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Guinea Pig GnRH: Localization and Physiological Activity Reveal That It, Not Mammalian GnRH, Is the Major Neuroendocrine Form in Guinea Pigs

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The isolation of GnRH cDNA from guinea pig hypothalamus predicted a novel form of GnRH with two unique amino acid substitutions relative to all known forms of this essential decapeptide. The predicted substitution at amino acid 2 in guinea pig (gp) GnRH was particularly intriguing because of the proposed importance of position 2 for binding and activation of the GnRH receptor. In the present study, gpGnRH was synthesized, and a specific antibody was generated and used to assess translation of the gpGnRH transcript. The localization of intensely labeled gpGnRH-positive cell bodies and processes in tissue sections through the preoptic area and hypothalamus argue that gpGnRH is the major neuroendocrine form of GnRH in guinea pigs. Guinea pig GnRH stimulated LH release in guinea pigs and increased LH output from guinea pig pituitary fragments, thus demonstrating biological activity in this species. In contrast, gpGnRH demonstrated little ability to stimulate LH release in rats, a species known to possess the highly conserved mammalian GnRH receptor. These findings suggest that: (1) the amino acid substitutions in gpGnRH impede binding to and/or activation of the mammalian GnRH receptor, and (2) the unique amino acid substitutions in gpGnRH are accompanied by changes in the guinea pig GnRH receptor. (*Endocrinology* 143: 1602–1612, 2002)

GnRH, ALSO REFERRED TO AS LH releasing hormone, is the primary hypothalamic signal that regulates pituitary gonadotropin release. GnRH release is pulsatile (1), and the parameters of this pulsatile release influence GnRH receptor gene expression and gonadotropin subunit gene expression in the pituitary as well as the ratio of circulating titers of LH and FSH (2). In males, GnRH is essential for testosterone production and spermatogenesis, and in females it is important for the LH surge required for ovulation.

Our interest in the steroid regulation of GnRH gene expression led us to the guinea pig (*Cavia porcellus*) as an attractive animal model for these studies. Guinea pigs, like primates and unlike other lab rodents, have a relatively long reproductive cycle (16–17 d) with a true luteal phase (3). Thus, they afford the opportunity to examine GnRH gene expression during progesterone dominant (luteal phase) and E2 dominant (follicular phase) endocrine states. Our initial studies in the guinea pig isolated GnRH cDNA from an expression library generated from guinea pig hypothalamus (4). Surprisingly, the nucleotide sequence of the guinea pig GnRH (gpGnRH) transcript predicted a GnRH decapeptide that differed from the expected mammalian form by two amino acid substitutions. Moreover, the predicted amino acid substitutions were unique among all currently known forms of GnRH. The high levels of gpGnRH transcript measured in hypothalamic extracts from individual guinea pigs suggested that gpGnRH replaces mammalian GnRH (mGnRH) as the main neuroendocrine form of the decapeptide in this species. This serendipitous finding challenged the prevailing belief that mGnRH is the primary neuroendocrine form of GnRH in all mammals.

Remarkably, the predicted amino acid sequence of gpGnRH indicates that it is the first peptide of the family (Fig. 1) in which histidine is not present in position 2 of the decapeptide (various forms of GnRH are reviewed in Ref. 5, also see Refs. 6 and 7). The predicted nonconservative substitution of tyrosine for histidine occurs in what has been considered the invariant N-terminal sequence of the decapeptide. In addition, in gpGnRH valine replaces the leucine present in position 7 of mGnRH. This conservative change represents a second unique substitution relative to all forms of GnRH identified to date. However, unlike the change in amino acid 2, some variation in amino acid 7 has been documented previously among the currently known forms of GnRH decapeptide (Fig. 1).

The substitution of tyrosine for histidine at amino acid 2 in gpGnRH is of particular interest because all vertebrate GnRH receptors are thought to require the basic conserved NH_2 and COOH terminal sequences of GnRH for receptor binding and activation (8). The relationship among biological activity, receptor affinity, and residue substitution has been investigated in detail for mGnRH (9). Data from these studies have suggested that the histidine in position 2 of the decapeptide may be important for binding and/or activation of the mGnRH receptor (8). Reduced activity of synthetic forms of GnRH that contain substitutions at amino acid 2 have been demonstrated. The limited data available regarding tyrosine^2 analogs have revealed reduced LH-releasing activity in rat

Abbreviations: gpGnRH, Guinea pig GnRH; mGnRH, mammalian GnRH; PLSD, protected least significant difference.

	1	2	3	4	5	6	7	8	9	10
Guinea Pig:	pGlu	**Tyr**	Trp	Ser	Tyr	Gly	**Val**	Arg	Pro	Gly
Mammal:	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly
Chicken II:	pGlu	His	Trp	Ser	**His**	Gly	**Trp**	**Tyr**	Pro	Gly
Salmon:	pGlu	His	Trp	Ser	Tyr	Gly	**Trp**	**Leu**	Pro	Gly
Chicken I:	pGlu	His	Trp	Ser	Tyr	Gly	Leu	**Gln**	Pro	Gly
Frog (Rana):	pGlu	His	Trp	Ser	Tyr	Gly	Leu	**Trp**	Pro	Gly
Seabream:	pGlu	His	Trp	Ser	Tyr	Gly	Leu	**Ser**	Pro	Gly
Medaka/pejerrey:	pGlu	His	Trp	Ser	**Phe**	Gly	Leu	**Ser**	Pro	Gly
Herring:	pGlu	His	Trp	Ser	**His**	Gly	Leu	**Ser**	Pro	Gly
Catfish:	pGlu	His	Trp	Ser	**His**	Gly	Leu	**Asn**	Pro	Gly
Dogfish:	pGlu	His	Trp	Ser	**His**	Gly	**Trp**	**Leu**	Pro	Gly
Lamprey III:	pGlu	His	Trp	Ser	**His**	**Asp**	**Trp**	**Lys**	Pro	Gly
Lamprey I:	pGlu	His	**Tyr**	Ser	**Leu**	**Glu**	**Trp**	**Lys**	Pro	Gly
Tunicate I:	pGlu	His	Trp	Ser	**Asp**	**Tyr**	**Phe**	**Lys**	Pro	Gly
Tunicate III:	pGlu	His	Trp	Ser	**Leu**	**Cys**	**His**	**Ala**	Pro	Gly

FIG. 1. The 15 currently identified forms of GnRH. Guinea pig GnRH has unique amino acid residues in position 2 and 7 *(boxed)* relative to all known forms of the decapeptide. Amino acid residues that vary from mammalian GnRH are *underlined* and *bold*. As depicted, amino acid residues in positions 1, 4, 9, and 10 are invariant in all forms of GnRH.

pituitary cultures (10) and a decrease in binding affinity at the mGnRH receptor (11).

In the present study, we describe a specific antiserum generated to gpGnRH (TF 60) that is used to assess translation of the previously identified gpGnRH transcript and to localize the peptide in the guinea pig brain. Moreover, the relative abilities of gpGnRH and mGnRH to stimulate LH release are compared in guinea pigs and rats, a species known to possess the highly conserved mGnRH receptor, using *in vivo* and *in vitro* paradigms.

Materials and Methods

Animals

Twenty-one male Hartley White guinea pigs were purchased from Elm Hill Breeding Laboratories (Chelmsford, MA) (500–550 g), and 26 male Sprague Dawley rats (Crl:CD(SD)IGS BR) were purchased from Charles River Laboratories, Inc. (Wilmington, MA) (200–225 g). Animals were individually housed in a facility managed by the Division of Laboratory Animal Medicine. Guinea pigs were exposed to a 12-h light/12-h dark cycle (lights on at 0600 h). Guinea pig chow and water were available *ad libitum,* and their diet was periodically supplemented with fresh vegetables. Rats were exposed to a 14-h light/10-h dark cycle (lights on at 0400 h) with rat chow and water available *ad libitum*. Protocols for these studies were approved by the Institutional Animal Research Committee, and all experiments were carried out according to the Guide for the Care and Use of Laboratory Animals.

