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IN THE CENTRAL ROLE OF GnRH IN THE REGULATION OF REPRODUCTIVE FUNCTIONS: MECHANISM OF ACTION AND PRACTICAL RESULTS

GYORGY KERI, ANIKO HORVATH, KAROLY NIKOLICS and ISTVAN TEPLAN
1st Institute of Biochemistry, Semmelweis University, Medical School, Budapest, Hungary

Summary—The regulation of reproductive functions is influenced by several hormones. The hypothalamic hormone GnRH has a central role in reproduction. Several superactive and inhibitory analogs of GnRH synthesized in our laboratory showed use in animal breeding.

GnRH was suggested to be degraded by the hypothalamus itself and also by the pituitary. We have used cultured fetal hypothalamic cells, and cultured pituitary cells to study the storage, the release and the action of this neuropeptide. It was found that neither the cultured hypothalamic nor the pituitary cells degrade GnRH, and pretreatment of these cultured cells with steroids do not induce GnRH degradation. The basal GnRH secretion from the cultured hypothalamic cells was increased by steroid pretreatment while the neurotransmitter induced GnRH release did not change. At the pituitary level it was found that GnRH regulates its own effect by priming or desensitizing gonadotropin release depending on the pretreatment time or the concentration of GnRH. The self-regulatory action of GnRH follows a similar pattern for both LH and FSH release.

INTRODUCTION

The regulation of reproductive functions is a very complicated system which is influenced by many hormones, factors and unknown parameters along the hypothalamo–pituitary–gonadal axis. The gonadotropin releasing hormone (GnRH) has a central role in this regulatory system. The tonic and phasic release of GnRH from the hypothalamus determine the basic pattern of reproductive functions.

Several seemingly contradictory results accumulated in the literature show that the steroids exert both stimulatory and inhibitory action on GnRH release. On the other hand GnRH regulates its own effect of gonadotropin release. The explanation of these results can probably be found in the timing of the reproductive functions.

SUPERACTIVE GnRH ANALOGUES

The isolation and structure determination of GnRH opened the possibilities of its use for the regulation of reproductive processes. Investigations directed toward the development of highly potent and long-acting GnRH analogues were initiated. These substances were expected to be effective in the treatment of human infertility and in animal breeding. Japanese and U.S. groups succeeded in synthesizing analogues of GnRH that were extremely active in releasing LH and FSH. These were about 50–100 times more active than the natural decapeptide and released gonadotropins during 4–6 h following their injection compared to the 30–60 min effect of GnRH itself.

Substitution of Gly^6 by bulky, apolar, aromatic D-amino acids is the basic change for obtaining superactive long-acting GnRH-peptides. The substitution of Gly^{10} by alkylamides further enhances the potency of such compounds. The most potent GnRH superagonists of today have approx 150–200-fold activity compared to GnRH.

VETERINARY APPLICATIONS OF GnRH SUPERAGONISTS

The GnRH superagonists prepared in our laboratory were used in several species, however, most experiments were carried out in cattle. Therefore, in briefly summarizing the results, we shall concentrate on this field. Table 1 shows data obtained in about 15–20 different farms and inseminating stations with a considerable number of cows.

Different types of applications were tried in the cows. Most important, however, is the treatment of cows with the GnRH-analogue before insemination for inducing a higher rate of ovulation and consequently, breeding efficiency. As can be seen from the table, this treatment led to increase (13%) in conception rate in very large number of animals. GnRH superagonists appear to serve important functions in veterinary medicine for treatment of reproductive disorders. Most important of these, today, is the induction of ovulation in female animals.

Table 1. Veterinary application of D-Phe^6-GnRH-NHEt (Ovurelin) in a representative series of experiments for the treatment of reproductive disorders in cattle

Expt	Application	Conception/Total	%	Control %
1.	Ovarian cysts	53/74	71	—
2.	Anoestrus	39/56	70	—
3.	"Repeat breeder" syndrome	48/80	60	—
4.	Involution	31/42	78	63
5.	Treatment for inducing ovulation before insemination	351/730	48	35
	Total	522/982	53	

Control values in experiments 1–3 are 0% as pretreatment value.
Dose of GnRH analog-50–100 μg/animal i.m.

