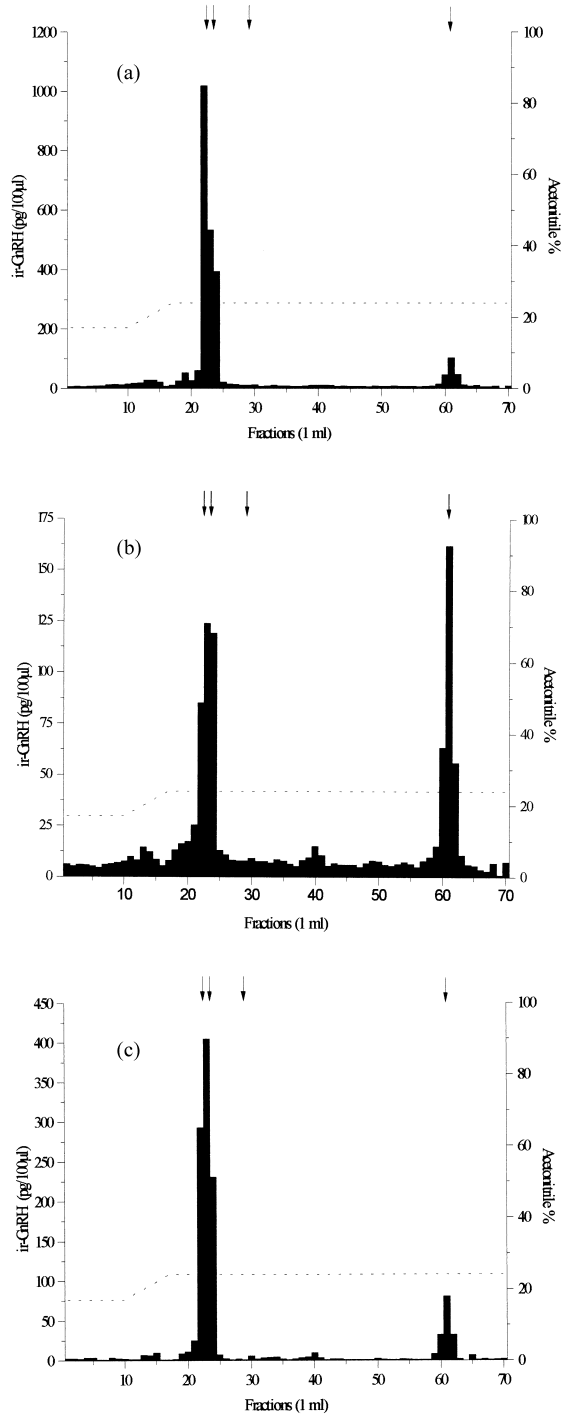

Fig. 1. RP-HPLC of extracts from preoptic–hypothalamic region analyzed with RIAs with the following antisera: (a) PBL ≠45, (b) PBL ≠49 and (c) cII 678. Arrows indicate the elution position of four different GnRH standards. The left arrow shows the elution position of mGnRH. The second arrow represents cIGnRH, the third is cIIGnRH, and the last one is sGnRH.

coeluting with synthetic sGnRH. These fractions were analyzed with two specific sGnRH RIA systems (s1668 and sGnRH≠2 antisera). Both yielded curves parallel to those of sGnRH synthetic standard (Fig. 3a and b).