Peptide synthesis and antibody generation

Guinea pig GnRH synthesis was performed using Fast Moc TM chemistry on a model 431A, version 1.12 software, peptide synthesizer (Applied Biosystems, Foster City, CA). The peptide was cleaved from the resin using a mixture of phenol, ethanedithiol, thioanisole, and trifluoroacetic acid. Samples were purified, reconstituted, and sent for Matrix-assisted laser desorption/ionization mass spectrum (HT Laboratories, San Diego, CA) for conformation of peptide synthesis. Guinea pig GnRH was purified to greater than 95% by HPLC in the Protein Core Facility in the Department of Physiology at Tufts Medical School. An extended form of gpGnRH, containing lysine coupled to the C terminus, was conjugated to BSA via glutaraldehyde following the procedures described by Coligan (12). This preparation was then shipped to Cocalico Biologicals (Reamstown, PA) for polyclonal antibody production. Two male New Zealand white rabbits housed at their facility were injected with the extended peptide, boosted, and bled (13). All bleeds were sent to us for characterization of the polyclonal antisera.

Localization of gpGnRH in brain

Characterization of antiserum to gpGnRH-RIA. RIA was performed as previously described (14–16). Guinea pig GnRH was iodinated using the chloramine T method (14). Iodinated mGnRH was purchased from NEN Life Science Products (Boston, MA). Binding curves were set up with various dilutions of the two polyclonal antisera generated to gpGnRH (antisera TF 59 and TF 60 generated at Cocalico Biologicals) and an antiserum generated to mGnRH (Ab-R1245 obtained from Terry Nett, Colorado State University, Fort Collins, CO). Because antiserum TF 60 exhibited a very high level of specificity for gpGnRH (in RIA at a dilution of 1:400,000, cross-reactivity with mGnRH was calculated at $<0.001\%$ for TF 60 and 28% for TF 59), it was used for the studies described. Standard curves were set up with synthetic gpGnRH or mGnRH and antiserum TF 60 to gpGnRH (1:50,000 working dilution, 1:400,000 final dilution) or antiserum to mGnRH (Ab-R1245, 1:30,000 working dilution, 1:120,000 final dilution). Antiserum TF 60 was also used in RIA to assess levels of gpGnRH present in hypothalamic extracts from guinea pigs and rats.

Characterization of antiserum to gpGnRH: immunocytochemical protocols. Six guinea pigs and three rats were deeply anesthetized with Nembutal (100 mg/kg, ip, Abbott Laboratories, North Chicago, IL). They were perfused intraventricularly with a fixative containing 2% acrolein (Polysciences, Inc., Warrington, PA) and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Brains were removed from the skull, postfixed for an additional 30 min and coronally sectioned at 50 μm on a Vibratome (Technical Products International, St. Louis, MO). Brain sections were placed sequentially into one of six vessels. This collection method allowed adjacent sections to be incubated with antisera for gpGnRH (TF 60) at dilutions of 1:70,000 and 1:150,000 and mGnRH (DiaSorin, Inc., Stillwater, MN) at a dilution of 1:30,000. For controls, sections were incubated with gpGnRH antiserum (1:70,000) or mGnRH antiserum (1:30,000) preabsorbed with synthetic gpGnRH or mGnRH at a concentration of 0.05 mM (0.0591 mg/ml).

The details of the immunocytochemical protocol have been described previously (17). In brief, following a pretreatment to remove residual aldehydes and decrease background staining, tissues were incubated with antisera or preabsorbed antisera for 48–72 h at 4 C. Bound antibody was detected with a Vectastain Elite ABC kit, rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) followed by the chromagen diaminobenzidine (0.25 mg/ml in 0.05% hydrogen peroxide).

The number of cells labeled with antisera to gpGnRH (TF60 at a dilution of 1:70,000) or mGnRH (DiaSorin, Inc. at a dilution of 1:30,000) were counted in sets of five matched adjacent sections through the preoptic area and 12 matched adjacent sections through the anterior, medial, and posterior hypothalamus of the guinea pig brain.

Assessment of physiological activity

Catheterization. The catheterization protocol was similar to that described previously in guinea pigs (17) and rats (18). Guinea pigs were approximately 6–9 wk old at the time of surgery. They were anesthetized with xylazine (5 mg/kg, im, Burns Veterinary Supply, Inc., Rockville Center, NY) and ketamine hydrochloride (50 mg/kg, im, Fort Dodge Animal Health, Fort Dodge, IA). Rats were approximately 7–12 wk old at the time of surgery. They too were anesthetized with xylazine (6–6.7 mg/kg, im) and ketamine (75–85 mg/kg, im) and received 0.1 mg/kg buprenorphine hydrochloride (im Reckitt & Colman Products Ltd., Hull, UK) to ease postoperative pain. A beveled SILASTIC brand catheter (0.020" inner diameter, 0.037" outer diameter, Dow Corning Corp., Midland, MI) was inserted into the right jugular vein, threaded into the right atrium of the heart, and the free end of the catheter exited from the back of the neck. Catheters were flushed daily (with heparinized saline) to maintain patency and accustom the animals to the blood sampling routine.

In vivo GnRH administration and blood sampling

A cross-over design was used for GnRH administration such that each animal was tested with equivalent doses of gpGnRH and mGnRH. On the day of infusion, fresh aliquots of gpGnRH and mGnRH were diluted in saline so that the desired dose was delivered in a volume of 0.2 ml. Animals were allowed a minimum of 48 h to recover after surgery before GnRH infusion and sample collection. A minimum of 6 d for guinea pigs and 3 d for rats was allowed between the first and second GnRH infusion.

Baseline blood samples were collected at least 1 h before infusion of GnRH. For guinea pigs, blood samples (0.5 ml) were collected at 10, 20, 40, and 60 min after GnRH infusion. For rats, blood samples (0.3 ml) were collected at 15, 30, 60, and 120 min after GnRH infusion. After each blood sample, the catheter was flushed with saline and heparinized saline. Blood samples were centrifuged for 20 min at 1000 × *g* at 4 C and plasma was stored at −30 C until subsequent LH RIA.

In vitro perifusion

Guinea pigs were killed for perifusion experiments a minimum of 14 d after the last GnRH infusion. Because of their large size (723 ± 38 g body weight), they were anesthetized with Nembutal before decapitation. The pituitary was rapidly removed, placed in a dish with medium that had been warmed and oxygenated, and the anterior pituitary was dissected into eight fragments (19). Tissues were perifused with Medium 199 without phenol red (no. 11043–023, Life Technologies, Inc., Rockville, MD) with the addition of 25 mM HEPES (15630-080, Life Technologies, Inc.), 0.5% BSA (A-7030, Sigma-Aldrich Corp., St. Louis, MO), 90 U/ml bacitracin (B-0125, Sigma), and 25 μg/ml gentamicin (no. G-1272, Sigma). Pituitary fragments from a single animal were placed into a 500-μl chamber in the AcuSyst-S cell culture system (Cellex Biosciences Inc., previously Endotronics, Inc., Coon Rapids, MN) with warmed (37 C) and oxygenated (95% O_2/5%CO_2) medium flowing at a rate of 0.2 ml/min (12 ml/h). Effluents were collected every 5 min on ice and stored at −30 C until LH RIA. On each day of perifusion, two guinea pigs were killed and two chambers were run. One received pulses of gpGnRH, and the other received equivalent pulses of mGnRH. Pulses lasted 4 min and were administered at 60 min (0.5 μg/ml; 4.2×10^{-7} M), 120 min (1 μg/ml; 8.4×10^{-7} M), and 180 min (5 μg/ml; 4.2×10^{-6} M) after the pituitary fragments were placed in the chambers. At 240 min, tissues received a 30-min exposure to 60 mM KCl to confirm tissue viability.

