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Polygenic Expression of Gonadotropin-Releasing Hormone (GnRH) in Human?

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STOPA, E. G., S. A. SOWER, C. N. SVENDSEN AND J. C. KING. *Polygenic expression of gonadotropin-releasing hormone (GnRH) in human?* PEPTIDES 9(2) 419–423, 1988.—A non-mammalian lamprey-like gonadotropin-releasing hormone (lGnRH) has been detected in human hypothalami using a combination of immunocytochemistry, high performance liquid chromatography and radioimmunoassay. The hypothalamic distribution of immunopositive lGnRH neurons is similar to that observed for those containing the mammalian gonadotropin-releasing hormone (mGnRH), indicating a possible role for this newly identified peptide in the regulation of pituitary function. Our data suggest the existence of a separate gene for lamprey-like GnRH in humans. Confirmation of the exact nature and role of this newly detected form of GnRH will require future isolation and sequence analysis. The possibility that polygenic expression of a given peptide may be a common phenomenon even in higher mammals is discussed.

Polygenic expression Gonadotropin-releasing hormone Human studies Immunocytochemistry
High performance liquid chromatography Radioimmunoassay Hypothalamus LHRH

GONADOTROPIN-RELEASING hormone (GnRH) was first isolated from porcine [16,23] and ovine [2] hypothalamic extracts, giving rise to the popularly held view that a single form of GnRH (mGnRH) is present in all mammals [15]. Recent investigations have demonstrated molecular heterogeneity of the peptide responsible for gonadotropin-releasing activity across vertebrate phyla (Fig. 1) [9]. Multiple forms of GnRH have been shown within bird [11, 17, 18], reptile [19,20], amphibian [4, 13, 28], bony fish [3, 12, 21, 25, 31], and lamprey [27] brain. In addition, a form of GnRH different from mGnRH has been identified in sheep [10].

The present study was undertaken to test the hypothesis that a phylogenetically early, non-mammalian form of GnRH obtained from lamprey might be conserved during evolution, and may therefore be found in human brain. The lamprey is one of two living representatives of the most ancient class of vertebrates, the Agnatha, which evolved over 450 million years ago.

METHOD

Antisera

Two antisera generated against mGnRH were supplied by Dr. Robert Millar (R.M. 1076) and Dr. Victor Ramirez ($CRR_{11}B_{73}$). The two antisera generated against lGnRH (J.A.K. 1467, J.A.K. 1459) were provided by Dr. Judy A. King. The binding of R.M. 1076 antiserum requires residues Trp^3 to Pro^9 of mGnRH and demonstrates less than 0.01% cross-reactivity with lGnRH (J.A.K. and R.M., unpublished). Specificity analysis of Chen-Ramirez $R_{11}B_{73}$ has demonstrated that minor changes in the primary amino acid sequence of mammalian GnRH will cause dramatic reductions in immunoreactivity [6]. Antisera generated against lGnRH (J.A.K. 1467, J.A.K. 1459) demonstrate 0.02% and less than 0.01% cross-reactivity, respectively, with mGnRH (J.A.K. and R.M., unpublished).

Immunocytochemical Studies

Human tissues were obtained within 6–18 hours postmortem from two adult men and two women. The hypothalamus and preoptic area were fixed by immersion for 24 hours in 5% acrolein (PolySciences, Worrington, PA) in 0.1 M phosphate buffer (pH 7.2) and then serially sectioned at 80

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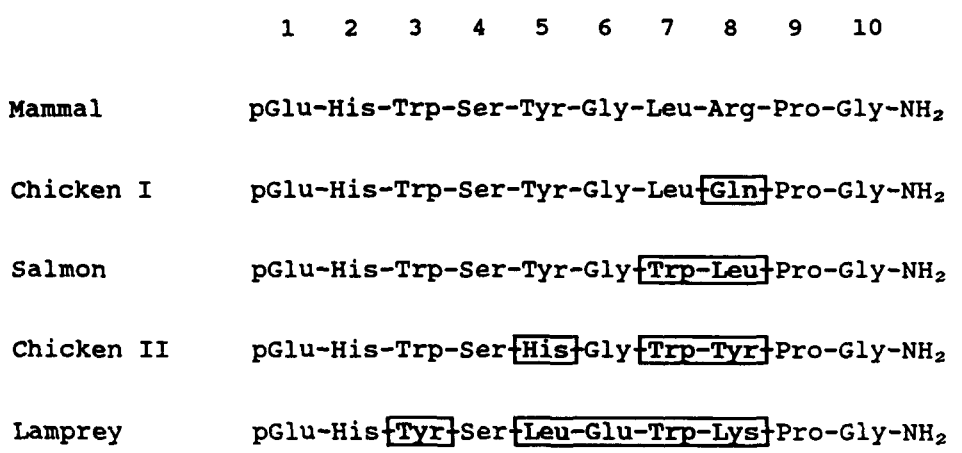


