Aspects of Hypothalamic Regulation of the Pituitary Gland

Its implications for the control of reproductive processes.

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In this article I give an account of my search for the hypothalamic regulatory hormones. Since my work on the hypothalamus has extended over 23 years, I omit reference to studies that did not contribute directly to my main objective, that is, demonstration of hormonal activity in hypothalamic extracts and the purification, isolation, and determination of the structures of hypothalamic hormones and their testing in biological and clinical settings.

I was attracted to hypothalamic endocrine research in 1954 while an undergraduate at McGill University in Montreal. A decisive stimulus was provided by the formulation by Harris and others [for example, (*1*)] of hypotheses relating to the hypothalamic control of secretion of the anterior pituitary gland (Fig. 1). Harris and others postulated that neurohumoral substances might originate in the median eminence of the tuber cinereum, reach the anterior lobe by way of the hypophysial portal system, and thus regulate pituitary secretion (*1*). About the same time, Sawyer *et al.* (*2*) demonstrated involvement of the central nervous system in the control of gonadotropin secretion. Without the work of these men my contributions would not have been forthcoming. It was clear that, despite a strong circumstantial case favoring hypothalamic control of the pituitary, the proposition would remain speculative until direct evidence for the existence of specific hypothalamic chemotransmitters controlling release of pituitary hormones could be demonstrated.

In the beginning it was not possible to prove the existence of, and isolate, hypothalamic hormones since there were no specific methods for the detection of their activity. Working on the problem of control of corticotropin (ACTH) secretion, Saffran and I reached the conclusion that the hypothalamic theory best explained most of the then existent experimental facts. Using isolated rat anterior pituitary fragments (Fig. 2), we devised a test system for measuring the release of ACTH (*3*). This in vitro pituitary system was simple and consisted of exposing symmetrical portions of the gland to test substances. This permitted compensation for any possible indirect effect or contamination with trophic hormones and proved decisive not only for demonstrating the existence of the corticotropin-releasing factor but also of hypothalamic hormones regulating the secretion of thyroid-stimulating hormone, or thyrotropin (TSH), growth hormone (GH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin. I still recall our exaltation when we found that hypothalamic or neurohypophysial extracts added to the anterior pituitary tissue caused an unequivocal increase in the release of ACTH (Table 1). We "knew" then that we had done it, that we had demonstrated experimentally for the first time the existence of a substance that stimulated the release of ACTH (*3–5*). We named this substance corticotropin-releasing factor (CRF). We still apply the term "factor" to those hypothalamic substances whose activity cannot be ascribed to a specific chemical structure. However, for those substances which have had their structures determined and which have been shown to be likely physiological regulators of secretion of respective anterior pituitary hormones, we employ the name "hormone" (Table 2).

In our early attempts to purify CRF we used mainly posterior pituitary powders, since large quantities of hypothalami were not readily available. We obtained evidence that CRF was a polypeptide (*5*); but, despite seven years of intensive effort, two with Saffran in Montreal and five with Guillemin in Houston, we were unable to isolate enough material for the determination of its structure. However, during that period (1955–1962) new in vivo assays for hypothalamic hormones and improved purification methods were developed. Techniques of gel filtration on Sephadex, which I learned at the Institute of Biochemistry in Uppsala with J. Porath, proved to be of particular value.

Arrangements were also made in 1962, after I moved to New Orleans, for the procurement of hundreds of thousands of hypothalami. Oscar Mayer & Co. donated about a million pig hypothalami. This enabled us to undertake a large-scale effort aimed at the purification of adequate amounts of material to permit chemical characterization. In addition to CRF, we systematically investigated purified fractions for the presence of TRH, LH-RH, FSH-RH, GH-RF, PIF, and MIF (*6–15*), since the discovery of CRF opened the way to the demonstration of these other releasing factors.

Thyrotropin-Releasing Hormone

Our next great effort was devoted to the isolation and identification of TRH. We first demonstrated the presence of TRH in pig, beef, and human hypothalami using in vitro assays based on the release of TSH from rat pituitary glands and in vivo assays based on iodine-131 release from thyroid glands of mice (*6, 12, 13, 15*). Then with the help of Bowers and Redding I undertook the purification of bovine and porcine TRH. In 1966 we isolated 2.8 milligrams of TRH from 100,000 pig hypothalami (*16*) by Sephadex gel filtration, phenol extraction, carboxymethylcellulose chromatography, countercurrent distribution, free-flow electrophoresis, and partition chromatography (Fig. 3). In our in vivo assay, 1 nanogram of this homogenous porcine TRH was active and in vitro, 0.01 ng stimulated TSH release (*16*). We also correctly reported that it had three amino acids—glutamic acid (Glu),

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histidine (His), and proline (Pro) in equimolar ratios (*16*)—establishing for the first time that TRH was a peptide. By mass spectra we detected a band due to the diketopiperazine of His-Pro and we also determined that an intact histidine was necessary for full biological activity of TRH, but unfortunately we did not take full advantage of these original early findings (*13, 16*).

Although the TRH problem could have been solved in 1966, three more years had to elapse for additional technological breakthroughs necessary to determine its precise structure. Since we lacked synthetic capabilities at that time, Merck Sharp & Dohme Laboratories synthesized for us eight tripeptides containing histidine, proline, and glutamic acid or glutamine, one of which was in the correct sequence, Glu-His-Pro. None of these, however, proved to have biological activity (*13*) and a complete series of possible analogs was not made. Somewhat discouraged by these negative results, I turned my attention to LH-RH, leaving the problem of the structure of TRH to the chemists with whom I was working. However, when Burgus and Guillemin announced in 1969 that they had found the same three amino acids in ovine TRH as I had 3 years earlier for porcine TRH (*16*), my enthusiasm for the program was rekindled and we intensified our efforts.

Fortunately, since I thought that the amount of TRH originally isolated would be insufficient to allow complete determination of structure, I took the precaution of obtaining about five additional milligrams of TRH from 250,000 pig hypothalami (*17*). Its structure then was systematically investigated by a series of degradation reactions (*17, 18*). First with Nair we established the correct amino acid sequence in New Orleans (*17*), and then in a parallel effort between my group and Enzmann and Bøler working in Folkers' laboratory in Austin, Texas, we were able to assign the correct structure to porcine TRH and synthesize it (*18–20*).

The structure was determined on the basis of (i) the amino acid sequence of TRH established in my laboratory (*17*); (ii) comparison of activity of synthetic analogs of Glu-His-Pro in assays carried out by Bowers and, independently, by Redding (*17, 19, 20*); (iii) mass spectra of natural and synthetic preparations (*18*); and (iv) synthetic modification and physicochemical comparisons of these synthetic analogs and natural TRH (*19, 20*). Thus, we attempted to modify both the amino and the carboxyl ends of Glu-His-Pro in order to generate TRH activity.