The procedure for rats was similar to that for guinea pigs with a few exceptions. Rats were lightly anesthetized with metofane before decapitation. Tissues were exposed to three 3.5-min GnRH pulses at hourly intervals containing 0.1 μg/ml (8.4×10^{-8} M, pulses 1 and 2) and 0.2 μg/ml (1.7×10^{-7} M, pulse number 3). Exposure to KCl was limited to 5 min.

LH RIA

The RIA for guinea pig LH was optimized previously in the lab (20). Briefly, the LH antiserum (CSU 120, generously provided by Dr. Terry Nett, Colorado State University) was used at a final dilution of 1:100,000. Guinea pig pituitary powder (21) was used as a reference prep for the standard curve, and iodinated rat LH was purchased from Covance Laboratories, Inc. (Vienna, VA). Plasma samples were assayed in singlets and perifusion samples were diluted and assayed in duplicate. The limit of detection for the LH assay was 11.2 U/tube (88.2% B/Bo) and the midrange of the assay was 59.7 U/tube. Intraassay variability ranged from 3.2–8.3% and interassay variation was calculated at 6.5%.

Rat LH was measured as previously described (18), using the rat LH assay kit provided by the NHPP and NIDDK containing LH antiserum NIDDK-anti-rLH-S-11 and reference preparation LH NIDDK-rLH-RP3. The limit of detection of the assay was 21 pg/tube (89% B/Bo), and the midrange of the assay was 121 pg/tube. Intraassay variation ranged from 2.7% to 6.8%, and the interassay variability was 4.8%.

Statistics

Comparisons of GnRH cell numbers in sections through the guinea pig brain incubated with antiserum to gpGnRH *vs.* antiserum to mGnRH were analyzed by a paired *t* test. *In vivo* response to infusions of gpGnRH and mGnRH were assessed by ANOVA. Comparisons of the *in vivo* and *in vitro* responses to the two forms of GnRH were made using two-way ANOVA with repeated measures and *post hoc t* tests and Fisher's protected least significant difference (PLSD; StatView, Cary, NC).

Results

Characterization of gpGnRH antiserum

Characterization by RIA. Initial binding curves demonstrated marked specificity of antiserum TF 60 for gpGnRH. As mentioned previously, the cross-reactivity of antiserum TF 60 with mGnRH in RIA at a final dilution of 1:400,000 was calculated at less than 0.001%. Therefore, antiserum TF 60 was used for the studies described. Displacement curves (Fig. 2A) demonstrate that iodinated gpGnRH bound to antiserum TF 60 is displaced by synthetic gpGnRH but not by synthetic mGnRH. In contrast, iodinated mGnRH bound to mGnRH antiserum Ab-R1245 is displaced by both synthetic mGnRH and synthetic gpGnRH (Fig. 2B), indicating that Ab-R1245 binds gpGnRH. RIA results with antiserum TF 60 revealed the presence of significant levels of gpGnRH in hypothalamic extracts from guinea pigs (Fig. 2C). No evi-

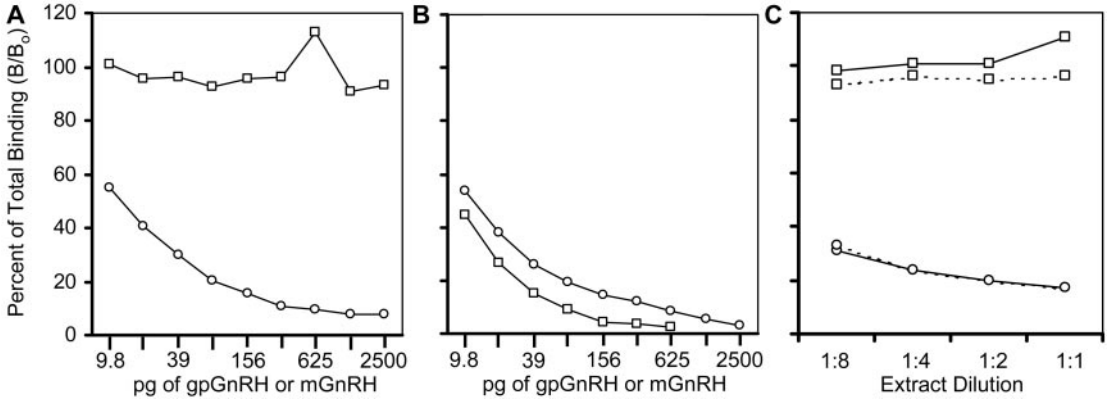

FIG. 2. Binding curves reveal specificity of gpGnRH antiserum TF 60. A, Iodinated gpGnRH bound to gpGnRH antiserum TF 60 is displaced by increasing amounts of synthetic gpGnRH (○) but not synthetic mGnRH (□). B, Iodinated mGnRH bound to mGnRH antiserum Ab-R1245 is displaced by increasing amounts of both synthetic mGnRH (□) and synthetic gpGnRH (○). C, Iodinated gpGnRH bound to gpGnRH antiserum TF 60 is displaced by extracts from guinea pig hypothalami (○) but not by extracts from rat hypothalami (□).