FIG. 1. The amino acid sequence of GnRH has been identified in the mammal [2, 16, 23], salmon [25], chicken (I and II) [11, 17, 18], and in the lamprey [27]. Compared to mammalian GnRH (nGnRH), chicken I GnRH differs by one residue, chicken II by three residues and salmon by two residues. The lamprey GnRH (lGnRH) differs by five amino acids when compared with mGnRH.

μm on a freezing microtome. The immunocytochemical procedures used have been described in detail [7,8].

HPLC

The median eminence and basal hypothalamus were dissected from ten brains following removal, snap-frozen and stored at −80°C until extracted and assayed. Each frozen tissue weighing 0.3–1.3 g was powdered in liquid nitrogen using a Beckman Omni-Mixer. The extraction method used was by Chang and Leeman [5], adapted by Sherwood *et al.* [26]. The tissue was added to acetone: 1 N HCl (100:3, v/v), 1 g frozen tissue to 10 ml. The extraction mixture was stirred for 3 hours with addition of dry ice in and around the container, then filtered (Whatman No. 4). Insoluble material was reextracted in acetone: 0.01 N HCl (80:20, v/v) in two-fifths of the volume of the original extraction fluid, stirred for 5 minutes and refiltered. The combined filtrates were extracted with petroleum ether. The final aqueous phase was centrifuged at 2500×g for 10 minutes. The aqueous phase was removed and stored at −80°C until chromatographed by HPLC.

The HPLC system consisted of a Model 6000A pump coupled to a U6K injector and a μBondapak C_{18} (3.9 mm × 30 cm) reverse phase column (Waters Associates, Milford, MA). A coulometric electrochemical detector (ESA Associates, Bedford, MA) set at +450 mV would oxidize both forms of GnRH, and the resulting current was recorded on an Omniscribe penchart recorder (Houston Instruments, TX). An isocratic mobile phase consisting of 7.40 g ammonium acetate and 3.04 g citric acid in 1 l of 19% acetonitrile/water (final pH adjusted to 4.6 with phosphoric acid), gave a retention time of 11.1 and 13.3 minutes for mGnRH and lGnRH respectively at a flow rate of 1.4 ml/min. Peak heights represent 25 ng of each synthetic peptide (Fig. 3). GnRH peaks in human brain extracts could not be distinguished electrochemically due to insufficient sensitivity.