Table 1. The CRF activity of pig hypothalamic preparations measured by stimulation of ACTH release in vitro from isolated rat pituitary fragments [From (*4*) and (*5*)]

Preparation	Dose (μg)	Percentage ACTH released (ratio experimental to control)	95 percent confidence limits
Oxycel nonadsorbed	10	340	220 to 520
Oxycel nonadsorbed	10	240	130 to 470
Oxycel adsorbed	10	240	130 to 430
Acetic acid insoluble	10	240	90 to 660

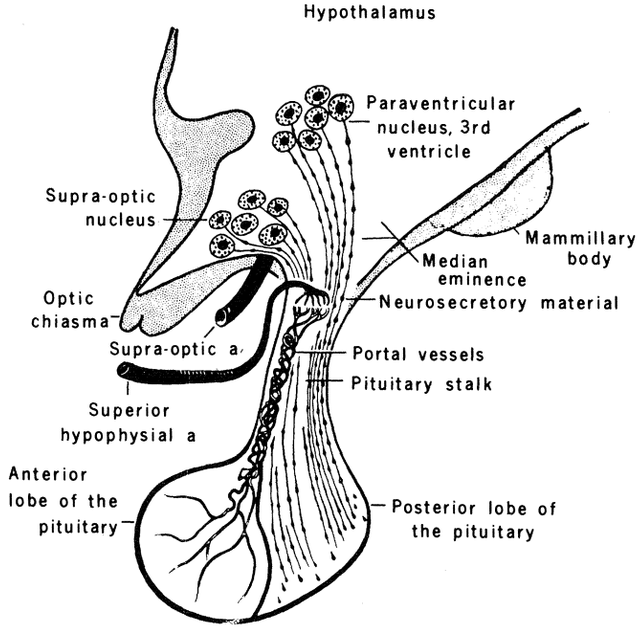


Fig. 1. Simplified schematic reconstruction of the hypothalamic - hypophysial nerve tracts and blood supply illustrating the principles of neurohumoral control of the anterior pituitary gland. [Courtesy of the *International Journal of Fertility*]

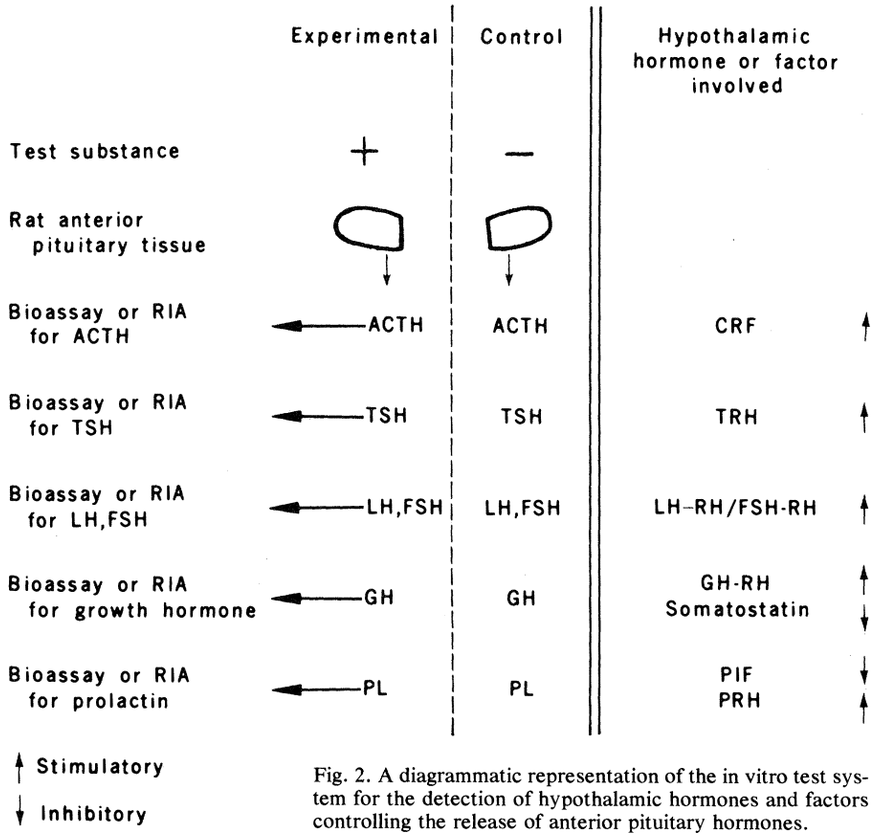


Fig. 2. A diagrammatic representation of the in vitro test system for the detection of hypothalamic hormones and factors controlling the release of anterior pituitary hormones.

Treatment of the methyl ester of Glu-His-Pro with anhydrous ammonia led predominantly to formation of (pyro)Glu-His-Pro-amide, and to generation of TRH activity (*19*).

Synthetic L-(pyro)Glu-L-His-L-Pro-amide gave R_F values identical to those of natural TRH in 17 chromatographic systems (*20*). Upon gel filtration on Sephadex G-25 columns in 0.2*M* acetic acid, natural porcine TRH and synthetic (pyro)Glu-His-Pro-amide displayed identical migration rates (Fig. 4). The structure of TRH was thus (pyro)Glu-His-Pro-amide, or 2-pyrrolidone-5-carboxylyl-His-Pro-amide (Fig. 5). The biological activity of synthetic TRH was the same as that of natural porcine TRH (*21*). It is somewhat ironic to realize that had Merck Sharp & Dohme Laboratories furnished us with Glu(NH_2)-His-Pro-NH_2, it would have partially cyclized to the active (pyro)Glu-His-Pro-NH_2 form after the synthesis, and we would in all probability have solved the problem of TRH structure 3 years earlier.

The structural work of Burgus and Guillemin (*22*) on ovine TRH paralleled that of our group, and they elucidated the structure of ovine TRH about the same time. Subsequent studies disclosed that bovine and human TRH probably have the same structure as the porcine and ovine hormone.

We have conducted physiological studies since 1962 with natural preparations of TRH (*12, 13, 16*) and confirmed and extended them by using synthetic TRH, demonstrating that the concentration of TSH in the plasma increased when TRH was administered intravenously, subcutaneously, intraperitoneally, or orally (*21*). Later we found that TRH also stimulates prolactin release in sheep (*23*). A direct action on the pituitary tissue in vitro in picogram doses was demonstrated in the pituitaries of rats, sheep, and goats (*24*). In pituitary tissue cultures, TRH stimulated the synthesis as well as the release of TSH. A dose-response relationship, both in vivo and in vitro, was also demonstrated; that is, increasing doses of TRH caused a progressively greater release of TSH (*21, 24*). Thyroxine (T_4) and triiodothyronine (T_3) blocked the stimulatory effect of TRH on TSH release (*13, 24*). This occurred not only in vivo but also with pituitary fragments in vitro, thus confirming that thyroid hormones must exert an action directly on the pituitary gland. We also suggested that among the physiological stimuli that may release TRH is exposure to mild cold (*25*). In attempting to characterize this hormone, we showed that it is rapidly inactivated in the bloodstream and studied its excretion and metabolism (*13, 25*). Recent results suggest that TRH, in addition to its effect on the pituitary, might have central nervous system (CNS) effects, possibly as a neurotransmitter or modulator (*26*). These results, and subsequent studies by other workers, helped to establish the physiological importance of TRH.

The first clinical studies with TRH, carried out in 1967 with Bowers and Gual, showed that natural porcine TRH stimulated TSH release in humans (*27*) as measured by both bioassay and radioimmunoassays (Fig. 6). After synthetic TRH became available, these findings were confirmed and extended by us and

Table 2. Hypothalamic hormones or factors controlling the release of pituitary hormones.

Hormone or factor	Abbreviation
Corticotropin (ACTH)-releasing factor	CRF
Thyrotropin (TSH)-releasing hormone	TRH
Luteinizing hormone (LH)-releasing hormone and follicle-stimulating hormone (FSH)-releasing hormone	LH-RH/ FSH-RH
Growth hormone (GH) release-inhibiting hormone	GH-RIH; somatostatin
Growth hormone (GH)-releasing factor	GH-RF
Prolactin release-inhibiting factor	PIF
Prolactin releasing factor	PRF
Melanocyte stimulating hormone (MSH)-release-inhibiting factor	MIF
Melanocyte-stimulating hormone (MSH)-releasing factor	MRF

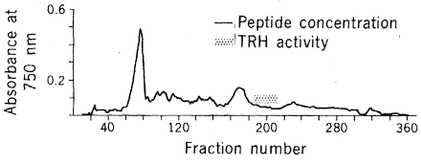
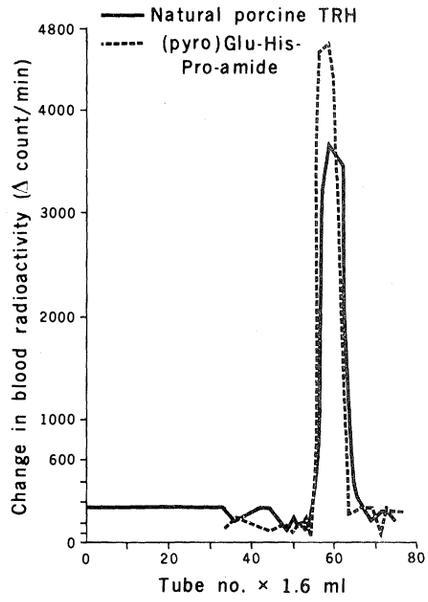