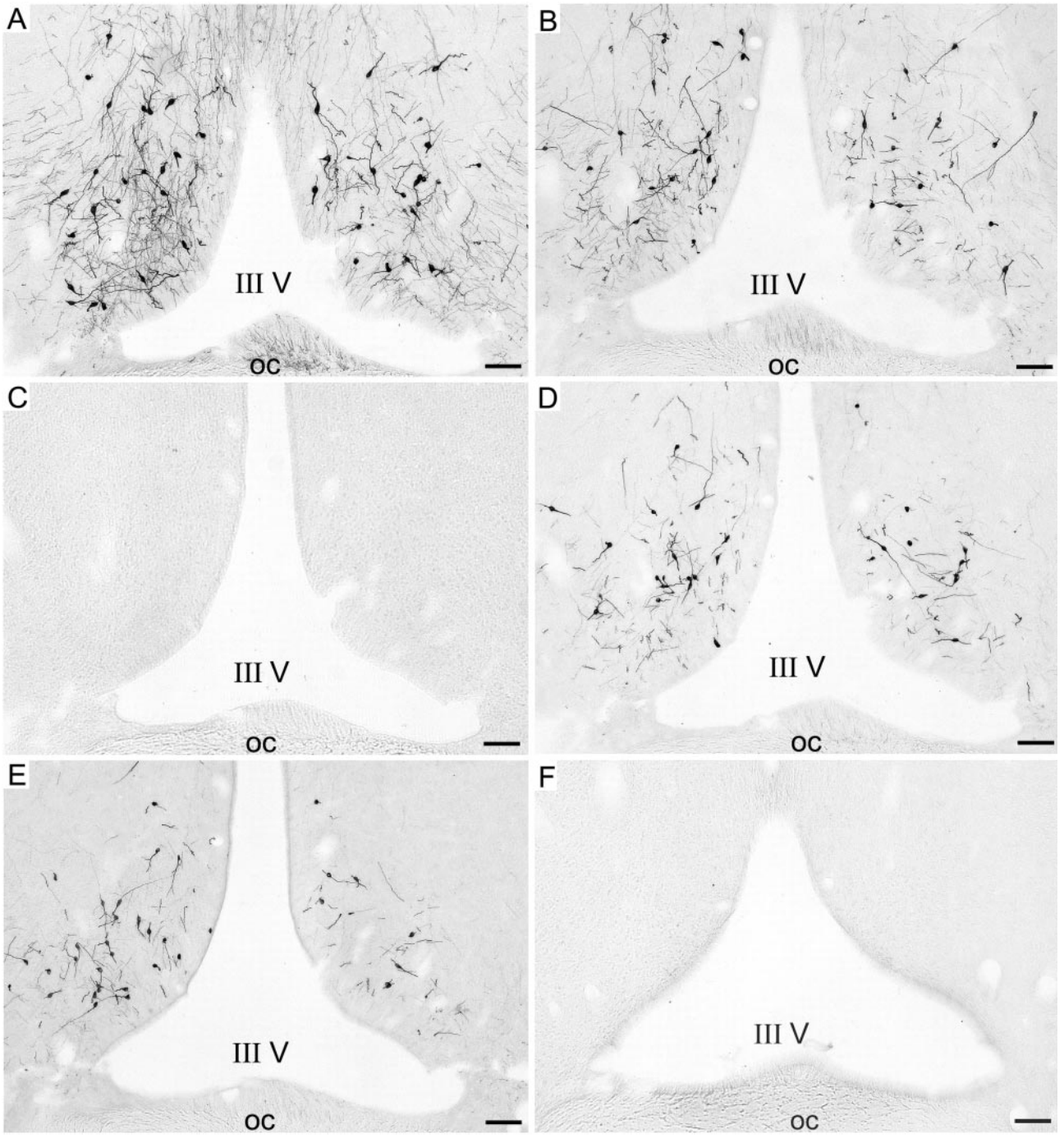

FIG. 3. Localization of gpGnRH and mGnRH in the guinea pig brain. GnRH neurons are shown in coronal sections through the rostral preoptic area at the level of the optic chiasm in guinea pigs. Incubation with the antiserum to gpGnRH, TF 60, at dilutions of 1:70,000 (A) or 1:150,000 (B) revealed intensely labeled cell bodies and processes. Preabsorption of TF 60 (1:70,000) with synthetic gpGnRH eliminated all immunoreactivity (C). Preabsorption of TF 60 (1:70,000) with mGnRH did not eliminate immunoreactivity (D). Incubation with a commercially available antiserum to mGnRH (DiaSorin, Inc., at 1:30,000) revealed immunoreactive cell bodies and processes (E), but preabsorption of the mGnRH antiserum with gpGnRH eliminated the reaction product (F). III V, Third ventricle; oc, optic chiasm. *Scale bar*, 100 μm.

dence of gpGnRH was detected in hypothalamic extracts from rats (Fig. 2C).

Characterization by immunocytochemistry. Incubation of tissue sections through the guinea pig brain with antiserum TF 60 (1:70,000 and 1:150,000 dilutions) revealed intense reaction product in cell bodies and processes. Examples of these tissue sections through the preoptic area and the medial basal hypothalamus and caudal median eminence are shown in Figs. 3, A and B, and 4, A, B, and C. Adjacent tissue sections incubated with a commercially available antiserum to mGnRH (DiaSorin, Inc.: 1:30,000 dilution; Figs. 3E and 4, D, E, and F) revealed fewer cell bodies and processes and less

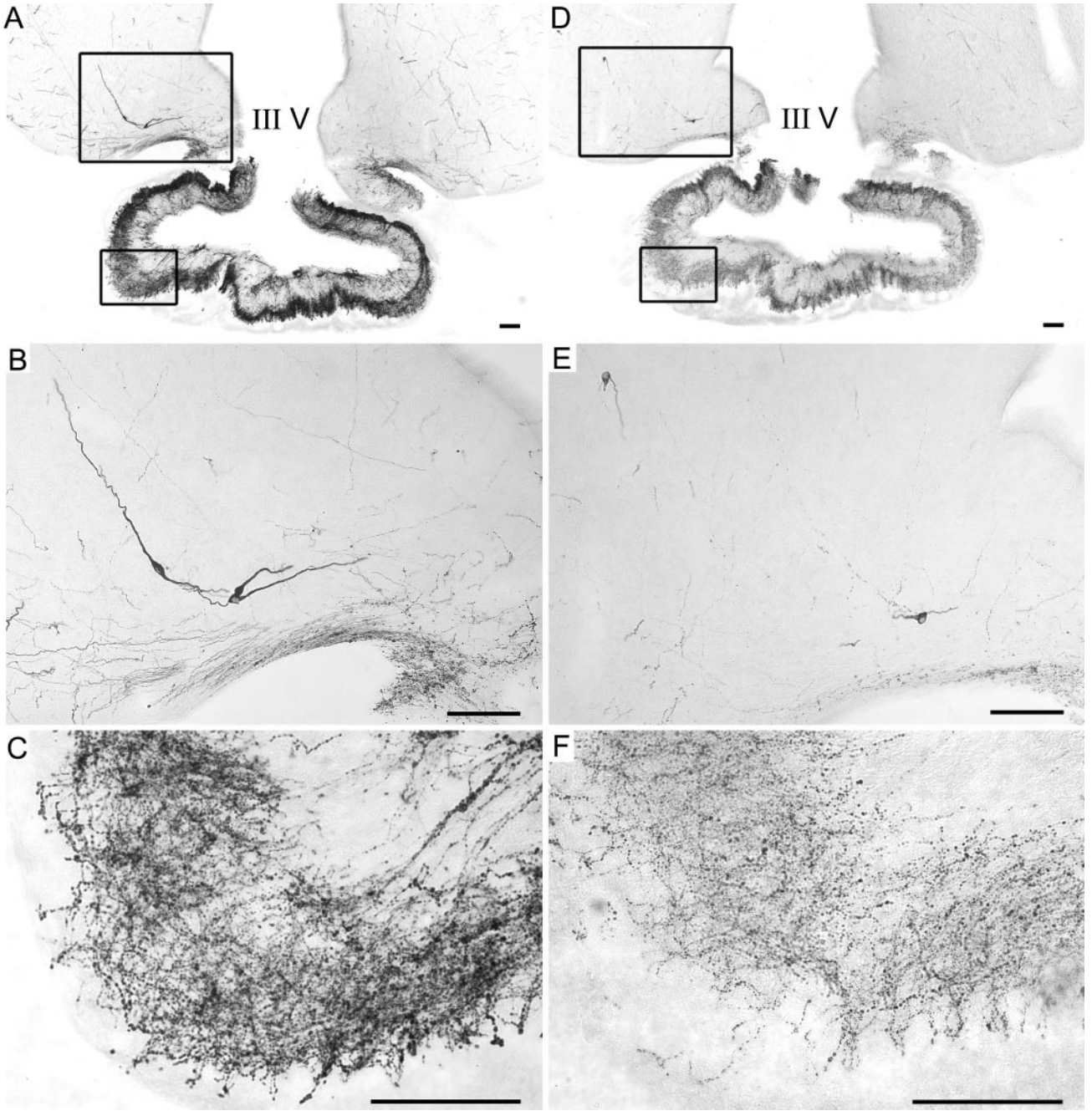

FIG. 4. GnRH immunoreactivity in the hypothalamus. Examples of GnRH immunoreactivity are shown in adjacent coronal sections through the caudal median eminence. Incubation with the antiserum to gpGnRH TF 60 at 1:70,000 dilution (A, B, C) revealed more intense immunoreactivity than that revealed with the antiserum to mGnRH (DiaSorin, Inc.) at 1:30,000 dilution (D, E, F). *Rectangles* in A and D indicate the regions of higher magnification shown in B, C, E, and F. Examples of GnRH-positive neurons in the medial basal hypothalamus are shown in B and E. Immunoreactive axons and terminals in the caudal median eminence are shown in C and F. III V, Third ventricle. *Scale bar*, 100 μm.