INHIBITORY GnRH ANALOGUES

The deletion of Histidine^2 from the decapeptide resulted in a completely inactive compound. This, although in high concentration, proved to be a competitive inhibitor. During the past decade nearly 1000 inhibitory analogues of GnRH have been synthesized. Substitutions in positions 1,2,3,6 and 10 of the decapeptide cause inhibitory activity. The best inhibitory compounds contain 4 or 5 D-amino acids as substitutions and therefore they are still too expensive for general contraception, even in low doses. It was demonstrated that GnRH antagonists cause rapid decrease in available GnRH binding sites in the pituitary [1]. Thus, GnRH antagonists, beside being potential contraceptives, may provide a tool to study the GnRH-gonadotropin relationship.

Our laboratory has also participated in the development of new GnRH antagonists. In collaboration with Tulane University, New Orleans and the Department of Anatomy of the Medical School in Pecs, we designed a number of new peptides and tested them for antiovulatory activity. Table 2 gives the most important structural changes made by us. The last two compounds are very important milestones in the development of GnRH antagonists. D-Lys^6 containing peptide is the first which causes low-dose long-term inhibition of ovulation, while the D-Arg^6 containing peptide is the first orally active GnRH-antagonist. Although, at present, these compounds are too expensive for wide use, the remarkable development in the synthesis of these peptides is very promising for the future.

STUDIES ON GnRH METABOLISM

GnRH secreting cultured fetal hypothalamic cells do not degrade GnRH

It was suggested by several authors that GnRH is degraded by the hypothalamus itself and also by the pituitary in order to regulate the storage and release [2]. GnRH is predominantly synthesized in the medial preoptic area of the rat brain but because of axonal transport it is largely accumulated in the nerve terminals of the median eminence, where it is secreted into the portal circulation and binds with specific receptors of the anterior pituitary. Several observations suggested that GnRH is degraded by specific peptidases of the pituitary gland, thus regulating the levels of the hormone. Pituitary homogenates were found to have a high capacity for degradation of GnRH. GnRH superagonists were found to be resistant to degradation by homogenized pituitary tissue. The finding that pituitary plasma membranes degraded GnRH but not resistant derivatives of the peptide, raised the possibility that membrane-bound specific peptidases would inactivate the hormone.

We have investigated the possible degradation of GnRH by intact pituitary tissue as described in detail elsewhere [2]. Trypsin and collagenase treated pituitary cells dispersed mechanically, and fresh pituitaries cut into eight segments were incubated with

Table 2. Development of GnRH antagonists

	Compound/amino acid number										Dose (mg) for 100% inhibition of ovulation
	1	2	3	4	5	6	7	8	9	10	
1975:		D-Phe	Phe			D-Phe					3.0
1976:		D-Phe	D-Trp			D-Phe					1.0
1977:		D-Phe	D-Trp			D-Lys					0.25
						isophtaloyl dimer					
		D-Phe	D-Trp			D-Lys					
1978:	D-Glp	D-Cpa	D-Trp			D-Phe					0.12
1979:	Ac-D-Cpa	D-Cpa	D-Trp			D-Phe					0.05
1980:	Ac-D-Cpa	D-Cpa	D-Trp			D-Phe				D-Ala	0.01
1981:	Ac-D-Trp	D-Cpa	D-Trp			D-Lys				D-Ala	0.002†
1982:	Ac-D-Nal/2/	D-Cpa	D-Trp			D-Arg				D-Ala	0.001‡

Abbreviations: D-Cpa is D-4-chloro-phenylalanine; D-Nal(2) is D-naphtyl-2-alanine. Principal substitutions are underlined.
*Subcutaneous dose/rat on proestrous day.
†Also causes inhibition of ovulation after 14 days at a dose of 5 μg/rat.
‡Also effective orally, 2 mg/rat dose leads to 100% inhibition of ovulation.

tritiated GnRH or unlabelled GnRH. Degradation of GnRH could not be detected by either method during incubations with any of the pituitary cell cultures or fresh tissue. The results suggest that pituitary degradation is not involved in the regulation of circulating levels of GnRH.

GnRH beside stimulating the release of LH and FSH from the pituitary can also promote sexual behaviour by direct action on the hypothalamus and mesencephalic central gray and has been suggested to serve as a neuromodulator facilitating sexual behaviour in these regions [3]. GnRH is localized predominantly in nerve-endings and it has been hypothetized to act as neuromodulator or neurotransmitter. It has also been reported that GnRH is present in extrahypothalamic brain-loci to influence the firing rate of neurons variously situated in the brain [4]. In addition to being present in nerve-terminals and therefore available to other neurons by normal synaptic mechanisms, GnRH axons may also have access to both CSF and plasma and the peptide may be distributed within the brain by these routes as well. Due to this the regulation of extracellular GnRH level by degradation of the hormone may have a very important physiological mechanism. It has been suggested that peptidases may be important regulators of the extracellular level of GnRH in brain tissue *in vivo* [5]. It was demonstrated by several authors, that GnRH is rapidly degraded by peptidases present in both soluble and particulate fractions of the rat brain including the hypothalamus [6].