Fig. 3 (left). Partition chromatography of porcine TRH from free-flow electrophoresis on a column of Sephadex G-25 (0.9 by 76 cm). The upper phase of the solvent system consisted of *n*-butanol, acetic acid, and water (4:1:5 by volume). Fraction size, 1 ml; void volume 20 ml. [From Schally *et al.*(*13*), p. 515; courtesy of *Recent Progress in Hormone Research*] Fig. 4 (right). Gel filtration of natural porcine TRH (160 μg) and synthetic (pyro)Glu-His-Pro-amide (200 μg) on Sephadex G-25. Column size, 1.1 by 123 cm; solvent, 0.2*M* acetic acid; fraction size, 1.6 ml. The biological activity of effluents was followed by bioassay for TRH. [From (*136*)]



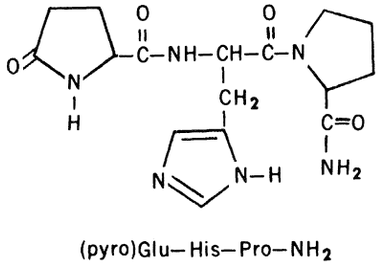
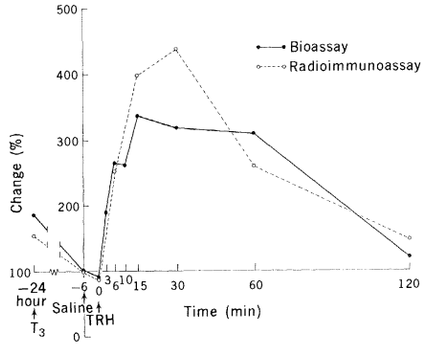


Fig. 5 (left). Molecular structure of thyrotropin-releasing hormone (TRH). Fig. 6 (right). Changes in plasma TSH concentrations after the injection of TRH to cretins: at −24 hours 25 μg of T_3 was given orally, and at time zero, 300 μg of porcine TRH was given intravenously. Plasma samples were measured by both bioassay and radioimmunoassay. [From (*21*)]



others (*28–31*). I have found it particularly gratifying that TRH is useful clinically for the differentiation between hypothalamic and pituitary hypothyroidism and for the diagnosis of mild hyper- and hypothyroidism.

After identification of TRH, we redoubled our efforts on the LH and FSH releasing hormone.

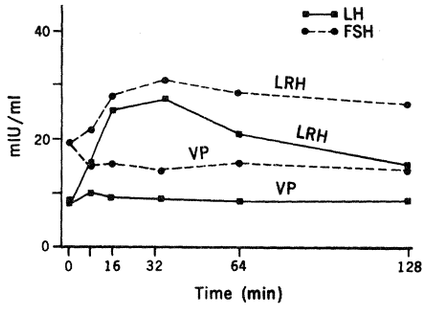
LH-RH and FSH-RH

It has long been known that the reproductive activity of animals is influenced by seasonal and external environmental factors such as nutrition, light, and temperature and that aberrations in the menstrual cycles of women can occur as a result of adverse environmental and psychological stimuli and emotional disturbances (*31*). In the late 1920's, after the involvement of the pituitary in the processes of reproduction, systematic investigations were initiated on the link between the hypothalamic region of the CNS and the secretion of pituitary gonadotropins. On the basis of experiments involving electrical stimulation of the hypothalamus, interruption of the blood vessels between the hypothalamus and the anterior pituitary by sectioning of the hypophysial stalk, and the transplantation of the pituitary to various sites, Harris proposed the hypothesis of neurohumoral regulation of gonadotropin secretion (*1*). Sawyer *et al.* (*2*) also adduced evidence for involvement of the CNS in control of the secretion of gonadotropins by demonstrating neuropharmacological stimulation and inhibition of ovulation with centrally acting stimulants and blocking agents.

The question that remained to be resolved was how information perceived in the CNS would be communicated to the pituitary. It was our aim to find that link between the hypothalamus and the pituitary insofar as the control of reproductive functions was concerned.

Although strong evidence for the existence of LH-RH and FSH-RH in hypothalamic extracts of rats and domestic animals was provided in the early 1960's (*7, 8, 11, 32–40*), it was thought that these activities were due to two different substances. We were able to demonstrate that materials with the properties of peptides purified from beef and pig hypothalami stimulated LH release not only in vivo but also in vitro (*7, 8*). The in vitro study (*8*) was the first demonstration that hypothalamic materials release LH by a direct action on the pituitary. With purified LH-RH at our disposal, we initiated work on how the interaction between LH-RH and sex steroids regulates gonadotropin secretion.

At first we postulated that the inhibitory effect of contraceptive steroids on gonadotropin release was exerted mainly on the hypothalamus (*41, 42*), but subsequently with Arimura, Sawyer, and Hilliard (*31, 43, 44*) we were able to prove that estrogens, progestins, and androgens also suppressed in part the response to LH-RH at the pituitary level. Later, I confirmed by in vitro studies this



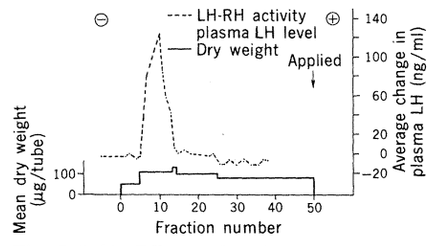
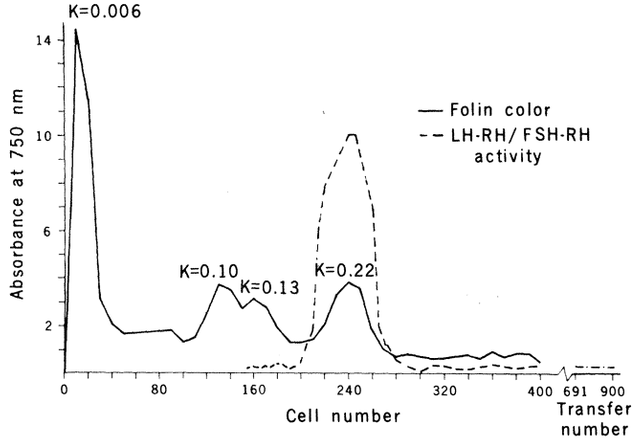


Fig. 7 (left). Effects of an intravenous injection of 0.7 mg of porcine LH-RH on the concentrations of LH and FSH in a normal woman, on day 9 of the menstrual cycle. *VP*, vasopressin control. [Based on Kastin *et al.* (*49*)] Fig. 8 (right). High-voltage electrophoresis of 2.3 mg of LH-RH in 0.18*M* pyridine acetate buffer, *p*H 6.3. A vertical column (0.9 by 97.6 cm), with external cooling at 5°C, was packed with cellulose powder. After the electrophoretic separation at 2570 V, 20 mA for 18 hours, the column was eluted with buffer, and 1.3-ml fractions were collected. [From (*50*)]

Fig. 9. Countercurrent distribution (III) of 55.4 mg of LH-RH from countercurrent distribution (II) in a system of *n*-butanol, acetic acid, and water (4 : 1 : 5 by volume). The lower phase was 3 ml and the upper phase 5 ml. The number of transfers was 900. Folin-Lowry analyses were done on 100-μl portions of the lower phase. Assays for the location of LH-RH activity were carried out on 2-μl portions of the lower phase per rat. *K*, partition coefficient. [From (*51*)]