intense reaction product relative to that observed with antiserum TF 60. There was, however, no convincing evidence to suggest that specific subgroups of the population of GnRH neurons were revealed with gpGnRH antiserum and not mGnRH antiserum. In tissue sections through the preoptic area, mGnRH antiserum revealed approximately 60% of the number of GnRH positive cell bodies detected in adjacent tissue sections incubated with gpGnRH antiserum (P = 0.009) (Fig. 5). In more caudal tissue sections through the hypothalamus, mGnRH antiserum revealed approximately 75% of the number of GnRH-positive cell bodies detected with gpGnRH antiserum TF 60 (P = 0.102, NS). Preabsorption of the TF 60 antiserum with synthetic gpGnRH before incubation eliminated reaction product in the guinea pig brain (Fig. 3C). In contrast, preabsorption of TF 60 with mGnRH did not eliminate immunoreactive cell bodies and

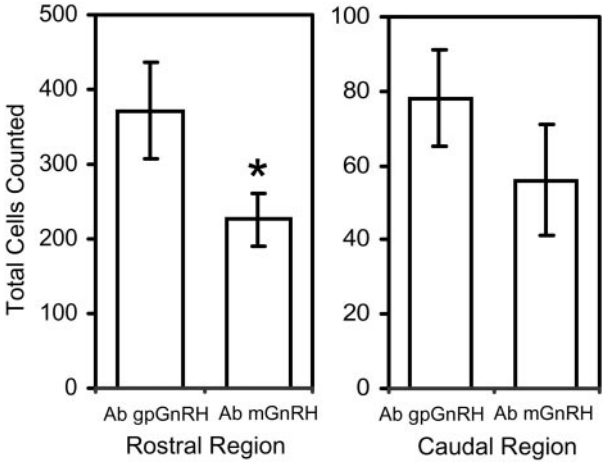

FIG. 5. Mean number of GnRH-positive cell bodies. Data from cell counts of 5 matched sections through the preoptic area (rostral cells) and 12 matched sections through the hypothalamus (caudal cells) are shown. In each case, more GnRH-positive cell bodies were detected with antiserum TF 60 to gpGnRH relative to the number detected with the antiserum to mGnRH. Differences in positive cell number were statistically significant in sections through the preoptic area (rostral cells; *, $P = 0.009$).

fibers (Fig. 3D). Preabsorption of the mGnRH antiserum with synthetic gpGnRH (Fig. 3F) completely eliminated immunoreactivity in the guinea pig brain.

In the rat brain, pale labeling of some cell bodies and processes was observed in tissue sections incubated with gpGnRH antiserum, TF 60, at a dilution of 1:70,000 (Fig. 6A). Immunoreactivity was completely lost when the antiserum was further diluted to 1:150,000 (not shown) or when it was preabsorbed with either mGnRH (Fig. 6B) or gpGnRH (not shown). Intensely labeled GnRH-positive cells and processes were observed in adjacent rat brain sections incubated with the mGnRH antiserum (Fig. 6C). The mGnRH antiserum clearly revealed more GnRH-positive cell bodies and processes than were observed following incubation with the gpGnRH antiserum TF 60.

Localization of gpGnRH in guinea pig brain

The pattern of immunoreactivity observed with gpGnRH antiserum TF 60 was examined in every sixth 50-μm section through the guinea pig brain extending from the diagonal band of Broca rostrally through the caudal hypothalamus. Antiserum TF 60 revealed intensely labeled GnRH-positive cell bodies throughout the diagonal band of Broca, rostral preoptic area, preoptic area (Fig. 3, A and B), and septal nuclei, and within the anterior, medial (Fig. 4, A and B), and posterior divisions of the hypothalamus. Within the hypothalamus, some GnRH-positive cell bodies appeared to be located within the arcuate nucleus, whereas other positive cell bodies were located in lateral, ventral, and dorsal aspects of the hypothalamus. Immunoreactive processes were noted in the regions mentioned above as well as in the amygdala and the median eminence (Fig. 4, A and C).

Physiological activity of gpGnRH

Activity of gpGnRH in guinea pigs. In vivo studies demonstrated the ability of gpGnRH to release LH in guinea pigs.

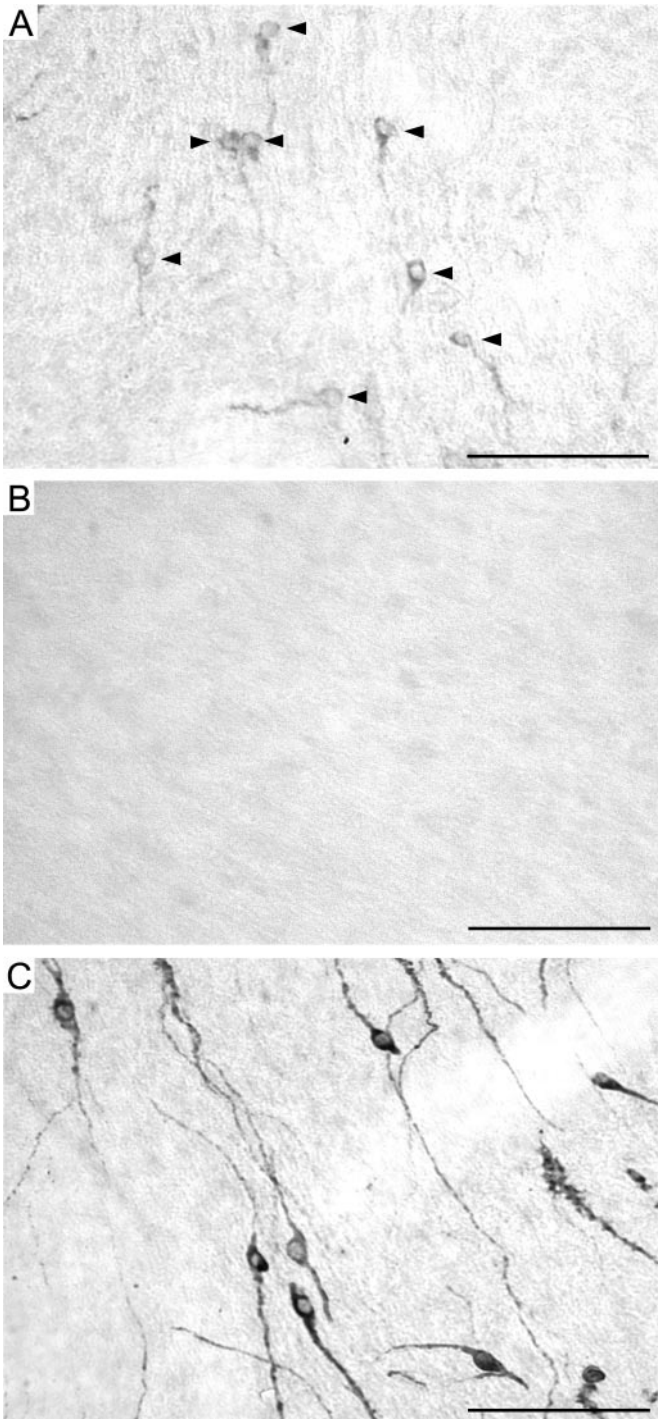

FIG. 6. Localization of GnRH in the rat. GnRH neurons are shown in coronal sections through the preoptic area in the rat. Tissue sections incubated with antiserum to gpGnRH, TF 60, at a dilution of 1:70,000 revealed only faint reaction product (A, *arrowheads* point to cell bodies). Preabsorption of the gpGnRH antiserum, TF 60 with mGnRH eliminated reaction product in the rat brain (B). Adjacent sections incubated with antiserum to mGnRH (DiaSorin, Inc.) at 1:30,000 revealed intensely labeled cell bodies and processes (C). *Scale bar,* 100 μm.