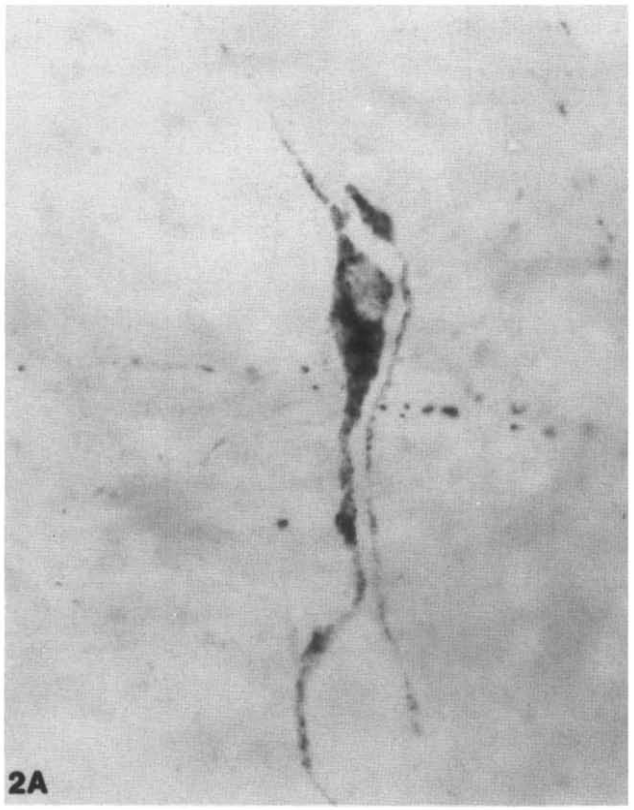
Radioimmunoassay

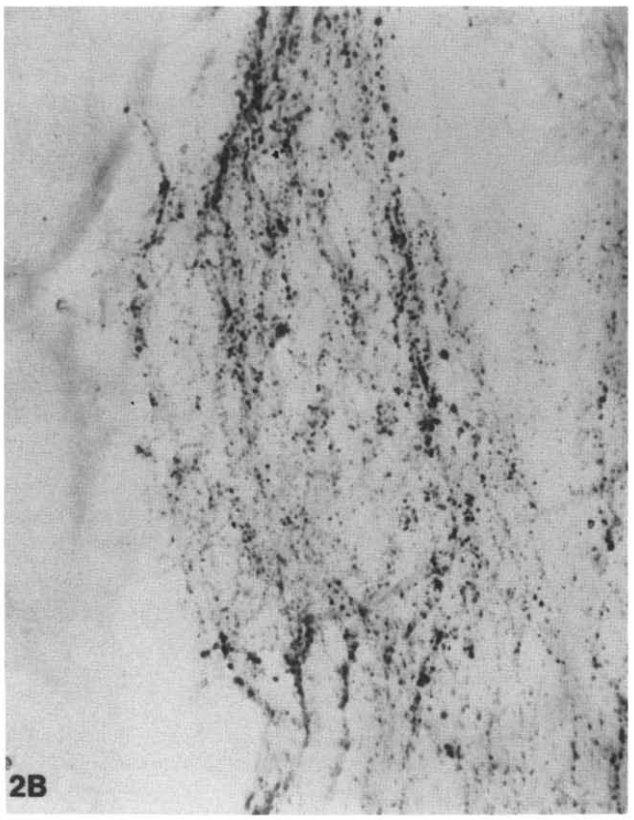
Immunoreactive lamprey GnRH (using antisera J.A.K. 1467) and mammalian GnRH (using antisera R.M. 1076) were eluted as shown in Fig. 3. Fractions (1.4 ml) were collected and aliquots (100 μl) assayed in duplicate for immunoreactive mGnRH and immunoreactive lGnRH. The synthetic standard fractions were also assayed (mGnRH=1183 mol.wt.; lGnRH=1227 mol.wt.).

RIA was performed as previously described [14] except using either synthetic mGnRH or lGnRH as the iodinated tracer and standard. The antisera were used at dilutions of 1:120,000 for the mammalian RIA (R.M. 1076), 1:50,000 for the lamprey RIA (J.A.K. 1467) and 1:9,000 for lamprey RIA (J.A.K. 1459). The antibody binding ranged between 10–32% for R.M. 1076 of ^{125}I-mGnRH; 55–56% for J.A.K. 1467 of ^{125}I-lGnRH; 64–67% for J.A.K. 1459 of ^{125}I-lGnRH. RIA sensitivities were 4.0 pg (R.M. 1076), 19.5 pg (J.A.K. 1467), and 4.9 pg (J.A.K. 1459). The GnRHs were iodinated using a modification of the chloramine-T method and purified by Sephadex G-25 followed by QAE-Sephadex A-25 chromatography followed by Sep-Pak C_{18} (10 ml 0.2% formic acid/pyridine pH 3.3; 5 ml of 80% acetonitrile at a flow rate of 4 ml/min).