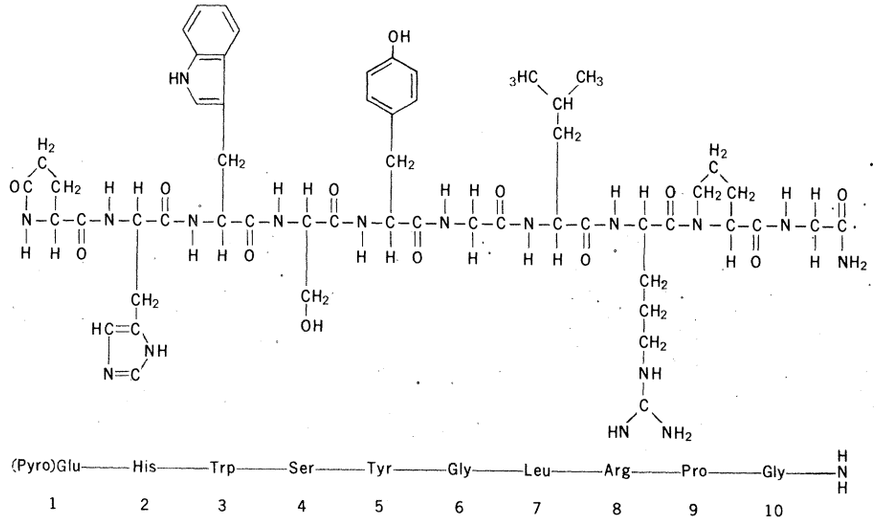


Fig. 10. Molecular structure of LH- and FSH-releasing hormone (LH-RH/FSH-RH).

inhibitory effect (negative feedback) of steroids on the pituitary (*45*). Several laboratories, including ours (*31, 44, 46*), obtained evidence that estrogens and progesterone can also exert a positive feedback at the pituitary and the hypothalamus, and augment the pituitary responsiveness to LH-RH. These results may be correlated with events in the human menstrual cycle and the estrous cycle of animals. Thus, an increase in estrogen concentration in plasma which precedes the ovulatory surge of LH in animals and women appears to augment the pituitary responsiveness to LH-RH. Conversely, the large amounts of estrogen and progesterone which are secreted after ovulation may lower pituitary responsiveness to LH-RH. With the aid of LH-RH, we also found that clomiphene exerts a central effect on the hypothalamus (*47*).

In view of the relative purity and apparent absence of visible toxicity of porcine LH-RH, I decided to test it in humans. These studies, conducted in 1968 and 1969 in collaboration with Kastin and Gual in Mexico (*48, 49*), unequivocally established that highly purified LH-RH released LH and FSH in men and women under a variety of conditions (Fig. 7). Realizing that LH-RH might be useful clinically, we intensified our efforts to establish the structure of LH-RH. As in the case of TRH, tens of thousands of hypothalami had to be laboriously extracted, concentrated, and purified to obtain enough material for a chemical characterization. The first isolation of 800 μg of LH-RH/FSH-RH from ventral hypothalami of 165,000 pigs was achieved by 12 successive purification steps that included extraction with 2*N* acetic acid, gel filtration on Sephadex, phenol extraction, chromatography and rechromatography on carboxymethylcellulose, free-flow electrophoresis, countercurent distribution, partition chromatography in two different solvent systems, and high-voltage zone electrophoresis (Fig. 8) (*50*). During the purification, the LH-RH and FSH-RH activities were followed by bioassay in vitro and in vivo. The LH and FSH released were determined by bioassays and later by radioimmunoassays.

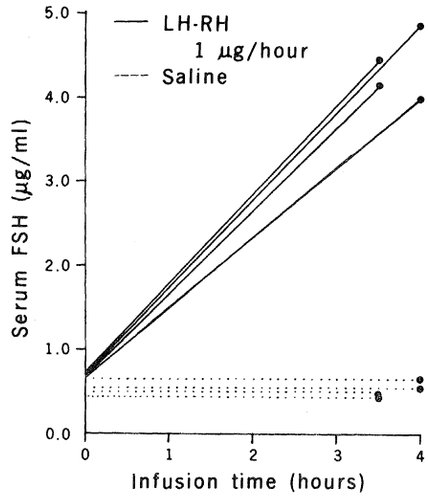


Fig. 11. Concentrations of FSH in the serum of immature male rats after prolonged intravenous infusion of synthetic LH-TH. The FSH is expressed as NIAMD standard Rat FSH-RP-1. [Based on Arimura *et al.*(*59*)]

Subsequent isolation of 11-milligram amounts of LH-RH/SH-RH from 250,000 pig hypothalami was carried out mainly by the countercurrent distribution technique (Fig. 9) (*51*). In all the isolation steps, the LH-RH activity and FSH-RH activity were located in identical fractions. They could not be separated by additional partition chromatography in ten different solvent systems (*50, 51*). The amino acid composition determined after hydrolysis with 6*N* HCl at 110°C showed the presence of the following nine amino acid residues: His, 1; Arg, 1; Ser, 1; Glu, 1; Pro, 1; Gly, 2; Leu, 1; and Tyr, 1 (where Arg, Ser, Gly, Leu, and Tyr are, respectively, arginine, serine, glycine, leucine, and tyrosine) (*50*). Since hydrolysis in 6*N* HCl leads to destruction of tryptophan (Trp), the analysis for this amino acid was carried out after acid hydrolysis in the presence of thioglycollic acid, or by alkaline hydrolysis; one Trp residue was found. Thus, the molecule of LH-RH/FSH-RH consisted of ten amino acids (*52*). Experiments with proteolytic enzymes showed that LH-RH/FSH-RH was a polypeptide (*52*). The LH-RH and FSH-RH activities were simultaneously abolished by incubation with some endopeptidases (chymotrypsin, papain, subtilisin, and thermolysin) but not by incubation with exopeptidases (leucine aminopeptidase, aminopeptidase M, and carboxypeptidase A and B) (*52*). That the hormones were not inactivated by the Edman procedure and that no amino acid could be detected by the dansyl method indicated a blocked NH_2-terminus. Inactivation by pyrrolidone carboxylyl peptidase suggested that the NH_2-terminus was occupied by pyroglutamic acid.

In the initial attempt to determine the structure of LH-RH/FSH-RH with Matsuo and Baba, we utilized the combined Edman-dansyl procedure coupled with the selective tritiation method for COOH-terminal analyses (*53*). These procedures were used directly on the digestion products of LH-RH with chymotrypsin and thermolysin without prior separation of fragments. Additional data

Table 3. Effect of natural (N) and synthetic (S) LH-RH/FSH-RH in humans and animals. [After Schally and Arimura (*135*)]

Species	Effect and route of administration
Rat	Release of LH and FSH in vivo and in vitro (N or S); stimulation of spermatogenesis (S); stimulation of follicular maturation (S); ovulation (N or S). Injected intravenously, into the carotid artery, or subcutaneously, or given orally, intravaginally, cutaneously,* or intraventricularly†
Mice	Release of LH; ovulation (S). Injected subcutaneously
Golden hamster	Release of LH in vivo (N). Injected intravenously
Nutria	Release of LH in vivo (N). Injected intravenously
Rock hyrax	Release of LH in vivo (S)
Rabbit	Release of LH in vivo (N or S); ovulation (N or S)
Mink	Ovulation (S)
Spotted skunk	Ovulation (S)
Sheep	Release of LH and FSH in vivo (N or S). Injected intravenously, subcutaneously, or intramuscularly; ovulation (S)
Pigs	Release of LH in vivo (S). Injected intravenously or intramuscularly; ovulation (S)
Cattle	Release of LH and FSH in vivo (S). Injected intramuscularly, into the carotid artery, or subcutaneously; ovulation (S)
Impala	Release of LH in vivo (S)
Horses	FSH release > LH release, ovulation (S). Injected subcutaneously
Monkeys	Release of LH (N or S)
Humans	Release of LH and FSH (N or S); stimulation of spermatogenesis (S); ovulation (N or S)
Pigeons and chickens	Premature ovulation (S) and release of LH. Injected into the carotid artery, intravenously, or intramuscularly
Fish‡	Release of gonadotropins in vivo (N or S). Injected intravenously
Newts	Release of LH and FSH in vivo (S)
Frogs	Spermiation (S). Injected subcutaneously

*In dimethyl sulfoxide. †In the third ventricle. ‡Brown trout and carp.

were provided by high resolution mass spectral fragmentation of LH-RH/FSH-RH. On the basis of these results, we proposed the decapeptide sequence for LH-RH/FSH-RH shown in Fig. 10. The correctness of this structure was confirmed by additional conventional structural analyses involving the separation of chymotryptic fragments (*54*) after the cleavage of NH_2-terminal pyroglutamyl residue in pyrrolidone carboxylyl peptidase as well as by synthesis of this material by means of the solid phase methods (*55–57*). Synthetic LH-RH/FSH-RH possessed the same properties as the natural material (*56, 57*). Thus, in rats it stimulated the release of LH and FSH in vitro and in vivo (*58, 59*) (Fig. 11). The time courses of LH and FSH release in vitro induced by natural or synthetic LH-RH were identical (Fig. 12) (*58*). Simultaneous studies demonstrated that in human beings synthetic LH-RH also increased the plasma concentrations of LH and FSH (*56, 57, 60*) (Fig. 13).