Guinea pigs infused with 0.3, 1, and 10 μg of synthetic gpGnRH exhibited a dose-dependent response to the treatments (Fig. 7A). Following infusion of the two lower doses,

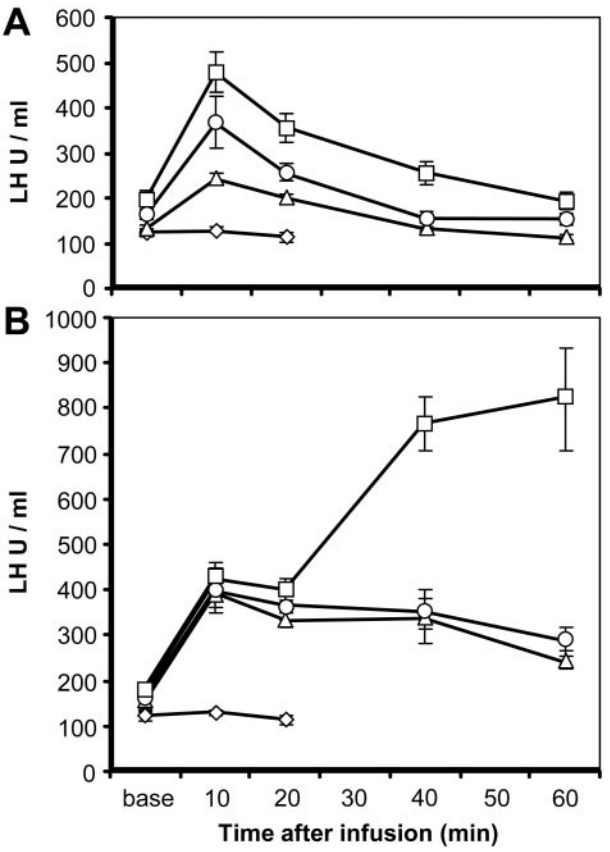

FIG. 7. *In vivo* bolus infusion of gpGnRH *vs.* mGnRH in guinea pigs. Plasma LH levels (U/ml) measured in sequential blood samples taken from guinea pigs 10, 20, 40, and 60 min after infusion of gpGnRH (A) or mGnRH (B). The doses of GnRH are as follows: ◇ = 0, saline vehicle; △ = 0.3 μg; ○ = 1 μg; □ = 10 μg. LH measurements are displayed as the mean ± SEM. Each *point* represents LH measurements from five to six animals.

LH levels peaked at 10 min and returned to baseline by 40 min. After infusion of the highest dose, LH levels peaked at 10 min and returned to baseline by 60 min.

In contrast to the response to gpGnRH, all three doses of mGnRH resulted in a similar LH response at 10 min (Fig. 7B, $P = 0.8278$, NS), and LH levels failed to return to baseline at 60 min. Following the 10-μg infusion, LH levels at 40 and 60 min were significantly increased relative to the levels measured at the 10-min time point (ANOVA F = 19.844, $P < 0.0001$; Fisher's PLSD, $P = 0.0006$, 10 min *vs.* 40 min; $P = 0.00075$, 10 min *vs.* 60 min). Comparisons of the response to the 1-μg infusion of mGnRH and gpGnRH (Fig. 7, A and B) revealed that, although both peptides release similar amounts of LH at 10 min, LH levels differed at the 20-, 40-, and 60-min time points (ANOVA, F = 4.629, $P = 0.0041$; Fisher's PLSD, $p_{20\ min} = 0.0117$, $p_{40\ min} = 0.0004$, and $p_{60\ min} = 0.0026$). Comparison of the response to 0.3-μg infusions of the two forms of GnRH (7, A and B) suggests that the LH response to mGnRH is more robust in guinea pigs (ANOVA F = 4.447, $P = 0.0057$; Fisher's PLSD, response to gpGnRH *vs.* mGnRH $P < 0.01$ at 10, 20, 40, and 60 min).

In vitro perifusion studies verified the ability of gpGnRH to release LH from guinea pig pituitary fragments (Fig. 8). The LH response to equivalent doses of gpGnRH and mGnRH were not significantly different. Pituitary fragments exposed to mGnRH appeared to demonstrate higher baseline LH levels relative to those exposed to gpGnRH.

Activity of gpGnRH in rats

Guinea pig GnRH did not effectively stimulate LH release in rats (Fig. 9A). Rats infused with 0.1, 0.3, and 1 μg of gpGnRH showed only minimal increases in LH release (Fig. 9A) relative to the levels released in response to equivalent doses of mGnRH (Fig. 9B). Infusion of 0.05 μg of gpGnRH (Fig. 9, A and B) did not stimulate LH release (ANOVA, $P = 0.7311$, NS), although the same dose of mGnRH elicited a robust LH response (ANOVA, F = 14.986, $P < 0.001$).

When rat pituitary fragments were stimulated with gpGnRH, the levels of LH released were only slightly higher than baseline levels (Fig. 10). In contrast, administration of mGnRH induced a robust LH response. Moreover, baseline levels of LH release from pituitary fragments that received gpGnRH were lower than those that were exposed to mGnRH (Fig. 10). As shown in Fig. 10, KCl failed to stimulate LH release in rat pituitary fragments that received gpGnRH. These data are consistent with the lack of response of the rat pituitary to stimulation by gpGnRH. Previous data from Elskus *et al.* (22) revealed that the ability of male rat pituitary fragments to respond to KCl stimulation after several hours of perifusion was dependent on prior stimulation with mGnRH.

Discussion

Localization of gpGnRH in guinea pigs

The generation of a specific antiserum to gpGnRH enabled us to demonstrate that the previously identified gpGnRH transcript (4) is translated in guinea pig brain. Use of the antiserum in RIA revealed the presence of high levels of gpGnRH in hypothalamic extracts from guinea pig brains. Moreover, immunocytochemical analyses enabled the precise localization of gpGnRH positive cell bodies and processes.

As described, immunoreactive cell bodies were localized within the diagonal band of Broca; preoptic area; septal nuclei; and the anterior, medial, and posterior hypothalamus. Immunoreactive processes were apparent in these same regions as well as in the amygdala. As expected, labeled axons and terminals were most abundant in the caudal median eminence. The localization of gpGnRH-positive cells and processes are in general agreement with early descriptions of immunoreactive GnRH in the guinea pig brain (23, 24); however, the intensity of the immunoreactivity and number of GnRH-positive cell bodies and processes observed in the present study appear to be significantly greater than that described in earlier studies (23, 24) and relative to previous observations in our own laboratory (17, 20). Both more sensitive immunocytochemical protocols and the availability of a specific antiserum to gpGnRH undoubtedly enhanced the level of immunoreactive GnRH observed in the guinea pig brain in the present study.

The two antisera to mGnRH used in the present studies (Ab-R1245, DiaSorin, Inc.) demonstrated significant cross-reactivity with gpGnRH. This was not surprising because