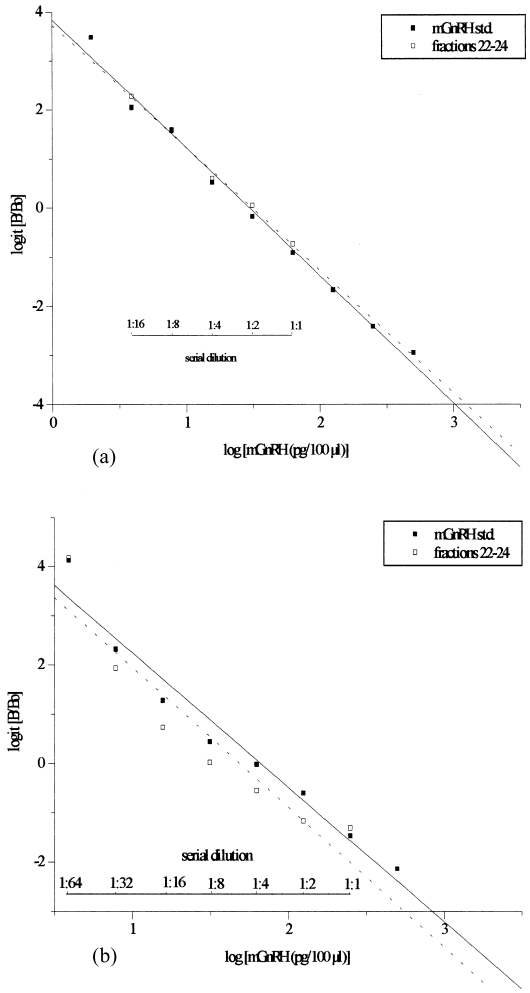

Fig. 2. Parallelism of serial dilutions of fractions 22–24 from RP-HPHLC extract from preoptic–hypothalamic region in comparison with synthetic mGnRH. (a) mGnRH 1076 and (b) EL-15 antisera. The slopes of both curves were not significantly different from those of synthetic standard.

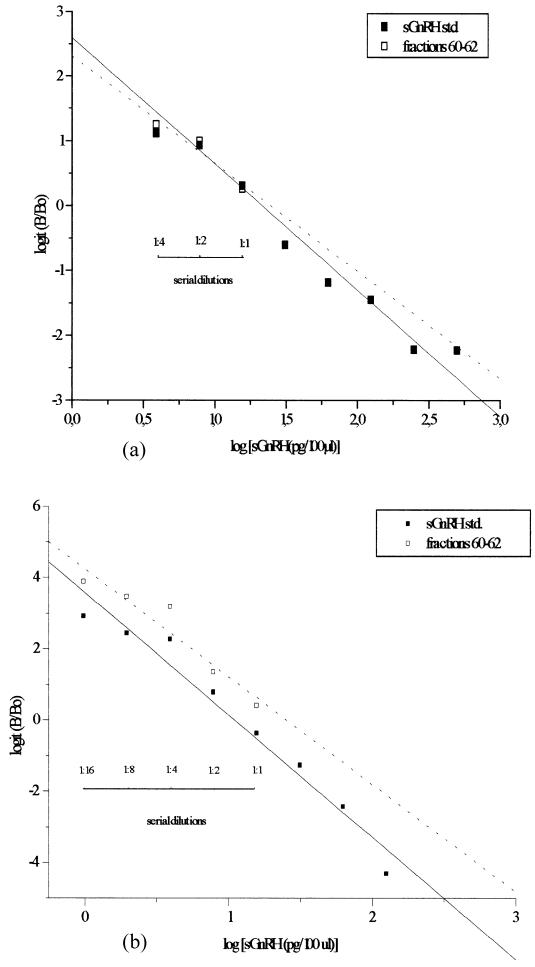

Fig. 3. Parallelism of serial dilutions of fractions 60–62 from HPLC extract of preoptic–hypothalamic region with respect to synthetic sGnRH, using (a) sGnRH 1668 antiserum and (b) sGnRH≠2 antiserum. The slopes of both curves were not significantly different from those of synthetic standard.

Retention time and quantification of immunoreactive GnRH peaks are summarized in Table 2.

3.2. Olfactory bulbs

A similar screening was performed to characterize ir-GnRH fractions in these structures. Three ir-GnRH peaks were revealed (Fig. 4a, b and c). The first one (fractions 21–23) eluted in the same position as mGnRH synthetic. The second ir-GnRH peak (fractions 27–28), coeluted with cIIGnRH synthetic standard and the last one eluted in a position similar to that of sGnRH (fractions 49–50).

Unfortunately ir-GnRH levels in all ir-GnRH fractions were low and serial displacement studies were not so clear. Nevertheless the three ir-GnRH fractions appeared displacing radioiodinated GnRH in homologous assays (EL-15, cII675 and s1668 RIA systems, data not shown).

Retention times and quantification of ir-GnRH peaks of olfactory bulbs extracts are showed in Table 3.

4. Discussion

The analysis of preoptic–hypothalamic region extract from ‘capybara’ revealed two ir-GnRH peaks. These peaks have chromatographic and immunological features resembling those of mGnRH and sGnRH synthetic standards.

Characterization of RP-HPLC fractions using: cII678, PBL ≠45 and PBL ≠49, revealed two ir-GnRH peaks coeluting with mGnRH and sGnRH synthetic standards. Parallelism of serial fractional dilution showing ir-mGnRH and ir-sGnRH to standards curves was seen in each case using specific antisera (mGnRH 1076 and EL-15 antisera for mGnRH and sGnRH 1668 and sGnRH≠2 antisera for sGnRH). In both cases (mGnRH and sGnRH assays) serial dilution of pooled fractions with specific antisera reinforced RP-HPLC and RIA data.