Changes in the activity of GnRH degrading enzyme(s) with castration and gonadal steroid injection and during the estrous cycle suggested that GnRH degrading enzymes may have a functional significance in controlling the amount of GnRH available for release from the hypothalamus. It was also suggested that peptidases released into the medium under *in vitro* incubations of the rat medial basal hypothalamus (MBH) may be important regulators of the extracellular levels of GnRH in brain tissue *in vivo* [6]. We have investigated the possible degradation of GnRH by the hypothalamus using cultured fetal rat hypothalamic cells.

Fetal hypothalamic cells were kept in culture for more than 2 weeks and GnRH released into the medium was measured by highly sensitive RIA from the third day. GnRH secretion started on the sixth day and the amount of GnRH released into the medium was about the same during the following days (200 pg/10^6 cells/24 h). The experiments were carried out on the 10th day of culture, because according to the morphological picture and the metabolic enzyme activities the cells became fully active during days 8–9.

In order to measure the specific function of the cells we investigated whether the GnRH released from these cells can be stimulated with neurotransmitters and other agents which may have a function in the regulation of GnRH release. Figure 1 shows

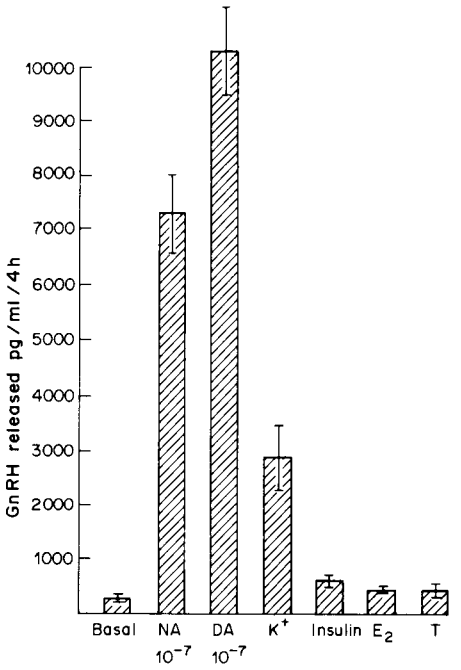

Fig. 1. Effect of various agents on GnRH release by fetal hypothalamic cells. Data are the mean values of triplicate cultures ± SEM. NA—noradrenaline, DA—dopamine, E_2—estradiol, T—testosterone.

the effects of noradrenaline (NA), dopamine (DA), insulin, estradiol (E_2) and testosterone (T) on GnRH release. The effect of high concentration of potassium (59 mM), which has a depolarizing effect on the plasma membrane, was also measured. We found that both neurotransmitters stimulated GnRH release from these cultured cells: K^+ also stimulated GnRH release although to a smaller extent. Insulin and the steroids had no effect on basal GnRH release.

In order to investigate whether the GnRH secreting fetal hypothalamic cells degrade GnRH or not we incubated the cells for various time periods with specifically tritium labelled GnRH and aliquots of the medium were analyzed by HPLC, as shown in Fig. 2. These intact hypothalamic cells do not degrade

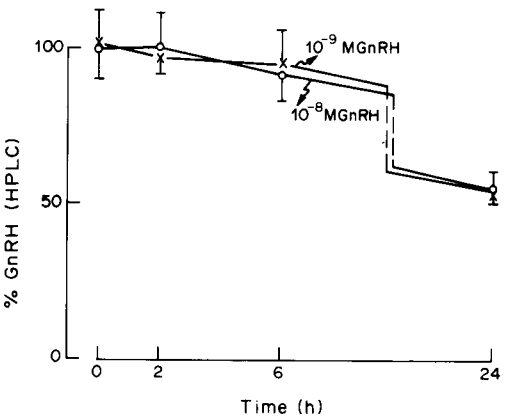

Fig. 2. Exogenous GnRH metabolism in fetal hypothalamic cell culture. Results are expressed as percent of controls incubated without cells under identical conditions.