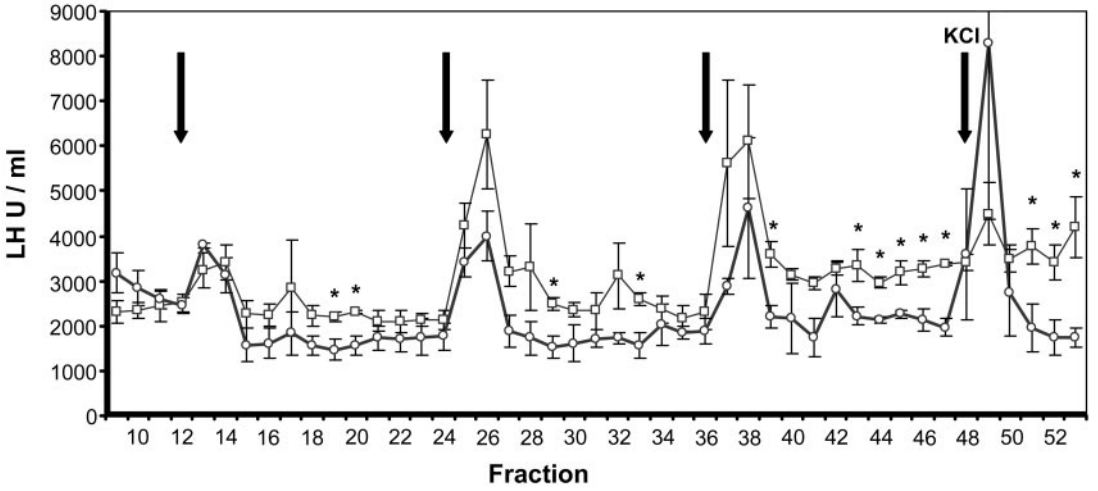

FIG. 8. *In vitro* perifusion of guinea pig pituitary fragments exposed to gpGnRH *vs.* mGnRH. Compiled profiles of LH output from guinea pig pituitary fragments exposed to gpGnRH (○) or mGnRH (□). The mean LH value and SEM were calculated for each 5-min fraction. Fragments were exposed to GnRH at fractions 12, 24, and 36 *(arrows).* Stimulation with KCl occurred at fraction 48. An *asterisk* indicates a significant difference ($P < 0.05$) between the mean LH values measured in fragments treated with gpGnRH, compared with mGnRH. The data are compiled from three perifusion experiments.

mGnRH antisera have routinely been used to detect GnRH in guinea pigs (20, 23–27). The level of immunoreactivity observed in guinea pig tissues incubated with gpGnRH antiserum was clearly increased relative to that observed with antiserum to mGnRH. The number of GnRH neurons counted, intensity of the fiber networks observed, and density of axons and terminals in the median eminence were all markedly increased in tissue sections incubated with gpGnRH antiserum relative to adjacent sections incubated with an antiserum generated to mGnRH.

Although the available data cannot definitively rule out the presence of mGnRH in the guinea pig hypothalamus, both the RIA data and the immunocytochemical data argue that gpGnRH is the predominant neuroendocrine form of GnRH in guinea pigs. When the gpGnRH antiserum was preabsorbed with gpGnRH, no immunoreactivity was detected in the guinea pig brain. In contrast, when the gpGnRH antiserum was preabsorbed with mGnRH, GnRH-positive cell bodies and processes were clearly evident. The intensity of labeling was somewhat reduced in tissue sections incubated with gpGnRH antiserum preabsorbed with mGnRH relative to those incubated with gpGnRH antiserum alone. A review of the immunocytochemical data presented suggests that the reduced level of immunoreactivity might be explained by cross-reactivity of some component of the polyclonal gpGnRH antiserum with mGnRH at the dilution used rather than the presence of mGnRH in guinea pig brain. These data are consistent with the results of the immunological analyses of Gao *et al.* (28) that demonstrated that the major form of GnRH in guinea pig hypothalamic extracts differed from mGnRH.

Localization of gpGnRH in rats

Immunocytochemical analyses revealed little convincing evidence of the presence of gpGnRH in the rat brain. Incubation of tissue sections through the rat brain with antiserum to gpGnRH at a dilution of 1:70,000 revealed only pale la-

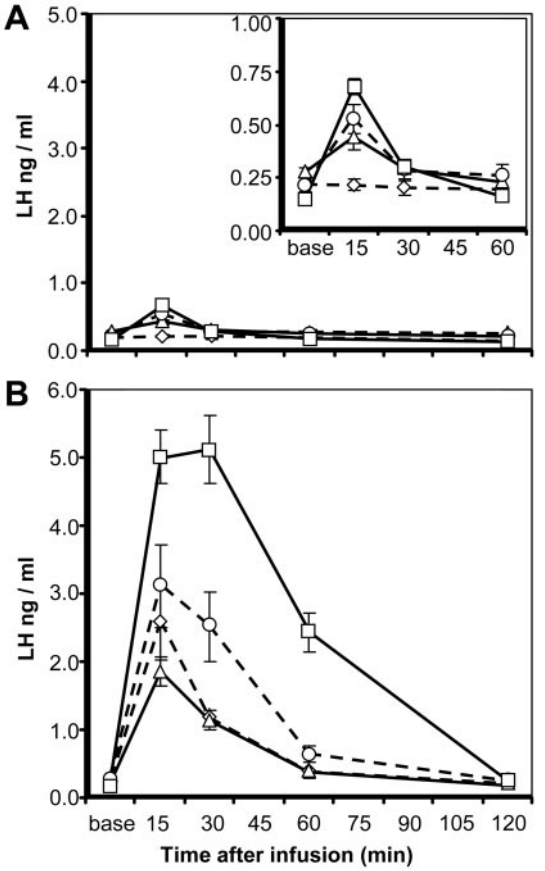

FIG. 9. *In vivo* bolus infusion of gpGnRH *vs.* mGnRH in rats. Plasma LH levels (ng/ml) measured in sequential blood samples taken from rats 15, 30, 60, and 120 min after infusion of gpGnRH (A) or mGnRH (B). The doses of GnRH are as follows: ◇, *broken line* = 0.05 μg; △, *solid line* = 0.1 μg; ○, *broken line* = 0.3 μg; □, *solid line* = 1 μg. LH measurements are displayed as the mean ± SEM. Each *point* represents LH measurements from five to seven animals.

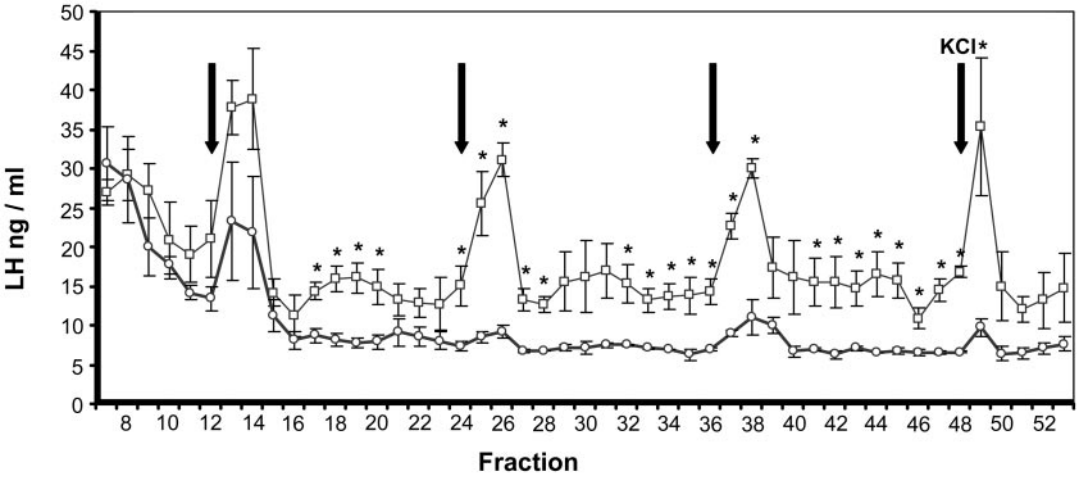

FIG. 10. *In vitro* perifusion of rat pituitary fragments exposed to gpGnRH *vs.* mGnRH: compiled profiles of LH output from rat pituitary fragments exposed to gpGnRH (○) or mGnRH (□). The mean LH value and SEM were calculated for each 5-min fraction. Fragments were exposed to GnRH at fractions 12, 24, and 36 *(arrows).* Stimulation with KCl occurred at fraction 48. An *asterisk* indicates a significant difference ($P < 0.05$) between the mean LH values measured in fragments treated with gpGnRH, compared with mGnRH. The data are compiled from three perifusion experiments.

beling of cell bodies and a low level of reaction product in axons and terminals of the median eminence. Further dilution of the gpGnRH antiserum (1:150,000) or preabsorption of the gpGnRH antiserum with mGnRH completely eliminated immunoreactivity in the rat brain. As discussed above, these data too would be consistent with a cross-reactivity of some component of the polyclonal gpGnRH antiserum with mGnRH at the lower dilutions used in immunocytochemistry. Consistent with this interpretation, hypothalamic extracts from rat brain failed to reveal detectable levels of gpGnRH in RIA.