Because both natural LH-RH and the synthetic decapeptide corresponding to its structure possessed major FSH-RH as well as LH-RH activity, we took the bold step of proposing that one hypothalamic hormone, designated LH-RH/FSH-RH, could be responsible for this dual effect (*56, 57*). This concept is now supported by many physiological as well as immunological data. The LH-RH decapeptide represents the bulk of FSH-RH activity in the hypothalamus and it appears to be the principal FSH releasing hormone. Our subsequent studies in collaboration with J. Reeves and those of others established that in addition to its effect in rats and humans, LH-RH greatly enhances the release of LH and FSH in other mammals, including mice, nutria, rabbits, golden hamsters, mink, spotted skunk, impala, rock hyrax, sheep, cattle, pigs, horses, and monkeys (Table 3) (*31, 57, 61*). In most of these species LH-RH can also induce ovulation. LH-RH was also found to be active in nonmammalian species such as chickens and pigeons, and even in some species of fishes such as brown trout and carp and in amphibia such as newts and frogs (*61*). These studies in mammals, birds, fish, and amphibia indicate that species-specificity does not occur with LH-RH. We also obtained evidence that LH-RH can increase the synthesis of LH and FSH in addition to their release (*62*), and that prolonged treatment with LH-RH after hypophysectomy and transplantation of the pituitary stimulates spermatogenesis in male rats and follicular development in female rats (*63, 64*). In another study with Rennels, we demonstrated that LH-RH increases the extrusion of secretory granules from LH gonadotrophs in rats with persistent estrus (Fig. 14) (*65*). Studies with synthetic ^{3}H-labeled LH-RH proved that LH-RH is rapidly degraded in blood by enzymatic cleavage of the (pyro)Glu-His group and is excreted in the kidneys (*66*). Its half-life is about 4 minutes in man.

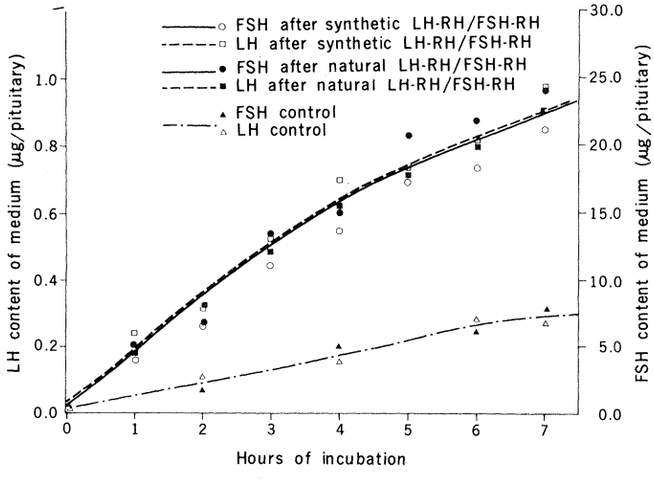
Table 4. Effect of intravenous injection of 1 ml of antiserum to LH-RH (No. 742) on serum LH and FHS concentrations and ovulation in cycling rats. One milliliter of normal rabbit serum or rabbit antiserum to LH-RH was injected intravenously into the rats at 0900 hours on the day of proestrus and blood was collected at 1630 hours for assays of serum LH and FSH. [From (*69*)]

Treatment	Number of rats		Number of ova*	LH (ng/ml)*†	FSH (ng/ml)*‡
	Injected	Ovulated			
Normal rabbit serum	4	4	12 ± 1.3	58.0 ± 13.2	720 ± 108
Antiserum to LH-RH	4	0	0	0.8 ± 0.13§	145 ± 21§

*Mean ± standard error. †Expressed in terms of NIH standard LH-S-17. ‡Expressed in terms of NIAMD standard Rat FSH-RP-1. §$P < .01$ by Student's t-test as compared with the corresponding LH and FSH concentrations of the serums from the rats injected with normal rabbit serum.

Immunological and Immunohistochemical Studies

Production of antiserums to LH-RH by Arimura *et al.* (*67*) and by others permitted the establishment of radioimmunoassays (RIA) and the performance of a variety of immunological studies (*68–73*). Male rabbits that were actively immunized with LH-RH and had generated its antibodies developed testicular atrophy associated with aspermatogenesis (*67*). Castrated rats actively immunized with LH-RH showed parallel decreases in serum LH and FSH concentrations associated with an increase in serum antibody titer to LH-RH. Administration of gamma globulin directed against LH-RH (anti-LH-RH gamma globulin) to castrated rats prevented the increase in serum LH and FSH concentrations nor-



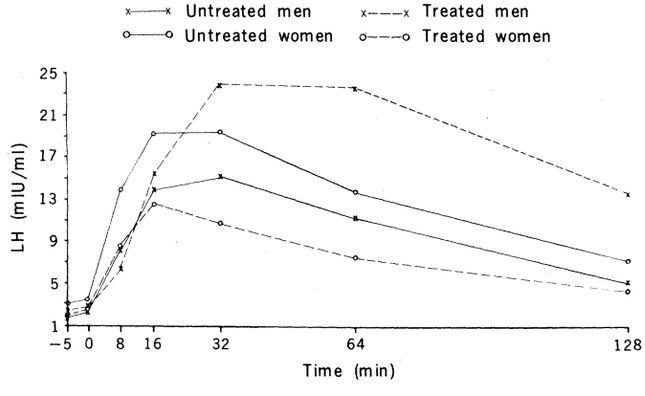


Fig. 12 (left). Release of LH and FSH from pituitaries of male rats (10 pituitary halves per beaker in 10 ml of Krebs-Ringer bicarbonate glucose), containing 4 ng of natural or synthetic LH-RH per milliliter. The ordinates were adjusted to compensate for the content of LH and FSH. LH is expressed as NIH standard LH-S-17. The FSH is expressed as NIAMD standard. [From (*58*)] Fig. 13 (right). Mean plasma concentrations of LH after intravenous administration of synthetic LH-RH equivalent to 38 μg to four groups of subjects: untreated men, untreated women, men previously treated with 1.0 mg of ethinyl estradiol for 3 days; women previously treated with oral contraceptive (Lyndiol) for 1 week. [From (*60*)]

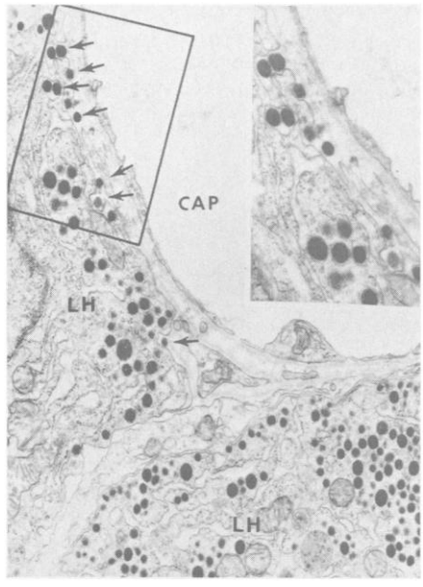


Fig. 14. Luteinizing hormone gonadotroph of a rat in persistent estrus killed 15 minutes after an injection of 200 ng of synthetic LH-RH. Arrows indicate massive extrusion of secretory granules into pericapillary space (*CAP*) (×26,300; inset, ×38,000). [From (*65*)]

mally seen after such operation, and the development of "castration" cells in the pituitary (*68*). Passive immunization of normal cycling rats or hamsters with LH-RH arrested follicular maturation, prevented the preovulatory surge of LH and FSH, blocked ovulation (Table 4), and reduced serum estradiol concentrations (*69, 70*).

