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Guinea Pig Gonadotropin-Releasing Hormone: Expression Pattern, Characterization and Biological Activity in Rodents

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Gonadotropin-releasing hormone · Gonadotropin-releasing hormone analogues · Gonadotropins · Reproduction · Comparative neuroendocrinology · Guinea pig

Abstract
Gonadotropin-releasing hormone (GnRH) is a decapeptide widely known for its role in regulating vertebrate reproduction by serving as a signal from the hypothalamus to pituitary gonadotropes. The first form of GnRH to be identified was isolated from mammals (mGnRH) and the same form has been reported for all mammals studied, which includes marsupials and placental mammals. Later, another variant, chicken GnRH-II (cGnRH-II) was shown to be expressed together with mGnRH in the brains of all jawed vertebrates, including mammals such as rats, monkeys and humans. Our objective was to characterize a third form of GnRH that was isolated previously as mRNA from guinea pigs (gpGnRH), but has not been reported for any other mammal to date. Furthermore, the gonadotropic activity of gpGnRH has not been fully characterized. Our results, using chromatographical and immunological methods, show for the first time that gpGnRH is expressed together with mGnRH in some rodents (wild guinea pig and capybara), but not in others (mouse and hamster). Also, the gonadotropic activity of gpGnRH and mGnRH was tested in two different rat cell culture systems. Although there have been reports that the salmon(s) form of GnRH is present in mammals, we did not detect sGnRH in capybara, wild guinea pigs, hamsters, rats or mice. Taken together with previous reports, the present results support the idea that the expression of multiple GnRH variants in a single species is a common pattern in most vertebrate groups.

Introduction

In vertebrates, gonadotropin-releasing hormone (GnRH) plays a central role in the development and maintenance of reproductive function. To date, 15 GnRH molecular variants have been sequenced from vertebrate and protochordate nerve tissue [1, 2]. In addition to this

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molecular diversity, most vertebrate species express more than one GnRH form [1–3]. The multiplicity of GnRH isoforms has been demonstrated in all seven classes of vertebrates: jawless fish [4–6], cartilaginous fish [7], bony fishes [1–3, 8], amphibians [9, 10], reptiles [11], birds [12, 13] and mammals [14, 15]. However, some of the identified GnRHs do not appear to control the pituitary gonadotropins, and their function remains uncertain.

In mammals, the first GnRH molecule described was isolated and sequenced from porcine and ovine brain tissue [14, 15] and it is now known as mammalian GnRH (mGnRH) or GnRH-I. Its structure is identical in pig [14], sheep [15], tree shrew [16], mouse [17], rat [18], rhesus monkey [19] and human [20]. A post-translational modification of mGnRH, hydroxyproline^{9} mGnRH ([Hyp^{9}]GnRH), was reported in mammals [21, 22].

For marsupials and placental mammals, there are reports of a second form of GnRH, which is called GnRH-II or chicken GnRH-II (cGnRH-II), because this form was first isolated from chicken brains. The structure is [His^{5},Trp^{7},Tyr^{8}]GnRH [13].

Chicken GnRH-II is expressed together with mGnRH in the brain of tree shrew and marsupial species studied to date [16, 23–25]. Moreover, the expression of cGnRH-II, in addition to mGnRH, has been described in the central nervous system of placental mammals like rodents [26, 27], primates [28–31] and even humans [26, 32]. Also, it has been demonstrated that human and mouse neuronal cell lines coexpress mGnRH and cGnRH-II [33]. In this context, previous studies have reported indirect evidence for a third and unknown GnRH form with the chromatographic and immunological characteristics of salmon GnRH (sGnRH), in the brain of an ancient mammal, the capybara [27, 34], and in rat, cow and human [35]. Quanbeck et al. [36] reported the presence of another third form of GnRH in fetal monkey brain.

Also, a distinct molecular form has been identified, using molecular biology techniques, in brain tissue of guinea pig *Cavia porcellus* [37] and named gpGnRH ([Tyr^{2},Val^{7}]GnRH). The presence of this peptide was demonstrated by immunoreactivity in guinea pig hypothalamic extracts [38].

In our experience, mGnRH and gpGnRH elute in the same position in the reverse phase-high performance liquid chromatography (RP-HPLC) system generally used by others and us for screening. Therefore, the first goal of this study was to use sequential RP-HPLC purification and radioimmunoassays (RIAs) with polyclonal antisera to determine if both variants are coexpressed in a selection of rodents. This study was done on the basis of preliminary results obtained in the capybara *Hydrochoerus hydrochaeris* [39], and extended by using wild guinea pig *Cavia aperea,* laboratory rat *Rattus norvegicus,* mouse *Mus musculus,* and hamster *Mesocritus auratus* brains. Also, the gonadotropic activity of synthetic gpGnRH and mGnRH was compared using two in vitro rat pituitary cell culture systems.

Materials and Methods

Animals

Five different rodent species, both wild and domestic, were used for our experiments.

Seventeen adult capybaras *(H. hydrochaeris)* of both sexes were captured and taken to the laboratory. They were anesthetized by a combination of ketamine hydrochloride (40 mg/kg) and sodium thiopental (60 mg/kg). Entire brains were removed after dissection; the total weight of the brains was 453 g.

Eight sexually mature wild guinea pigs *(C. aperea)* of both sexes were captured with trap-cages. They were immediately taken to the laboratory and killed by decapitation. Entire brains were removed (22.3 g), immediately frozen on dry ice and stored at –70°C until the beginning of the peptide extraction. Wild animals were captured by licensed animal officers from the Province of Entre Ríos (Argentina).

Nine hundred and sixty adult rats, *R. norvegicus* (Sprague-Dawley, IBYME strain), 204 adult mice *M. musculus,* and 774 adult hamsters *M. auratus* of both sexes were used. The rats and hamsters were quickly killed by decapitation. Intact brains (without cerebellum) from rats (1,096 g) and hamsters (791 g) were frozen on dry ice and stored as previously described [22]. Complete brains (with cerebellum) from mice were purchased (unstripped mouse brains; Pel-Freez Biologicals, Rogers, Ark., USA). Rat brains were collected during a 10-month period from the IBYME strain and stored at –80°C until peptide extraction. Fifty hamsters were obtained from the IBYME strain and the rest of them kindly provided by Dr. David Lovejoy (Department of Zoology, University of Toronto, Ont., Canada).