Similar results were obtained with extract from olfactory bulbs. Three ir-GnRH peaks were observed by RIA with polispecific antisera. However serial dilution experiments

Table 2
Retention time and quantification of ir-GnRH peaks in preoptic–hypothalamic extract after RP-HPLC with different antisera.

Tissue		Ret. time	ir-GnRH (pg/100 μl)[a]				Ret. time
PHR	Peak	(min)	M1076	EL-15	S1668	sGnRH≠2	GnRH-std[b]
	I	22–24	70	130	–	–	22 (M)
	II	60–63	–	–	20	10	60 (S)

[a] Immunoreactive GnRH in pooled fractions from each peak from extract of preoptic–hypothalamic region (PHR).
[b] Retention times of synthetic peptides which were run immediately after the biological tissue runs. (M), mGnRH; (S), sGnRH.

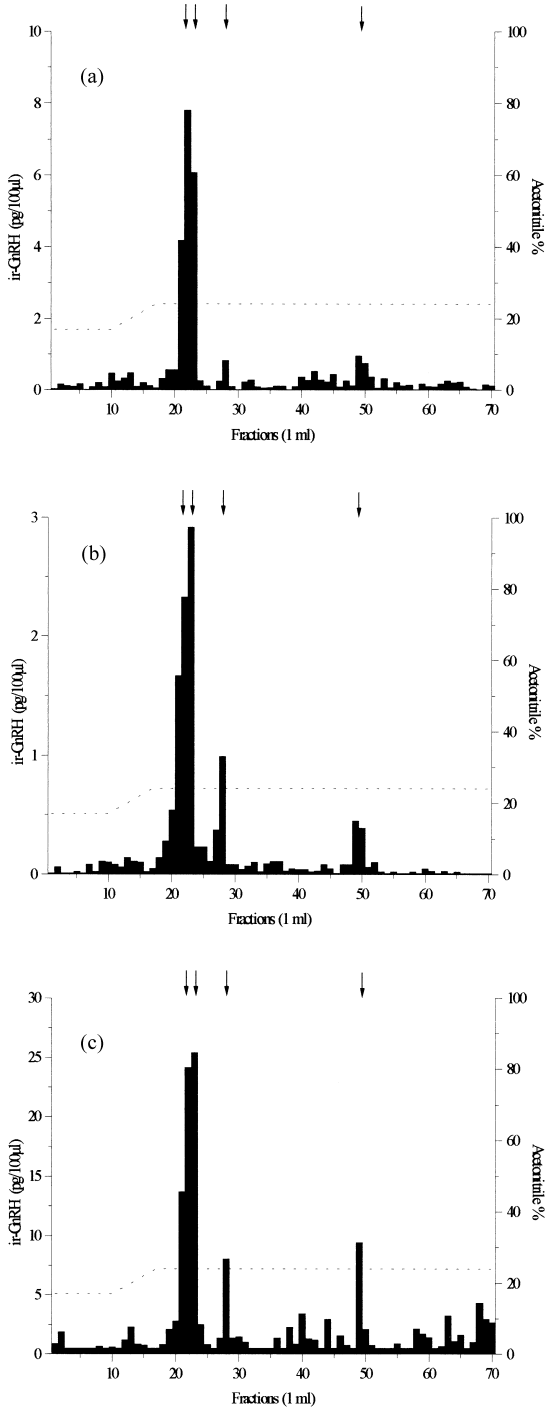

Fig. 4. RP-HPLC of the olfactory bulbs extract with (a) PBL ≠45, (b) PBL ≠49 and (c) cII 678 antisera. Arrows show the position of four different GnRH variants. See Fig. 1 for references.

with these ir-GnRH material were not convincing. This uncertainty was probably due to low GnRH levels. On the other hand, the presence of a third ir-GnRH peak in an elution position similar to that of cIIGnRH should be confirmed by further experiments.