We also showed that hypothalamic LH-RH is necessary for normal implantation and maintenance of pregnancy, since passive immunization with LH-RH in early pregnancy causes a delay in implantation of fertilized ova or termination of pregnancy in rats, depending on the time the antibody to LH-RH is injected (*71, 72*). Before and during the preovulatory surge of LH-RH release, Arimura *et al.* (*73*) detected by RIA peak concentrations of LH-RH in the peripheral plasma of women; others found these peaks in the blood of rats, sheep, rabbits, and monkeys. This indicates that this decapeptide is the mediator responsible for the release of the ovulatory quota of LH. These studies and others clearly established that LH-RH is the main link between the brain and the pituitary gland insofar as reproductive function is concerned.

The availability of antiserums to LH-RH made possible various studies on the localization of LH-RH by RIA or immunohistochemical methods. The bulk of LH-RH was localized in the median eminence and in the arcuate nucleus, and small but significant amounts were found in the preoptic and suprachiasmatic areas. In studies with Flerkó and Sétáló, we found that the pathway of LH-RH–containing nerve fibers in the median eminence of rats coincides with the course of the nerve fibers of the tuberoinfundibular tract (*74*), and that LH-RH is produced in neuronal cell bodies, especially in the medial preoptic and the suprachiasmatic area. However, other studies showed that extrahypothalamic brain areas also contain LH-RH and may be involved in its synthesis. This could suggest that, in addition to being the regulator of the release of LH and FSH, LH-RH might act as a central neuromodulator. LH-RH has indeed been shown to excite sexual behavior in rats (*75*). This is in agreement with the CNS effect of hypothalamic peptides which I helped Kastin demonstrate in 1971 (*76, 77*).

Analogs of LH-RH

The interest in possible veterinary and medical applications of LH-RH stimulated us and others to synthesize many hundreds of LH-RH analogs. Between 1972 and 1977 our laboratory synthesized more than 300 analogs by the use of rapid solid-phase techniques (*61, 78, 79*). Our aims were: (i) to develop analogs with prolonged biological activity, so that they would be more useful therapeutically than LH-RH itself; and (ii) to obtain inhibitory (antagonistic) analogs that could form the basis of new birth control methods. The studies of these peptides have shed much light on the relation between biological activity and structure.

Early results showed that the NH_2-terminal tripeptide and tetrapeptide fragments of LH-RH as well as the COOH-terminal nonapeptide and octapeptide of LH-RH have very little or no LH-RH activity (*61, 80*). Thus, very active small fragments cannot be obtained from LH-RH. In general, amino acids in position 1 and from 4 to 10 appeared to be involved only in binding to the receptors and in exerting conformational effects. However, histidine and tryptophan probably exert a functional effect in addition to providing receptor-binding capacity, since simple substitutions or deletions in positions 2 or 3 greatly decrease or abolish LH-RH activity. Coy in our laboratory (*81–83*), and others (*84–87*), showed that some analogs substituted in position 6 or 10, or both, are 10 to 60 times more potent than LH-RH and also possess prolonged activity; of these, the most interesting were [D-Phe^6]-LH-RH, [D-Trp^6]-LH-RH, [D-Ala^6,des-Gly^{10}]-LH-RH ethylamide, [D-Leu^6,des-Gly^{10}]-LH-RH ethylamide, and [D-Ser(Bu^t)^6,des-Gly^{10}]-LH-RH ethylamide (where Bu^t is tertiary butyl). These superactive LH-RH analogs cause a prolonged release of LH and FSH (*78, 79, 81–86*).

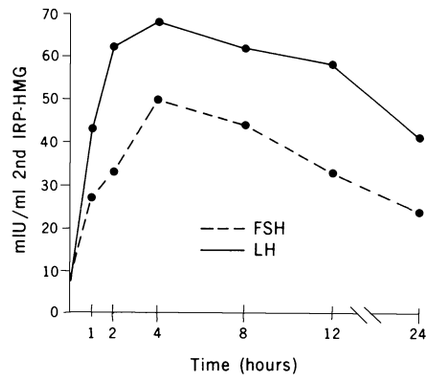


Fig. 15. Plasma LH and FSH concentrations in a 34-year-old woman with amenorrhea and galactorrhea after intramuscular administration of 250 μg of D-Leu-6-LH-RH ethylamide (second IRP-HMG is reference preparation). [From (*101*)]

However, it has also been recently demonstrated that long-term treatment with pharmacologic doses of these analogs or with large amounts of LH-RH can cause temporary and reversible impairment of reproductive functions. Thus, long-term administration of 1 to 10 micrograms of [D-Leu^6,des-Gly^{10}]-LH-RH ethylamide to mature female rats caused cessation of cycling and atrophy of the ovaries and uterus (*88*). Prolonged administration of the same analog to male rats resulted in a reduction of testicular LH-human chorionic gonadotropin (hCG) receptors and of testosterone levels (*89*). Pharmacologic doses of LH-RH or superactive analogs block implantation and terminate gestation in rats (*90, 91*). These paradoxical antifertility effects of LH-RH and its analogs appear to be directly related to hypersecretion of LH and, after nidation, to functional luteolysis or inhibition of progesterone secretion, and they have caused us and others to initiate investigations on their possible application as precoital (male and female) and postcoital contraceptives.

Clinical Uses of LH-RH and Its Superactive Agonistic Analogs

Luteinizing hormone-releasing hormone has been used diagnostically to determine pituitary LH and FSH reserve. It is not a complete diagnostic tool but, used alone, especially repeatedly, or in combination with the clomiphene test, it may be helpful in differentiating pituitary

and hypothalamic causes of hypogonadism (*61, 92*). LH-RH alone or in combination with human menopausal gonadotropin (hMG) or hCG has also been used therapeutically in Mexico, Chile, the United States, Israel, Sweden, Japan, and other countries (*93–96*) to induce ovulation in amenorrheic women. The use of LH-RH and its analogs can prevent superovulation and the resultant multiple births which are not uncommon after administration of hMG or hCG. In Argentina and England, LH-RH has also been used to treat oligospermia and hypogonadotropic hypogonadism in men (*97, 98*). We participated in many of these studies. Recently, LH-RH given intranasally was successfully used for treatment of cryptoorchidism (*99*). It was determined in collaborative studies carried out in Mexico, Brazil, Japan, England, Spain, and Germany (*100–105*) that single doses of the superactive analogs [D-Ala^6,des-Gly-NH$_2$^{10}]-LH-RH ethylamide, [D-Leu^6, des-Gly-NH$_2$^{10}]-LH-RH ethylamide, [D-Ser(But)^6,des-Gly-NH$_2$^{10}]-LH-RH ethylamide, or [D-Trp^6]-LH-RH can induce protracted stimulation of the release of LH and FSH lasting as long as 24 hours (Fig. 15). Consequently, these analogs should be more convenient and practical to use than LH-RH, which has to be given repeatedly each day (*96*). Moreover, these analogs are active not only after parenteral but also intranasal (Fig. 16), intravaginal, intrarectal, and oral administration if suitable doses are given (*61, 103–105*). No significant untoward side effects of LH-RH and analogs have been observed in humans. However, in spite of some positive results, our current knowledge about the use of these analogs for treatment of female and male infertility is inadequate and the therapeutic regimens are largely empirical. In view of the paradoxical antifertility effects of large doses of LH-RH and long-acting superactive analogs, caution must be exercised in devising clinical protocols. In order to fully exploit the potential of analogs of LH-RH for control of fertility at the level of the brain, we will need further work.