All procedures were performed according to protocols for animal use, as approved by the Institutional Animal Care and Use Committee (IBYME-CONICET) that follows NIH guidelines. In order to avoid unnecessary killing of animals, brains from rats, mice and hamsters were used also for peptide purification and sequencing of other GnRH isoforms [22]; ovaries, testes and livers were used by others for research.

Tissue Extraction

Frozen pooled brains from each species were powdered in a Waring blender with liquid nitrogen. Extraction of peptides was done as previously described [4]. Briefly, the tissues were homogenized in acetone: 1 *N* HCl (100:3, v/v) at 4°C, the mixtures stirred at 4°C for 3 h and filtered through Whatman No. 1 filter paper. The insoluble material was re-extracted in acetone: 0.01 *N* HCl (40% of the original volume), stirred for 5 min and re-filtered. Combined filtrates were treated with petroleum ether (bp 30–60°C) for five successive times. Extracts were concentrated under vacuum to <20 ml. For wild guinea pig, brain extracts were concentrated to approximately 1 ml.

RP-HPLC Purification

Brain extracts from each species were filtered and each extract was applied to a new SepPak column that consisted of 10 cartridges connected together (Waters, Milford, Mass., USA) in step 1 and eluted with mobile phases A (0.5% trifluoroacetic acid, TFA) and B (0.5% TFA-80% acetonitrile, ACN). Sixty 1-ml fractions were collected and an aliquot of 10 µl subjected to RIA. Immunoreactive GnRH (ir-GnRH) fractions were pooled, concentrated under vacuum and injected onto a Supelco (Supelcosil LC-18) analytical column using a Beckman 166 model liquid chromatograph. The sample was applied at the beginning of a 10-min isocratic period of 17% ACN in 0.25 *M* triethylammonium formate TEAF (pH 6.5); ACN was then increased to 24% over a 7-min period and held isocratically for 43 more min. The flow rate was kept at 1 ml/min and 1-ml fractions were collected. Aliquots of 10 µl from each fraction were used for RIA. ir-GnRH fractions that eluted in a similar position to mGnRH synthetic standard were selected and pooled for further purification on the same column using a 10-min isocratic period of 17% ACN in 0.13 *M* triethylammonium phosphate TEAP (pH 2.5); ACN was then increased to 24% as in the first case. Aliquots of 10 µl were taken from each 1-ml fraction for RIA.

Synthetic GnRH Standards

[Hyp^9]GnRH, gpGnRH and lGnRH-III peptides were synthesized using a solid phase method on a methylbenzhydrylamine resin as previously described [40]. mGnRH, cGnRH-II and sGnRH were obtained from Peninsula Laboratories Inc. (Belmont, Calif., USA).

GnRH RIA

RIAs were performed as previously described [2, 27] and standards were radiolabeled following established protocols [27, 41]. The RIA systems were as follows:

(a) GF-6 antiserum was raised by one of us (N.M.S.) in rabbits against sGnRH and used at 1:25,000 final dilution with synthetic mGnRH as tracer and standard; cross-reactivities were: mGnRH, 100%; gpGnRH, 106%; [Hyp^9]GnRH, 79%; cGnRH-I, 44%; cGnRH-II, 3.9%; lGnRH-III, 0.4%; sGnRH, 69% [28].

(b) B-7 antiserum was raised by one of us (N.M.S.) in rabbits against mGnRH and used at 1:5,000 final dilution with mGnRH as tracer and standard; cross-reactivities were as follows: mGnRH, 100%; gpGnRH, 104%; [Hyp^9]GnRH, 1.1%; cGnRH-I, <0.1%; cGnRH-II, <0.1%; lGnRH-III, <0.1%; sGnRH, <0.1% [28].

(c) PBL#49, a generous donation by Dr. W. Vale (The Salk Institute), was used at a final dilution of 1:150,000 with sGnRH as tracer and standard giving the following cross-reactivities: mGnRH, 93%; gpGnRH 5.77%, lGnRH-I, 2%; cGnRH-I, 333%; cGnRH-II, 33%; sGnRH, 100% [41].

(d) PBL#45, donated by Dr. W. Vale, was used at a final dilution of 1:250,000 with sGnRH as tracer and standard. PBL#45 showed the following cross-reactivities: mGnRH, 100%; gpGnRH 19.51%, lGnRH-I, 24%; cGnRH-I, 133%; cGnRH-II, 26%; sGnRH, 100% [41].

(e) BLA-5 was raised against lGnRH-I by one of us (N.M.S.) in rabbits and used at a final dilution of 1:5,000 with mGnRH as tracer and standard; cross-reactivities were: mGnRH, 100%; lGnRH-I, 319%; gpGnRH, <0.01%; cGnRH-I, 39%; cGnRH-II, 0.6%; sGnRH, 88% [42]. The present report is the first for cross-reactivity of gpGnRH synthetic standard with GF-6, B-7 and BLA-5 RIA systems.

Biological Activity of GnRH Peptides on LH and FSH Release

Synthetic mGnRH and gpGnRH peptides were tested for luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release in two in vitro rat anterior pituitary models.

Monolayer Cultures of Adenohypophyseal Cells

Anterior pituitary cells were obtained as previously described [43]. Two in vitro pituitary cell culture models were studied using 12-day-old female and adult males. Pituitaries were rapidly removed and placed in freshly prepared Krebs-Ringer bicarbonate buffer without Ca^{2+} and Mg^{2+}. Pituitaries were cut into small pieces and incubated in 0.2% trypsin for 30 min. After addition of DNase and limabean trypsin inhibitor, the fragments were dispersed into individual cells by gentle trituration and filtered through Nytex. Pituitary cells were plated in 96-well plates (50,000 cells/well) with Dulbecco's modified Eagle medium, supplemented with 10% horse serum, 2.5% fetal calf serum, 1% MEM nonessential amino acids, nystatin and gentamicin (medium 1). After 4 days in culture, cells were washed twice with serum-free DMEM-F12 medium containing 2.2 g/l $NaCO_3H$ and 0.1% BSA (medium 2) and then incubated for 1 h with the different GnRH variants, in a concentration range of $1 \cdot 10^{-11}$ to $1 \cdot 10^{-7}$ *M*. At the end of incubation, medium was removed and stored at –20°C until LH and FSH were analyzed by RIA after appropriate dilution with 0.01 *M* phosphate-buffered saline containing 1% egg albumin. Cell cultures were repeated 4–9 times, and each experimental group was done in quadruplicate. Results are expressed as percentage above the basal secretion (nonstimulated cultures) and analyzed by repeated measures ANOVA.