Our results show that similarly to other mammals the dominant form of GnRH in adult 'capybara' is mGnRH. In mammals and other vertebrates, neurons expressing the GnRH molecular variant related to gonadotropin secretion arise from the olfactory placode [28–32]. These neurons are distributed through the terminal nerve, the medial–septal, the preoptic area and the rostral hypothalamus. These neurons belong to the terminal nerve-septo-preoptic system [28,33]. This system is related to GnRH release to the pituitary portal system acting on gonadotropic cells of the adenohypophysis [1,28]. On the other hand, cIIGnRH was shown together with mGnRH in several species of metatherian and early evolved eutherian mammals [5–7,9,10]. In these animals, olfactory regions and areas of the ventral forebrain contain neurons and fibres immunoreactive to mGnRH. There is also a large population of ir-cIIGnRH cells in the midbrain [3,9,10].

Muske [28] proposed that gnathostomic vertebrates have two main GnRH systems with different embryonic origins. They are: a) the anterior, terminal nerve-septo-preoptic system, which is the main regulator of gonadotropin release and b) the posterior system formed by neurons in periventricular regions of the posterior diencephalon and/or midbrain.

During vertebrate evolution, the conservation of mesencephalic cIIGnRH is well known [3]. This form has been reported in cartilaginous fish [31], bony fish [22,23,25,27,34–36], amphibians [37–39], reptiles [40,41], birds [42–44], most of metatherian mammals [5–7] and early evolved eutherian species [7,9,10].

The above mentioned data suggest that there was a gene duplication in the earliest vertebrates. It is important to note that the conservation of structure and neuroanatomical location of cIIGnRH neurons would imply a very important nongonadotropic role of this variant. However the biological meaning and function of this form remain to be discovered [28]. Our data with extracts from olfactory bulbs would suggest that cIIGnRH is also being expressed in this mammal. More assays are needed to confirm these data. Moreover, further studies on extracts from mesencephalic region must be performed.

Table 3
Retention time and quantification of ir-GnRH peaks in the extract of olfactory bulbs chromatographed on RP-HPLC.

Tissue Olfactory Bulbs	Peak	Ret. Time (min)	ir-GnRH (pg/100 μl)[a]					Ret. time GnRH-std[b]
			M1076	EL-15	cII675	S1668	sGnRH≠2	
	I	21–23	10	10	–	–	–	22 (M)
	II	27–28	–	–	5	–	–	27 (CII)
	III	50–51	–	–	–	4	2	50 (S)

[a] Immunoreactive GnRH in pooled fractions from each peak (corresponding to the retention time shown in Fig. 4).
[b] Retention times of synthetic GnRH peptides. (M), mammalian GnRH; (CII), chicken GnRH II; (S), salmon GnRH.

Our data show the surprising fact that there are two coexisting GnRH variants in the anterior GnRH system of 'capybara'. These data suggest the following possibilities: a) both variants would be involved in the regulation of adenohypophyseal hormones; b) one of them, probably mGnRH would related to gonadotropin secretion whereas the other variant is expressed by nerve terminals ending in the pars nervosa; and c) one variant acts on pituitary regulation and the other would serve as a neurotransmitter or neuromodulator. Regarding these possibilities, we must point out that several forms of GnRH also stimulate the secretion of growth hormone in teleost fish [45–47]. Moreover, some variants have a differential gonadotropin-growth hormone-releasing activity [48]. This effect was recently shown in rats [49]. These findings support the idea of GnRH acting on two adenohypophyseal hormones.

There is also a worth mentioning fact. This is represented by the demonstration of ir-GnRH fibres entering the neural lobe in amphibians [50,51], mammals [52,53] and even in humans [52]. Even more, it appears that extension of hypophysiotropic neurosecretory systems (GnRH, GHRH and somatostatin) into the pituitary pars nervosa is characteristic of many mammalian species [53]. From that structure they could be released directly to the bloodstream and/or be involved to paracrine interactions within the neurohypophysis.

In our case, the presence of ir-sGnRH in extracts from preoptic–hypothalamic region opens a series of evolutionary questions. These questions are not only concerned with physiological aspects and differential actions of GnRH variants but with the fact that sGnRH has only been found in teleost fish [3]. Such an unusual presence provides abundant material for speculation.

New experiments must be done to characterize GnRH variants in mesencephalic extracts from 'capybara'. The neuroanatomical location of ir-mGnRH and s-GnRH cell bodies and fibres must also be determined.

Although final conclusions need isolation, purification and sequencing of the molecules, these results offer encouraging possibilities of further advances.

As far as we know, this is the first report showing immunological and chromatographic evidences for the presence of two GnRH molecular variants: mGnRH and a salmon GnRH-like form, in the forebrain of an eutherian mammal.

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