RESULTS

Neuronal cell bodies and fibers, immunoreactive with lGnRH antisera (J.A.K. 1467 and J.A.K. 1459), were dispersed throughout the preoptic area and basal hypothalamus in all brains examined. These immunoreactive neurons were predominantly bipolar (Fig. 2A) and exhibited a pattern of distribution that was similar to mGnRH containing neurons. The lamprey cells, however, were only one-third to one-fifth as numerous as those seen on comparable sections treated with antisera directed against the mammalian form of GnRH (R.M. 1076 and Chen-Ramirez $R_{11}B_{73}$). Many lGnRH immunoreactive fibers were seen within the median eminence (Fig. 2B). Absorption studies showed that no immunoreactivity was present in sections treated with lGnRH-directed antisera (J.A.K. 1467 and J.A.K. 1459) pre-incubated with synthetic lGnRH (10^{-6} M, 24 hours at 4°C). Pre-incubation of lGnRH antiserum (J.A.K. 1459) with a similar antigen excess (10^{-6} M) of mGnRH (Sigma) had no effect on the immunocytochemical reaction. No immunoreactivity was present in sections treated with mGnRH-directed antiserum (R.M. 1076) that was pre-incubated with mGnRH (10^{-6} M, 24 hours at 4°C).





High performance liquid chromatography (HPLC) was employed to further characterize the two forms of immunoreactive GnRH found in human hypothalamus. Synthetic mGnRH and lGnRH were separated and detected electrochemically (Fig. 3). Basal hypothalamic and median eminence extracts each contained two separate and distinct peaks of GnRH immunoreactivity as shown from one brain (Fig. 4). The first peak was immunoreactive with mGnRH antiserum but not with lGnRH antiserum. The second peak was immunoreactive with lGnRH antiserum but not with mGnRH antiserum. In all tissues examined, the concentration of immunoreactive mGnRH was approximately the same (average 7.3 ng/tissue) and three-fold higher than that of immunoreactive lGnRH (average 2.6 ng/tissue) in both the basal hypothalamic and median eminence extracts.

LGnRH-like and mGnRH immunoreactivity were consistently present in ten hypothalami examined with the exception of two hypothalami in which neither mGnRH or lGnRH-like molecules were detected and one hypothalamus in which only mGnRH was detected. The lack of immunoreactivity in these hypothalami is probably due to the loss of GnRH activity because of the small amount of tissue used for extraction. In those cases where lGnRH-like activity could be measured, results were repeated under strict experimental conditions using an HPLC column never exposed to any form of GnRH and a number of blank injections between samples to completely rule out the possibility of contamination by sample carry-over.

DISCUSSION

These data provide compelling evidence for the existence of a second form of GnRH in human brain using a combination of immunocytochemistry, HPLC separation and radioimmunoassay. The consistency of information obtained by these separate methodologies makes it unlikely that the immunoreactive molecules are cross-reacting proteins unrelated to the GnRH family. It is also unlikely that the lamprey-like GnRH seen in human brain is a degradation product since the lGnRH is not represented in the predicted mGnRH precursor amino acid sequence [1]. In nearly all mammals studied to date, only a single molecular form of GnRH has been isolated and only a single GnRH sequence has been detected in the mammalian genome [1,24]. The lGnRH differs by five amino acids from the mammalian form (Fig. 1). This significant structural difference may account for the inability of an mGnRH probe to recognize the lGnRH genome. Our data suggest the existence of a separate gene which codes for lamprey-like GnRH in man. In view of the essential role that the GnRH peptide has in reproduction, it is not surprising that an earlier form might be conserved in higher mammals. A GnRH which differs from mGnRH has been detected in sheep suggesting that other mammals may also have multiple forms of GnRH that have not yet been identified [10].

Of greater importance is the question of whether polygenic expression of a given peptide is in fact a common

FIG. 2. (a) High magnification (630×) photomicrograph of an immunoreactive lGnRH bipolar neuron within the preoptic region of human hypothalamus. (b) Sagittal section of human median eminence demonstrating numerous immunoreactive lGnRH fibers (250×).