LH and FSH RIA Measurements

LH and FSH were determined in media samples by RIA using kits provided by the NIDDK. Results were expressed in terms of RP3 rat LH and RP2 rat FSH standards. Assay sensitivities were 0.15 ng/ml for LH and 0.6 ng/ml for FSH. Intra- and interassay coefficients of variation for LH were 7.2 and 11.4% and for FSH 8.0 and 13.2%.

Results

In the first RP-HPLC system (TEAF pH 6.5), the elution positions of the different GnRH synthetic standards were: [Hyp^9]GnRH and lGnRH-III, 16 min; mGnRH, 20 min; gpGnRH, 21 min; cGnRH-II, 26 min, and sGnRH, 50 min. In contrast, in the second RP-HPLC system (TEAP pH 2.5), the elution positions of GnRH standards tested were: lGnRH-III, 8 min; [Hyp^9]GnRH, 11 min; mGnRH, 14 min; cGnRH-II, 20 min, and gpGnRH, 24 min.

Capybara

In the first RP-HPLC system (TEAF pH 6.5), two ir-GnRH peaks eluted close together in fractions 21–24 corresponding to the elution position of mGnRH/gpGnRH (fig. 1). Early eluting minor ir-GnRH fractions were observed in fractions 13–16; fraction 16 corresponds to the elution position of [Hyp^9]GnRH. No indications of

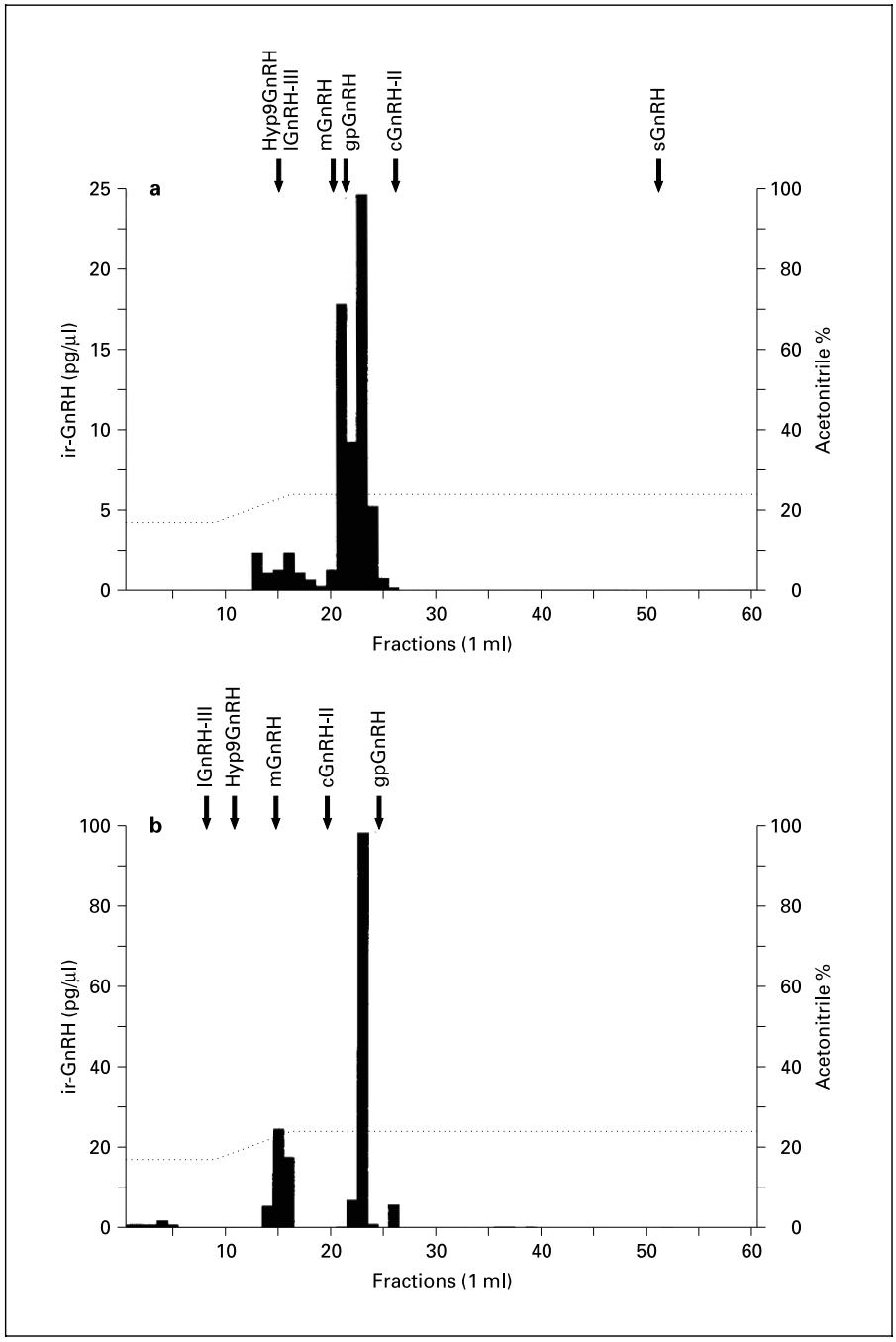

Fig. 1. RP-HPLC from capybara brain extracts: a chromatography with TEAF pH 6.5 assayed with GF-6 RIA system; b chromatography with TEAP pH 2.5 from ir-mGnRH peak from the first system analyzed with GF-6 antiserum. Percentage of acetonitrile is represented by dotted line. Arrows indicate the elution position of different GnRH standards.

ir-GnRH fractions corresponding to the elution positions of cGnRH-II and sGnRH were observed.