Physiological activity of gpGnRH in guinea pigs

Intra-atrial infusions of gpGnRH stimulated *in vivo* release of LH in guinea pigs confirming the physiological activity of this unique form of GnRH in this species. The magnitude of the LH response was clearly dose dependent. At each of the three doses, a rapid elevation of LH was noted at 10 min, and LH levels subsequently returned to baseline by the end of the sampling period.

As would be expected based on data from previous studies (29–33), guinea pigs did respond to mGnRH; however, the LH response to infusion of mGnRH differed from that of gpGnRH. The magnitude of the LH response to all three doses of mGnRH was similar at the 10-min time point, and mGnRH administration resulted in a prolonged elevation of circulating LH titers. LH levels failed to return to baseline during the sampling period even in response to the lowest dose of mGnRH. These data suggest that the LH response to mGnRH may be more robust than the response to the species-appropriate form of GnRH in guinea pigs.

The direct measurements of circulating LH levels in the present study are in agreement with the indirect assessments of circulating LH levels reported by Gao *et al.* (28) using the guinea pig Leydig cell bioassay (34). As in the present study, the bioassay data suggested an increased sensitivity of guinea pigs to mGnRH relative to gpGnRH and a prolonged elevation of LH levels after infusion of mGnRH. The dramatic rise in LH levels noted 40 and 60 min after administration of the highest dose of mGnRH to guinea pigs in the present study was not observed by Gao *et al.* (28). Whether this variation in the data may be attributed to a distinction between LH immunoreactivity and LH bioactivity remains to be determined.

Whereas the LH profile observed in guinea pigs after stimulation with mGnRH may relate to an inability of guinea pigs to effectively degrade mGnRH, this explanation seems unlikely because the enzymes that degrade GnRH are not specific for the decapeptide. A more likely explanation may be that the prolonged LH response reflects altered binding kinetics of mGnRH at the gpGnRH receptor. Formal studies of binding kinetics await characterization of the GnRH receptor in guinea pigs.

Guinea pig GnRH and LH release in rats

Guinea pig GnRH demonstrated little ability to release LH in rats, a species known to possess the highly conserved mGnRH receptor (8). This finding was not entirely surprising, given the amino acid substitutions in gpGnRH relative to mGnRH. As mentioned previously, amino acids 1 and 2 of the N-terminal segment of the decapeptide are conserved in all 15 currently known forms of GnRH with the exception of gpGnRH. Numerous studies of the ligand-receptor interaction for mGnRH have indicated that the histidine at residue 2 is one of three important amino acids (histidine^2, tryptophan^3, arginine^8) for the binding and activation of the mGnRH receptor (8, 35). An early study that examined the effects of replacing histidine^2 of mGnRH with tyrosine reported a 95% reduction in LH-releasing activity in rat pituitary cultures (10). A more recent study examined the mGnRH receptor and the changes in binding affinity that occur with substitutions in mGnRH (11). GnRH analogs in which histidine^2 was replaced by tyrosine required seven times more agonist than mGnRH to give an equivalent half-maximal response, suggesting that the amino acid substitution decreased affinity for the mGnRH receptor. Evidence of

decreased binding affinity was also suggested when phenylalanine or tryptophan were substituted for histidine[2] (11). These latter findings are consistent with reports of significant reductions in the activity of mGnRH analogs with these same substitutions (10, 36, 37). It is interesting to note that substitutions at amino acid 2 are a common feature of the GnRH peptide antagonists in current clinical use (38).

Of potential relevance with regard to gpGnRH is the finding that a mGnRH analog in which leucine[7] was replaced with valine (the amino acid present in position 7 of gpGnRH) favored FSH release (39). In addition, the lamprey III form of GnRH, which also contains a substitution at position 7 relative to mGnRH, has been shown to preferentially stimulate FSH release at the mammalian GnRH receptor (40, 41). These data suggest that future studies should explore the FSH-releasing activity of gpGnRH at the mGnRH receptor.

The gpGnRH receptor

Comparisons of the relative abilities of gpGnRH and mGnRH to release LH in rats and guinea pigs suggest that the gpGnRH receptor underwent a change in response to or concomitant with the alteration in the GnRH decapeptide. Rats possess the highly conserved mGnRH receptor (8), and they exhibited only a minimal LH response to gpGnRH. Although the GnRH receptor in guinea pigs has not yet been characterized, following infusion of gpGnRH, guinea pigs exhibited a marked increase in LH levels. Therefore, it is likely that in addition to a unique form of GnRH, guinea pigs possess a unique GnRH receptor with significant changes from the mGnRH receptor. It is important to note that the putative changes in the gpGnRH receptor do not eliminate its ability to bind or be activated by mGnRH. Rather as previously discussed, guinea pigs exposed to mGnRH demonstrated a robust and prolonged LH response. Given that guinea pigs appear to be more sensitive to mGnRH than gpGnRH, is it possible that guinea pigs actually maintain the mGnRH receptor and express a second receptor that binds gpGnRH? These issues await further clarification in future studies that identify and characterize the GnRH receptor(s) in guinea pigs and that examine GnRH ligand receptor-binding kinetics in this species.

Is gpGnRH the sole form of GnRH in guinea pigs?

The present study concentrated on the localization of the neuroendocrine form of gpGnRH. Whether guinea pigs synthesize additional forms of GnRH remains to be determined. It is likely that additional forms of GnRH will be identified in guinea pigs because multiple forms of GnRH have been identified in most vertebrates that have been examined (42–50).

A close relative of the guinea pig, the capybara (*Hydrochaeris hydrochaeris*), reportedly synthesizes three forms of GnRH including mGnRH, salmon GnRH, and chicken II GnRH (50, 51). The identification of mGnRH in this species resulted from the analysis of HPLC extracts from the capybara preoptic-hypothalamic region. These extracts revealed a peak that eluted with mGnRH and was capable of binding antisera generated to mGnRH (50). Because it is clear from the data presented here that some antisera to mGnRH demonstrate a significant level of cross-reactivity with gpGnRH, assessment of the possibility that the capybara synthesizes gpGnRH may require additional analysis. In this regard, it should be noted that the initial HPLC studies of GnRH in guinea pig hypothalamus (27) demonstrated a dominant form of GnRH in guinea pig tissue extracts that eluted with synthetic mGnRH. This form of GnRH was presumed to be mGnRH because it was immunoreactive with antiserum B6, a sequential-type GnRH antiserum generated to mGnRH in the Sherwood laboratory (27). Antiserum B6 is directed toward the last six amino acids of mGnRH. As discussed by the authors of the study, given its specificity, antiserum B6 would be capable of binding to forms of GnRH with alterations in amino acids 2, 3, or 4. Therefore, the substitution in amino acid 2 would not be expected to interfere with the ability of gpGnRH to bind antiserum B6.

The results presented here provide evidence of another example of an endocrine anomaly in guinea pigs (52) that may relate to the isolation of this species during evolution (53, 54). Whether this unique form of GnRH is also present in close relatives of the guinea pig that belong to the same suborder (*Hystricopmorpha*) remains to be determined. Moreover, potential novel and distinct properties of this unique, naturally occurring form of GnRH remain to be explored. The eventual identification and characterization of the gpGnRH receptor will provide an important opportunity for further elucidation of GnRH ligand-receptor interactions.

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