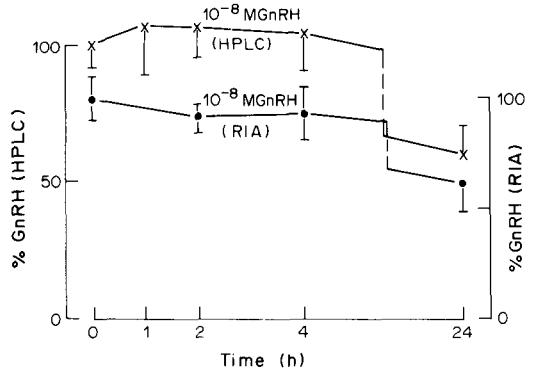

Fig. 3. GnRH (×——×) or [^{3}H]GnRH (●——●) content in mechanically dispersed fetal hypothalamic cell culture. Results are expressed as percent of controls incubated under identical conditions without cells.

GnRH during the first 4 h of incubation, but about 50% degradation occurs after 24 h of incubation. The rate of degradation did not change by increasing GnRH concentration in the medium.

To test whether a membrane bound peptidase was removed by enzymatic dispersion of the cells we measured GnRH degradation in a cell culture prepared by mechanical dispersion of the fetal hypothalamic cells. Figure 3 shows that these cells did not degrade exogenous GnRH during the first 4 h of incubation but about 40% degradation occurred after 24 h of incubation.

We also measured the basal, stimulated and exogenous GnRH levels by RIA and found that basal GnRH level did not change during 24 h of incubation while the stimulated and the exogenously given GnRH level decreased to about 50% after 24 h of incubation.

For comparison we measured GnRH degradation in a synaptosomal preparation and in a hypothalamus (HT) homogenate and we found that in both preparations GnRH was rapidly degraded. In our HPLC system the GnRH peak rapidly decreased while a peak close to the front (polar, small molecular weight fragments) increased simultaneously. On the other hand the intact cultured fetal hypothalamic cells prepared either by enzymatic or mechanical dispersion did not degrade GnRH during 4 h of incubation. If GnRH was incubated for 24 h with these cells about 50% degradation could be detected. However, this may not be a physiological mechanism for regulating GnRH level in the extracellular medium. We conclude that GnRH once secreted by intact hypothalamic cells is not degraded within the hypothalamus.

Pituitary desensitization by GnRH superagonists

Chronic administration of GnRH superagonists lowers the secretion of luteinizing hormone and follicle stimulating hormone [7]. In consequence, the level of gonadal steroids is also lowered. In addition it has been demonstrated by several authors that

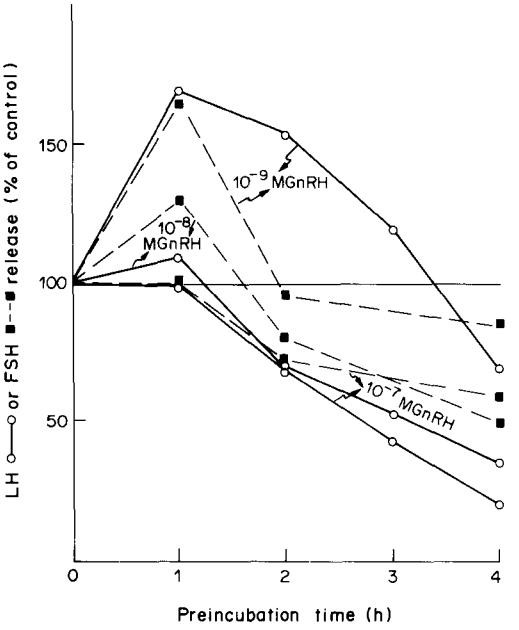

Fig. 4. Effect of preincubation time on subsequent GnRH induced LH and FSH release. Reincubation was performed with the same concentration of GnRH as used for preincubation for 1 h.

intermittent exposure of the pituitary to a submaximal dose of GnRH either *in vivo* or *in vitro* enhances gonadotropin release in response to subsequent GnRH stimulation [8]. We demonstrated that preincubating the cells for a shorter time-period with submaximal dose of GnRH caused a significant increase in subsequent GnRH induced LH release. This priming effect was decreased by increasing the concentration of GnRH during preincubation [9]. Preincubating the cells for a shorter time-period with a submaximal dose of GnRH (1 h) caused significant sensitization of both LH and FSH. The kinetic analysis of GnRH-induced priming and desensitization for LH and FSH release showed no significant differences (Fig. 4).

These results show that GnRH regulation of the sensitivity of pituitary gonadotrophs to a subsequent GnRH stimulus is biphasic, depending on the preincubation time and concentration of GnRH.

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