The ir-GnRH fractions 21–24 were pooled, concentrated and analyzed in the second step of purification (TEAP pH 2.5) because mGnRH and gpGnRH are separated in the second system unlike the first RP-HPLC system. Fractions from the second chromatography procedure were analyzed by RIA using two antisera: GF-6 (fig. 1b) and B-7 (data not shown). Both antisera revealed two ir-GnRH peaks, one in position 14–16, which is the position for synthetic mGnRH elution, and the other peak in position 22–23, which is close to the elution position of gpGnRH synthetic standard. Although the gpGnRH standard eluted one fraction after native

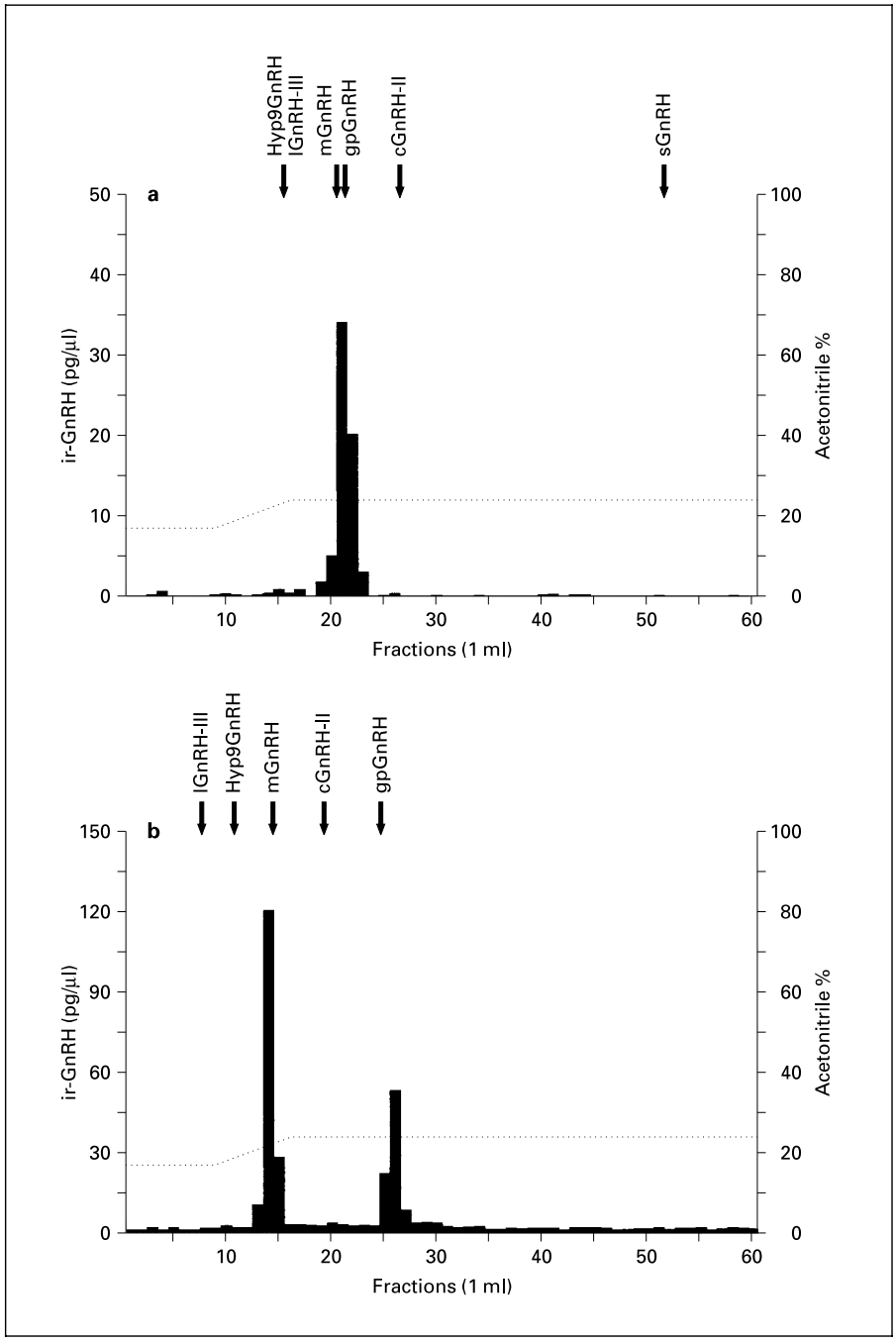

Fig. 2. RP-HPLC from wild guinea pig brain extracts: a chromatography with TEAF pH 6.5 assayed with PBL#49 antiserum; b chromatography with TEAP pH 2.5 from ir-m/gpGnRH peak from the first system analyzed with PBL#45 antiserum. Percentage of acetonitrile is represented by dotted line. Arrows indicate the elution position of different GnRH standards.

gpGnRH, this is explained by use of a different HPLC column and/or minimum changes in the chromatographic conditions for the two samples.

Wild Guinea Pig

Only one major ir-GnRH peak, which eluted in fractions 20–22, was found in wild guinea pig brain extracts. This peak corresponded to the elution position of mGnRH/gpGnRH. The fractions were assayed with PBL#49 (fig. 2a) and PBL#45 (data not shown). Neither antisera detected [Hyp^9]GnRH, cGnRH-II or sGnRH.

For further characterization, the ir-GnRH peak from the first RP-HPLC system was rechromatographed in a second system (TEAP pH 2.5) and analyzed with RIA