Inhibitory Analogs of LH-RH

The concept of antagonists of LH-RH, proposed by us in 1971 (*106*), was based on the assumption that replacement or deletion of some amino acids in LH-RH might result in analogs possessing features requisite for binding, but lacking those which are necessary for a functional effect. Such analogs would be competitive inhibitors of LH-RH; that is, they would be devoid of LH-RH activity, but by competing for attachment to the receptor site with endogenous LH-RH would lead to a decrease of LH and FSH secretion. From the early in-

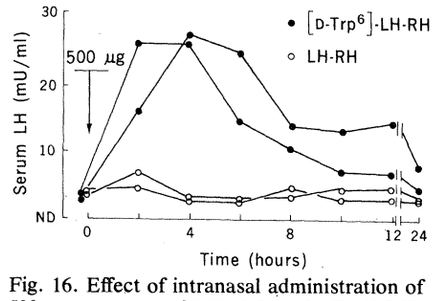


Fig. 16. Effect of intranasal administration of 500-μg amounts of LH-RH or [D-Trp^6]-LH-RH on serum LH concentrations in two men. The compounds were administered in 1 ml of saline by means of a Pasteur pipette. [From collaborative study with G. M. Besser and R. Hall]

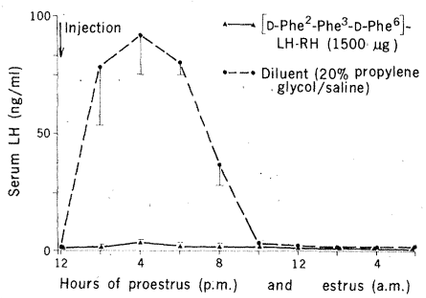


Fig. 17. Effect of a single subcutaneous administration of [D-Phe^2, Phe^3, D-Phe^6]-LH-RH (1.5 mg) on the preovulatory surge of LH in proestrous rats. The differences in LH levels between animals treated with diluent (20 percent propylene glycol in saline) and analog were significant at 1400, 1600, 1800, and 2000 hours ($P < .01$). The LH is expressed as NIH standard O-LH-S$_{17}$. [From (*109*)]

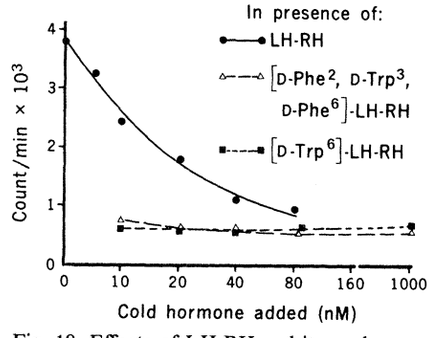


Fig. 18. Effects of LH-RH and its analogs on the binding of ^{125}I-labeled LH-RH by pituitary homogenates. The anterior pituitary was homogenized in Hepes buffer, *p*H 7.2. The homogenate equivalent to one pituitary in 0.5 ml was incubated with 25 μl of a solution (4.5 n*M*) of ^{125}I-labeled LH-RH (300 μCi/μg) for 30 minutes and at 4°C in the presence of LH-RH, [D-Phe^2,D-Trp^3,D-Phe^6]-LH-RH, or [D-Trp^6]-LH-RH. Each point represents the average of triplicate experiments. [From (*112*)]

activation studies on LH-RH (*50–52, 57*), we surmised the importance of His and Trp for the biological activity of LH-RH. However, the analogs based only on deletion of His or Trp were not very effective antagonists. The analog [des-His^2,des-Gly^{10}]-LH-RH ethylamide, made by Coy in our laboratory, was the first LH-RH inhibitor found to be active in vivo (*107*). Incorporation of a D-amino acid in the 6 position, in agreement with original report of Monahan *et al.* (*85*), also improved the inhibitory activity (*78, 79*). It was then determined by Rees *et al.* (*108*) that replacement of His in position 2 by D-Phe created more effective inhibitors than its deletion. Analogs such as [D-Phe^2,D-Phe^6]-LH-RH and [D-Phe^2,D-Leu^6]-LH-RH were synthesized (*78, 79*). The former was found to inhibit LH and FSH release for 6 to 8 hours after injection, and the latter to partially block ovulation in rats in doses of about 6 mg per kilogram of body weight (*78, 79*). [D-Phe^2,Phe^3,D-Phe^6]-LH-RH was a still more potent inhibitor, since given at noon on the proestrous day it suppressed the preovulatory LH (Fig. 17) and FSH surge, and completely blocked ovulation (Table 5) (*109*). The replacement of Trp by D-Trp in position 3 appeared to further increase the potency of inhibitory peptides (*110*). [D-Phe^2,D-Trp^3,D-Phe^6]-LH-RH is both longer-acting (nearly 10 hours in the rat) and more potent than [D-Phe^2,Phe^3,D-Phe^6]-LH-RH. Both of these inhibitors also inhibit ovulation in hamsters and rabbits, and suppress LH release in monkeys (*79, 111*). We have observed that [D-Phe^2,D-Trp^3,D-Phe^6]-LH-RH and the superactive agonist [D-Trp^6]-LH-RH compete with LH-RH for its pituitary plasma membrane receptors, displacing ^{125}I-labeled LH-RH more strongly than its parent hormone (Fig. 18) (*112*). Therefore, both stimulatory and inhibitory analogs of LH-RH may exert their action on the same pituitary plasma membrane receptors as those for LH-RH. Recently, in collaboration with Gonzalez-Barcena, we have determined that [D-Phe^2,D-Trp^3,D-Phe^6]-LH-RH significantly suppressed the release of LH and FSH in response to LH-RH in normal men (Fig. 19) (*113*). We believe that the progress being made in this area may eventually lead to development of new, safer birth control methods.

Growth Hormone-Release Inhibiting Hormone

In 1973, Brazeau *et al.* (*114*) isolated from sheep hypothalami and established the structure of a tetradecapeptide which

Table 5. Suppression of ovulation in rats by [D-Phe^2, Phe^3, D-Phe^6]-LH-RH. The rats had a 4-day estrus cycle and weighed 202.6 + 1.6 grams. Three subcutaneous injections of the diluent or the analog (1 mg per injection) were administered at 1200, 1430, and 1700 hours (C.S.T.). The diluent was 20 percent propylene glycol in saline. [From (*109*)]

Treatment	Number of animals	Number of animals ovulating	Number of ova*	Suppression (%)	*P*
Diluent	6	6	13.3 ± 0.8		
[D-Phe^2,Phe^3,D-Phe^6]-LH-RH	5	0	0.0 ± 0.0	100.0	.001

*Mean ± standard error.