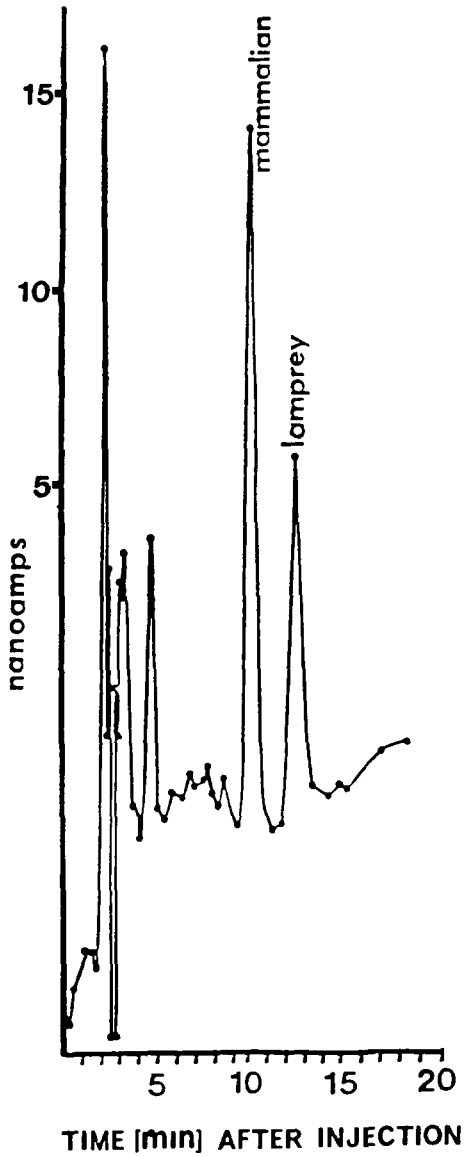


FIG. 3. HPLC chromatogram. Electrochemical separation of mGnRH and lGnRH following injection of synthetic peptides.

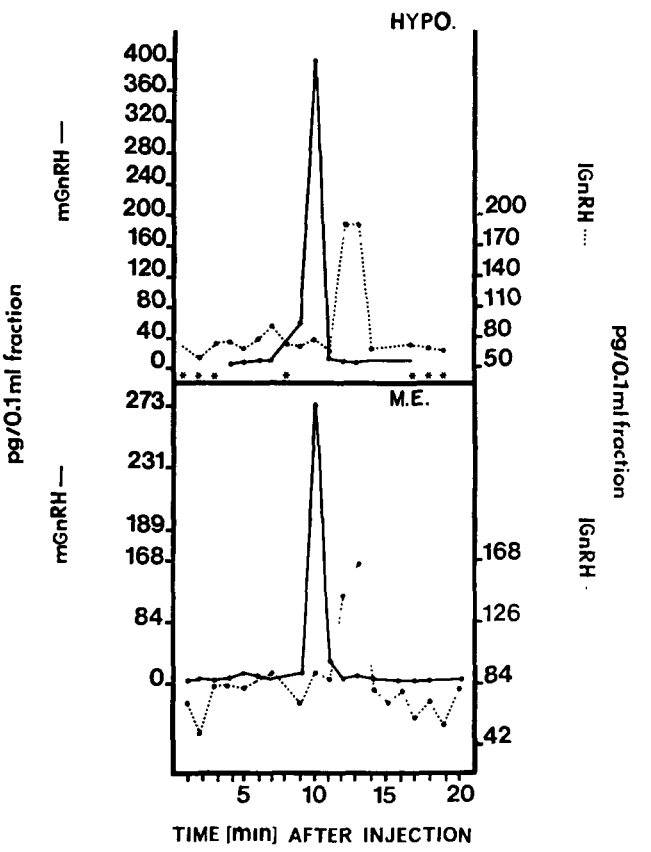


FIG. 4. Radioimmunoassay. Immunoreactive lamprey GnRH (O- - - -O) and mammalian GnRH (O——O) in basal hypothalamic (top) and median eminence extracts (bottom) following HPLC elution as shown in Fig. 3.

phenomenon. Such an argument is suggested by the multiple forms of GnRH [11, 17, 18] and somatostatin [30] observed in other vertebrates. As one ascends the evolutionary scale, consistent demonstration of multiple forms becomes increasingly difficult. This is best illustrated by the controversy which arose over the existence of different forms of insulin in the guinea pig [22,29]. Other molecular forms of a given peptide may be more primitive, less abundant and so similar that they are exceedingly difficult to detect. Complicating matters further is the issue of physiological state, which may also influence gene expression.

In summary, we have detected a non-mammalian form of GnRH in human brain which is immunologically identical to lamprey GnRH. Confirmation of the exact nature and role of this newly detected form will require future isolation and sequence analysis.

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