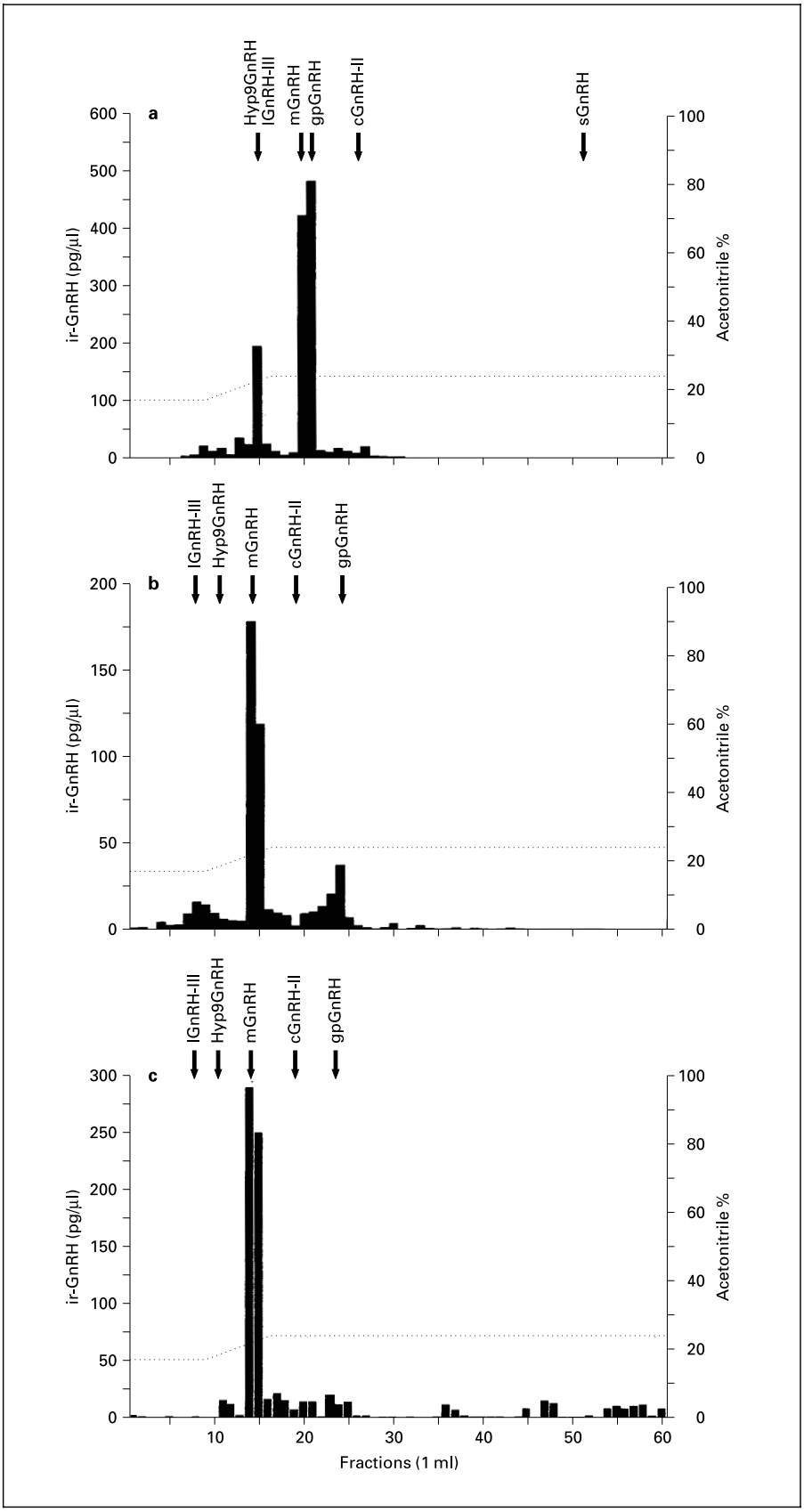

Fig. 3. RP-HPLC from rat brain extract: a chromatography with TEAF pH 6.5 assayed with PBL#49 antiserum; b chromatography with TEAP pH 2.5 from ir-m/gpGnRH peak from the first system analyzed with B-7, and c assayed with BLA-6 antiserum. Percentage of acetonitrile is represented by dotted line. Arrows indicate the elution position of different GnRH standards.

using two different antisera: PBL#45 (fig. 2b) and PBL#49 (data not shown). With both antisera, two major ir-GnRH peaks were detected. The first peak eluted in the same position with mGnRH synthetic standard in fractions 14–15, whereas the second peak eluted in fractions 25–26 similar to gpGnRH.

Rats

When rat brain extracts were analyzed by HPLC-RIA using PBL#45 (fig. 3a), two ir-GnRH peaks were observed in fractions 15 and 20–21. The first peak eluted in the same position as [Hyp^9]GnRH/lGnRH-III, whereas the second one eluted as mGnRH and/or gpGnRH. Evidence of cGnRH-II or sGnRH was not detected with this antiserum.

The ir-GnRH peak, which could have resulted from endogenous mGnRH and/or gpGnRH, was analyzed in the second RP-HPLC system (TEAF pH 2.5) and studied with three different antisera. Two ir-GnRH peaks were detected with B-7 (fig. 3) and PBL#49 (data not shown) antisera. The first peak eluted in position 14–15 together with mGnRH and the second one eluted in fraction 24, which is the elution position of gpGnRH synthetic standard. The peak at fraction 24 is small and hence difficult to interpret. However, a third analysis of the same fractions was performed with BLA-6 antiserum, which does not cross-react with gpGnRH and did not cross-react with fraction 24. This antiserum provides additional evidence on the identity of ir-gpGnRH peak (fig. 3c). BLA-6 did detect a single ir-GnRH peak in position 14–15 coeluting with the mGnRH standard.

Mice

Brain extracts from mice resulted in two peaks in HPLC fractions 16–18 and 20–21 when assayed with antiserum GF-6 (fig. 4a). The elution of these two peaks corresponded to the elution positions of [Hyp^9]mGnRH and mGnRH/gpGnRH, respectively. Antiserum GF-6 did not detect cGnRH-II or sGnRH in the extract.

When fractions 16–21 were pooled and reapplied for chromatography with the second system (TEAP, pH 2.5), two peaks eluted in fractions 11–12 and fractions 15–16 (fig. 4b). The elution of these two peaks corresponded to the elution positions of [Hyp^9]mGnRH and mGnRH, respectively. Antiserum G-6 did not detect any immunoreactive material in the positions associated with gpGnRH.

Hamsters

In the first RP-HPLC system from hamster's brain (TEAF pH 6.5), a major peak was observed between fractions 21–23 when analyzed with PBL#49 antiserum (data not shown). These fractions, eluting as mGnRH/gpGnRH, were reanalyzed in the second RP-HPLC system (TEAP pH 2.5). No evidence of gpGnRH was observed when fractions were tested with two different RIA systems with PBL#49 and B-7 antisera (data not shown).

Biological Activity of GnRH Peptides on LH and FSH Release in Rat Adenohypophyseal Cells

The release of LH (fig. 5a) and FSH (fig. 5b) from 12-day-old female rat pituitary cells after the addition of mGnRH showed a significant response in the concentration range of 10^{-11} to 10^{-7} *M* with a dose-response profile. In contrast, gpGnRH only stimulated LH release at 10^{-7} *M*, and to a lesser degree than mGnRH at the same concentration.

The gonadotropin-releasing effect of gpGnRH and mGnRH synthetic standards was also tested in adult male rat pituitary cell incubations. Whereas mGnRH induced significant release of both LH (fig. 6a) and FSH (fig. 6b) from 10^{-9} *M* and higher concentrations in a dose-response profile, no significant effect was observed with gpGnRH. Thus, there is a difference in sensitivity to mGnRH and gpGnRH by both LH and FSH gonadotropes.