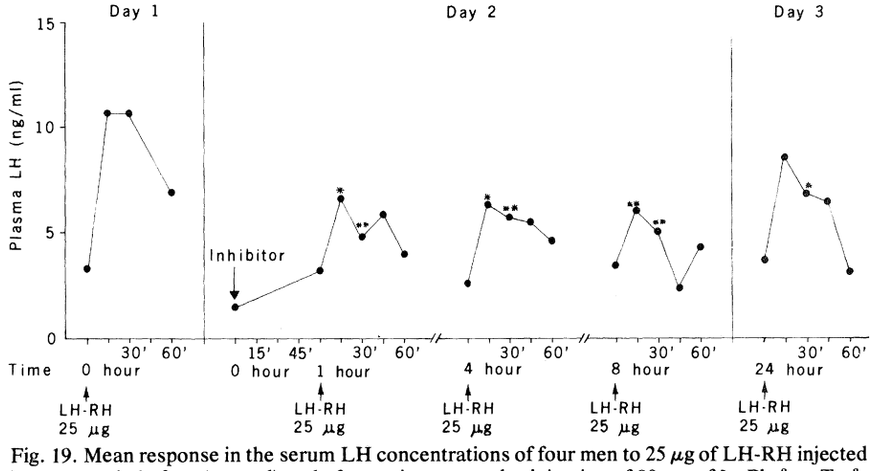


Fig. 19. Mean response in the serum LH concentrations of four men to 25 μg of LH-RH injected intravenously before (control) and after an intramuscular injection of 90 mg of [D-Phe^2, D-Trp^3, D-Phe^6]-LH-RH. Asterisks indicate values significantly ($P < .01$) different from the value at that time during the control period; CEA/IRE Sorin was used as the reference preparation. [From (*113*)]

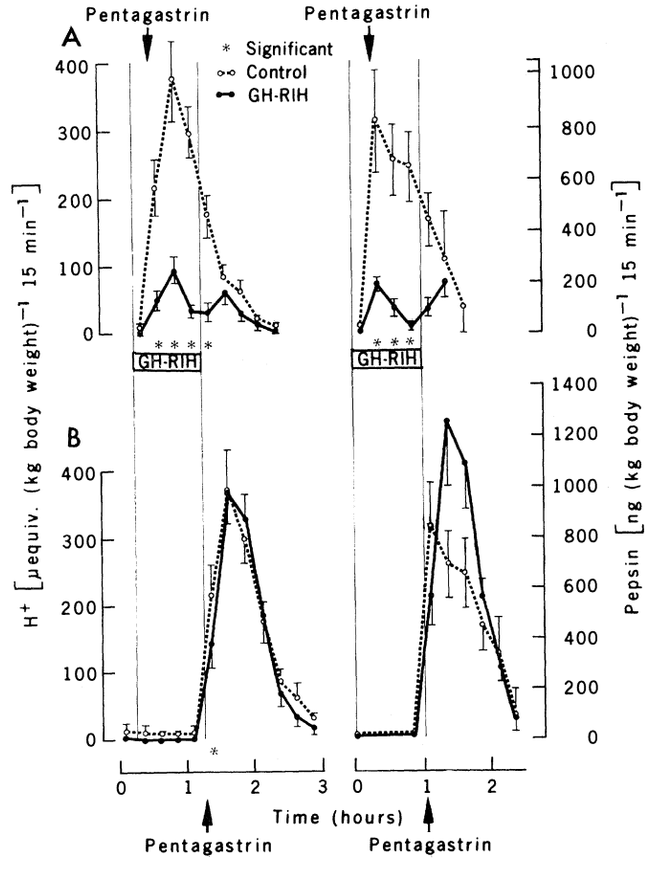


Fig. 20. The mean acid and pepsin responses to pentagastrin (6 μg/kg, subcutaneously) of cats with gastric fistulae in control experiments (○) compared in (A) with those when pentagastrin was injected during the infusion of GH-RIH (10 μg per kilogram per hour) (●) and in (B) with those when the injection of pentagastrin was preceded by the infusion of GH-RIH (○). Responses are expressed as the mean ± standard error. Asterisk indicates significant difference between the means at least at the 5 percent level. [From (*127*)]

they named somatostatin, or growth hormone-release inhibiting hormone (GH-RIH), which inhibited the release of GH in vitro and in vivo in rats. The presence of somatostatin in the hypothalamus was first observed by Krulich *et al.* (*115*). Somatostatin was synthesized by several groups, including ours (*116, 117*). Subsequently, we isolated and determined the structure of porcine somatostatin, showed the primary structures of native porcine and ovine somatostatin to be identical (*118*), and thus confirmed the existence of this peptide (*114*) in another species. We also found larger and more basic forms of somatostatin in pig hypothalami (*118*). These materials are biologically and immunologically active, possess different physicochemical properties from somatostatin, appear to have several amino acids including arginine attached to the NH_2-terminus, and may represent precursors of somatostatin. We have also found high concentrations of somatostatin in extracts of pancreas, stomach, and duodenum of the rat, as well as two types of immunoreactive somatostatin (*119*). In agreement with parallel studies by others, we found with Hall *et al.* (*92, 120*) and Besser *et al.* (*121*) that somatostatin inhibits the secretion of pituitary GH and TSH in human beings.

A physiological role of somatostatin in the regulation of GH and TSH secretion is supported by our observations with Arimura that passive immunization with antiserum to somatostatin increases basal GH concentrations, prevents the stress-induced decrease of GH in rats (*122*), and increases the TSH response to TRH (*123*). In collaborative clinical studies in England, also with Hall and Besser, parallel to those of others, we then determined that somatostatin suppresses the secretion of glucagon and insulin in humans (*124*). In joint investigations with Konturek we later established that somatostatin affects the exocrine pancreas as well, since it reduced the secretin-induced secretion of pancreatic fluid and bicarbonate (*125*). With Bloom *et al.* (*126*), Gomez-Pan *et al.* (*127*), and Konturek *et al.* (*128*) we made the original observations that somatostatin decreases the circulating levels of gastrin in men and dogs, and that it also exerts a direct antisecretory effect on gastric parietal and peptic cells, since it inhibits pentagastrin-induced gastric acid and pepsin secretion in cats (Fig. 20) and dogs. These studies established for the first time that this hormone can exert exocrine, as well as endocrine, effects. In our work with Konturek *et al.* (*125*), it was also determined that soma-

tostatin inhibited the release of secretin and cholecystokinin/pancreozymin from the duodenal mucosa.

Arimura in our laboratory was the first to generate antiserums to somatostatin and to establish an RIA for this hormone (*129*). These antiserums were used by us and Hökfelt *et al.* (*130*) for the localization of immunoreactive somatostatin in the brain, including hypothalamus, D cells of pancreas, the gastrointestinal mucosa, and other tissues by immunocytochemical methods. These studies support the view that somatostatin plays a role in the regulation not only of the pituitary but also of the pancreas, duodenum, and stomach.

Somatostatin itself is of little therapeutic value because it has multiple actions and a short biological half-life. Attempts are therefore being continued by us and others to produce analogs of somatostatin with prolonged activity and the ability to inhibit the release of some or only one hormone. We showed that [D-Ala^2,D-Trp^8]-somatostatin (*131*) has a potency 20 times greater than somatostatin on inhibition of GH release, but is only three times as potent in inhibiting pentagastrin-induced gastric acid secretion (*132*). Meyers *et al.* (*133*) in our laboratory and others (*134*) synthesized [D-Cys^{14}]-somatostatin and [D-Trp^8,D-Cys^{14}]-somatostatin which selectively inhibited GH and glucagon release more than insulin secretion. [D-Trp^8,D-Cys^{14}]-somatostatin has a ratio of 22 : 1 for the selective inhibition of glucagon over insulin, 100 : 1 for that of GH over insulin (*133*), and 3 : 1 for that of GH over gastric acid. Since potent analogs of somatostatin with selective activities can be prepared, and since promising results with long-acting analogs have already been realized in our laboratory, it is possible that future analogs may be useful in the treatment of such disorders as acromegaly, diabetic retinopathy, juvenile diabetes, peptic ulcers, and other diseases.

Conclusions and Perspectives

At the inception of my scientific career, the concept of hypothalamic control of anterior pituitary function was in its formative stage. It was my good fortune to have arrived on the scene at such a crucial time and to have helped place it on the solid foundation on which it now rests. At present, the validity of this concept stands proven by the isolation, structural identification, and synthesis of three hypothalamic regulatory hormones. The presence of at least six other hypothalamic hormones which stimulate or inhibit the release of pituitary hormones from the pituitary gland is now reasonably well established. It is likely that additional hypothalamic hormones will be found. Many clinical applications are now well established and more will come. The information gathered from both animal and human studies with natural and synthetic TRH, LH-RH, and somatostatin has provided us with new understanding and even more important, I believe, has opened vast new vistas for probing ever more deeply into these marvelously integrated systems of which the animate world is composed.

In any case, I hope that my work will be of practical use to humanity, and that I will be able to make new contributions in this field in the years to come.

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