Discussion

The crucial question in this study is whether mammals have a third form of GnRH in the brain. The best evidence of a third GnRH form to date is the gpGnRH isolated as mRNA from guinea pig brain. Our study examines whether gpGnRH peptide is restricted to one species or is widespread in mammals. The logical strategy is to examine other rodents that are both closely related to guinea pigs (wild guinea pig and capybara) and rodents that are distantly related (hamster, rat and mouse). We examined coexpression of mGnRH together with gpGnRH in brain tissue of different rodents. The elution position of ir-GnRH fractions compared to mGnRH and gpGnRH synthetic standards was followed using different chromatographic conditions. Sequential RP-HPLC analysis, similar to that originally described by Rivier [44] in 1978, was performed in two steps of purification because the elution position of GnRH variants changes using different mobile phases. In some cases, GnRH standards

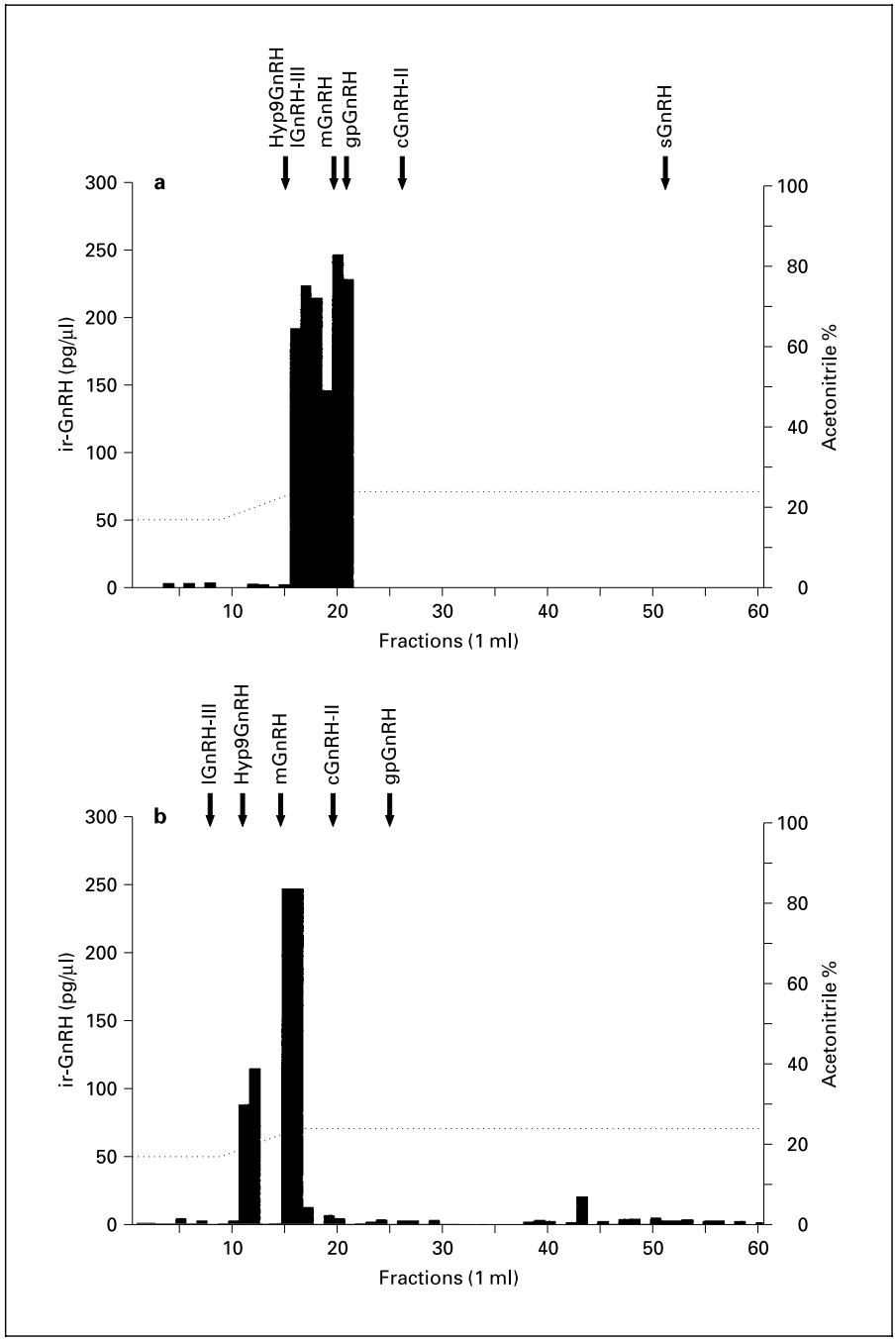

Fig. 4. RP-HPLC from mouse brain extract: a chromatography with TEAF pH 6.5 assayed with GF-6 antiserum; b chromatography with TEAP pH 2.5 of fractions 16–21 from the first system analyzed with GF-6 antiserum. The values for fractions 15 and 16 were off scale on the RIA and hence are >250 µg/µl. Percentage of acetonitrile is represented by dotted line. Arrows indicate the elution position of different GnRH standards.

eluting together in one system can be discriminated if other conditions are used [45, 46].

In the present study, RP-HPLC and RIA data show the presence of an immunoreactive fraction with chromatographic characteristics similar to those of both gpGnRH and mGnRH in brain extracts of different rodents: capybara, wild guinea pig and rats. However, the expression of ir-gpGnRH fractions was not evident in mouse or hamster brains.

In capybara, wild guinea pig and rat brain extracts, the dominant ir-GnRH fractions eluted in the same position as mGnRH/gpGnRH in the first RP-HPLC system (TEAF pH 6.5). When this peak was analyzed with the second system (TEAP pH 2.5), two ir-GnRH peaks were

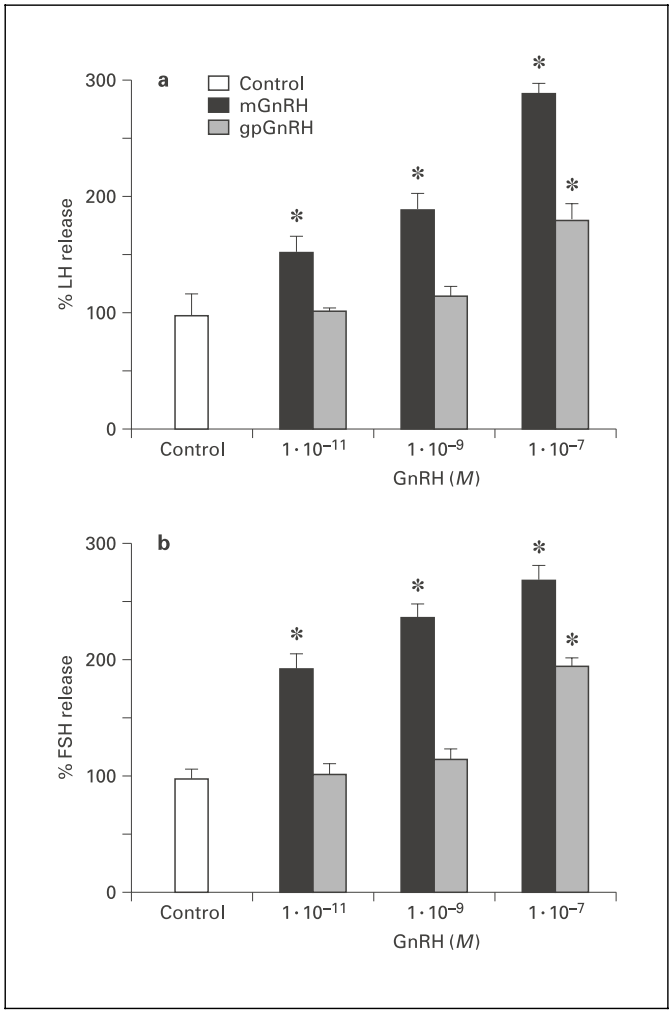

Fig. 5. Gonadotropin-releasing effect of mGnRH and gpGnRH on a LH and b FSH in 12-day-old female rat pituitary cells incubated in vitro. The values are expressed as percentage of basal response (control). Baseline without stimulation was LH 4.21 ± 0.85 and FSH 1.50 ± 0.11 ng/ml/50,000 cells. * $p < 0.05$ with respect to control value (n = 9).

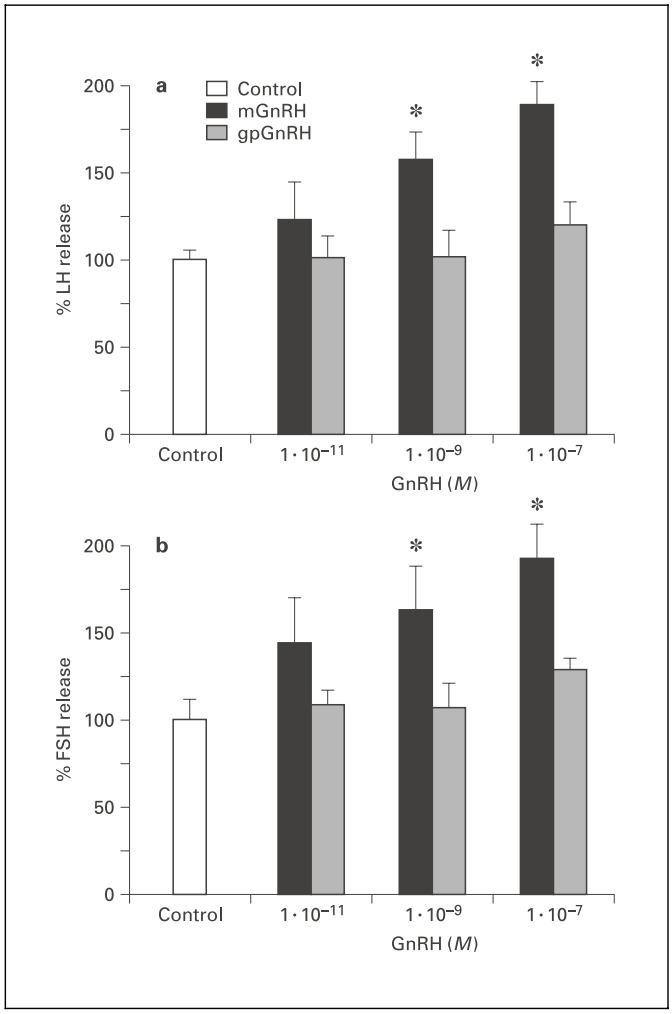

Fig. 6. Gonadotropin-releasing effect of mGnRH and gpGnRH on a LH and b FSH in adult male rat pituitary cells incubated in vitro. The values are expressed as percentage of basal response (control). Baseline without stimulation was LH 1.08 ± 0.12 and FSH 0.82 ± 0.12 ng/ml/50,000 cells. * $p < 0.05$ with respect to control value (n = 4).

revealed: an early eluting peak with chromatographic characteristics of mGnRH, and another peak eluting close to gpGnRH synthetic standard. A variety of antisera, some with known cross-reactivity against gpGnRH, were used to perform this study. Moreover, an antiserum that does not cross-react with gpGnRH [BLA-5] did not detect the putative gpGnRH in rat brain.

The original report describing the structure of gpGnRH by Jimenez-Liñán et al. [37] was performed using molecular biology techniques and the presence of the peptide inferred from gpGnRH-cDNA pre-pro-hormone. Recently, Gao et al. [38] reported complementary evidence on gpGnRH peptide expression in guinea pig using immunological methods on hypothalamic acid extracts. Also, the biological activity of gpGnRH was studied in vivo by administrating it to domestic guinea pigs and testing bioactive serum LH levels using an in vitro bioassay [38]. However, it is important to stress that the expression of gpGnRH has not been reported in any other mammal to date. Previous data by our group [22, 39] and the present study show that the elution position of gpGnRH is very similar to that of mGnRH in the first

RP-HPLC system used. Therefore, any previous indication of the presence of gpGnRH could have been masked by endogenous mGnRH.

In an earlier paper [47], we used domestic guinea pigs to show that normally one dominant ir-GnRH peak elutes from guinea pig brain extracts using the same first RP-HPLC system as in the present study. The fractions were tested with four antisera. However, in one out of seven HPLC runs, two immunoreactive peaks instead of one were detected in positions (fractions 19 and 22) close to mGnRH. In retrospect, it is likely that HPLC conditions were slightly different in the one run allowing separation of mGnRH and gpGnRH in the first system (TEAF pH 6.5). The pattern of elution in the 1990 paper [47] using guinea pig brains was very similar to the profile obtained with capybara brain extracts (fig. 1a). Our present results with wild guinea pig and those in 1990 suggest that the presence of mGnRH together with gpGnRH in the domestic guinea pig is a correct interpretation. This idea is supported by our results on the ir-mGnRH and ir-gpGnRH fractions eluting separately in the second RP-HPLC system (TEAF pH 2.5), in two other hystricomorph rodents (capybara and wild guinea pig).

The peak that elutes in the gpGnRH position from rat brain extracts is difficult to interpret because the peak is small. Also, the presence of gpGnRH in rats is not expected based on the pattern of evolution of the rodents. Within the order Rodentia, there are 3 suborders. One suborder (Myomorpha) contains the rat, mouse and hamster, whereas another suborder (Hystricomorpha) contains the guinea pig and capybara. In the present study the latter two species clearly express gpGnRH, whereas hamster and mouse in the other suborder do not have gpGnRH. The rat is anomalous as it is not only in the same suborder with hamster and mouse, but also in the same family (Muridae) with the mouse, which does not have gpGnRH. In the present study we report on 204 mice, but in a later study we used 670 mice and repeated the study. In both studies, mouse did not express gpGnRH. In rat, we suggest that the presence of gpGnRH is not definitively identified until mass spectrometry or sequencing is done.

In addition, an early ir-GnRH peak eluting as [Hyp^9]mGnRH/lGnRH-III was observed in the first purification step (TEAP pH 6.5) in capybara, hamster, mouse and rat brains with a profile similar to that shown by Kelsall et al. [47] in the domestic guinea pig. Some reports suggested that these early eluting fractions resulted from lGnRH-III [48, 49], but in a previous paper analyzing rat and hamster brain extracts we demonstrated by primary sequence that the earliest peak is [Hyp^9]GnRH [22].

Multiple GnRH molecular forms have been demonstrated in brain tissue from a single animal in all vertebrate classes. In most species cGnRH-II is expressed together with at least one of the other GnRH variants [1, 8].

In fish there are three GnRH neuronal systems: the terminal neurons that have sGnRH in most teleosts, the septo-preoptic-hypothalamic neurons that have one of several different forms of GnRH depending on the species, and the midbrain neurons that express exclusively cGnRH-II in all jawed species studied to date. The terminal neurons in the olfactory region have axons that are widely distributed throughout the brain, but they do not project to the pituitary [50]. This pattern with three forms of GnRH in the brain is very common in teleost fishes [51, 52]. Also, there appears to be three GnRH neuronal systems in mammals, at least in primates. The mGnRH neurons are in the olfactory region, preoptic area, hypothalamus, and around the lateral ventricle [1]; the cGnRH-II neurons are in the midbrain and the hypothalamic region [1, 28, 32]; and a third GnRH form that is not yet identified by structure is in the dorsal preoptic area, septum, amygdala, internal capsule and claustrum [36]. The third form is unlikely to be gpGnRH as we applied monkey brains of one species to the same two HPLC systems used here and did not detect a gpGnRH peak in the expected position of the second system [Sherwood and Terasawa, unpubl. data]. The axons of the mGnRH neurons project to both the median eminence and to other neurons, but the axons of the cGnRH-II neurons and the third form of GnRH neurons appear to project primarily to other neurons. The methodology followed in the present study does not allow us to determine the neuroanatomical location of gpGnRH-expressing neurons, and so it will be of some interest to determine the location of mGnRH and gpGnRH in the brain.

Our purpose in this study was not to detect the expression of cGnRH-II in any of the species, as we did not use antisera specific to cGnRH-II. Antisera GF-6, B-7 and BLA-5 show less than 4% cross-reactivity with cGnRH-II. However, PBL#49 and PBL#45 have 26–33% cross-reactivity with cGnRH-II. Thus, the amount of cGnRH-II in these brains is probably quite low or the PBL antisera would detect it. Also, only the first HPLC system would reveal cGnRH-II (at about fraction 26) because this fraction was not carried to the second HPLC system. However, other studies have demonstrated that cGnRH-II is present in rat [26], hamster [22], capybara [34] and mouse [Sherwood and Warby, unpubl. data], which reinforces the idea that multiple GnRH forms are present in a single

mammalian brain species. Additionally, the neuroanatomical locations of mGnRH and cGnRH-II neurons have been described using histochemistry in the mouse [53] and macaque [28, 31] brain.

Previous data obtained either in capybara by HPLC and RIA [26, 33] and in rat by immunohistochemistry [35] showed the expression of a salmon-like GnRH isoform, although this form has not been sequenced to date. However, in the present study we were not able to detect the expression of a late eluting ir-GnRH fraction corresponding to the elution position of this variant in capybara, wild guinea pig, rat, mouse or hamster brain extracts.

The biological activity of synthetic gpGnRH and mGnRH on LH and FSH secretion was studied in two in vitro rat cell culture models. Both systems appeared to behave similarly in terms of potency, but with different sensitivities. The prepubertal female gonadotrope is more sensitive for LH and FSH secretion as previously described [54]. For LH secretion, the pituitary cell cultures from 12-day-old females had higher sensitivity to mGnRH than to gpGnRH shown by a significant response elicited by mGnRH at a concentration 10,000 times lower than that of gpGnRH. This is in agreement with previously results by Gao et al. [38] for gpGnRH action on domestic guinea pig in vivo compared with mGnRH standard. FSH releasing potency was similar to that of LH. Although gpGnRH did not evoke a statistically significant response at low doses, it increased the FSH release at the highest dose. This is in accord with the high releasing activity of mGnRH compared with other natural isoforms except for cGnRH-II [55]. Because [Tyr^2]mGnRH is 8% [56] and [Ala^7]mGnRH is 5% [57] as potent as mGnRH in a rat pituitary cell culture assay, it was expected that gpGnRH ([Tyr^2,Val^7]GnRH) would have some gonadotropin-releasing activity. Also, valine is intermediate in size between alanine and leucine (Leu^7 is found in mGnRH). However, in adult rat male pituitary cells, gpGnRH did not show any gonadotropin-releasing effect.

In sum, our results support the idea that the expression of multiple GnRH variants within a single brain has become a common fact for mammals, adding more complexity to the understanding of the regulation of gonadotropins and other related pituitary hormones or neurotransmitters by GnRH.

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