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The Effect of Ovarian Hormones on LH-RH Release in vitro from the Hypothalamus of Immature Female Rats

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I. INTRODUCTION
At puberty, many important changes occur in an animal, most significant of these being the differentiation of the sex organs, and the capacity of the gonads to release sperm and ova, so that fertilization is made possible.

It has been shown that the pituitary hormones, LH and FSH, are important in this respect. These gonadotropins are regulated by circulating estrogens and androgens in the female and male, respectively, as well as by the hypothalamic releasing factor, luteinizing hormone-releasing factor (LH-RH). Part of this regulation is effected by a negative feedback action of the estrogens and androgens on the hypothalamic-pituitary unit, an action which has been demonstrated to be well developed well before puberty, in a number of animals—rat, ewe, and bovine, among others.

At puberty, changes in the LH and FSH levels occur. Plasma level determinations in the male and female rat show initially high levels of LH, but a decline to low levels some fifteen days before puberty. At puberty, a drastic rise in LH levels is noted. For FSH, the results are less clearcut. In the male rat, rising levels of FSH are noted at about thirty days. In the female rat, FSH levels rise to a peak at fifteen days of age, and then fall to lower levels by about day thirty (1,2). A study has also shown that LH-LH levels increase sharply, shortly before puberty, and drop soon after vaginal opening, in
the female rat (7).

One of the hypotheses that has been suggested to explain these elevated gonadotropin levels at puberty, is that a change in sensitivity of the negative feedback system occurs (2). The term, "gonadostat" has been proposed to designate the variability of this feedback sensitivity. According to this theory, at puberty, the sensitivity of the hypothalamic-pituitary system is decreased, so that the circulating estrogen or androgen levels can no longer suppress LH and FSH levels, allowing the gonadotropins to secrete, and cause follicular development or spermatogenesis.

One of the major countering hypotheses to this gonadostat hypothesis suggests that in the female rat, a positive feedback action of estrogen on the pituitary is activated. In this line of reasoning, estrogen levels are elevated due to an increased sensitivity of the ovaries to the gonadotropins, allowing for the estrogen to cause a surge in LH and FSH levels. Recent evidence shows that serum estrogen levels do increase shortly preceding the first preovulatory surge of gonadotropins. A concomitant increase in progesterone is similarly found, and it has been suggested that progesterone plays a role as a modifier in facilitating the estrogen positive feedback action (3).

Currently, this latter hypothesis is in more favor, since some studies indicate that pituitary responsiveness
to LH-RH decreases prior to puberty and that gonadotropin levels actually decrease shortly before puberty.

However, the mechanism through which these feedback actions change at puberty remains unknown, and the eventual finding might include a combination of these two hypotheses. As a result, other possible hypotheses still exist to account for the change in gonadotropin levels at puberty. Among these include changes in metabolism or metabolic clearance rate of gonadal steroids with age, or the possibility of other steroid involvements, such as dihydrotestosterone (DHT), in the case of the male rat.

Part A of this study will explore the effect of age on the LH-RH release and concentration levels in the hypothalami of prepubertal female rats. To this end, an in vitro perfusion system of hypothalamic tissue will be employed to examine the maturation of the LH-RH secretory mechanism.

Part B of this study is an extension of Part A, probing into the possible factors involved in the control of LH-RH release and concentration levels, and particularly looking at any circadian pattern in the LH-RH release or plasma LH levels of the 30-day-old immature female rat. Little study has been done with the immature rat in this area, although some related questions have been asked with regard to the adult female rat.
It has been reported by a number of investigators that in the adult rat, there is a daily neural signal for LH release (4). Furthermore, through the study of ovariectomized female rats, it has also been reported that estrogen can induce these surges (4, 6, 7, 15). This action of estrogen has been suggested to occur through two means: 1) by the stimulation of LH-RH release (8), or 2) by the modulation of the pituitary responsiveness to LH-RH to a more sensitive level (6, 7). Legen et al. (6) reported that pituitary responsiveness to LH-RH is increased at proestrus, in the female rat, treated with sodium pentobarbital, but that this change in the responsiveness of the pituitary is abolished with the ovariectomy of the animal, and cannot be restored by estrogen treatment. Baldwin et al. (7) confirms these results with sodium pentobarbital, but also finds that estradiol benzoate enhances the responsiveness of the pituitary to LH-RH in animals not previously treated with sodium pentobarbital.

More recently, Sarkar and Fink (8) have studied the levels of LH-RH measured in pituitary stalk blood in ovariectomized, estradiol benzoate or progesterone treated adult female rats. They find that the afternoon LH-RH concentrations are reduced in the animals which were ovariectomized and the animals which were ovariectomized and treated with progesterone, but not reduced in the ovariectomized and es-
trogen treated groups. All of the ovariectomies and treatments were done the day before sampling. They suggest that progesterone plays an inhibitory effect on the LH-RH concentration levels. However, the apparent dose-dependent action of progesterone, as well as other reports that progesterone can facilitate the release of LH, under certain light and hormone conditions (8,9,10), leaves the full understanding of the action of progesterone still a perplexing problem.

Other reports that the light schedule has a major effect on the estrogen induced LH surge (11,12) make it increasingly intriguing to examine, as this study proposes to do, how these mechanisms of pituitary responsiveness and increased LH-RH release might manifest themselves in the developing female rat.
II. MATERIALS AND METHODS
A. Iodination of the Hormone.

The use of the radioimmunoassay requires an iodinated tracer, i.e. the iodination of the hormone in question. For LH-RH, then, it is necessary to iodinate the decapeptide:

\[
p\text{Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH}_2.\]

The existence of the tyrosine group makes it possible to iodinate easily. The following reaction takes place:

\[
\begin{align*}
\text{HO-} & \quad \text{Na}^{125\text{I}} \quad \text{HO} \\
\text{CH}_2\text{CHO} & \quad \text{Chloramine T} \\
\text{NH} & \\
\end{align*}
\]

This method, introduced by Greenwood (13) requires the reaction of the oxidizing agent, Chloramine T (10 \(\mu\)l of a 2 mg/ml 0.05M Phosphate Buffer), radioactive sodium iodide (1mCi/10 \(\mu\)l), and the peptide in question (2.5 \(\mu\)g in 10 \(\mu\)l H\(_2\)O). The reaction is then terminated by the immediate addition of sodium metabisulfite (20 \(\mu\)l of 4 mg/ml 0.05M Phosphate Buffer), a reducing agent. The mechanism for the labelling reaction requires the generation of an active iodine molecule in the form of H\(_2\)O\(^{125\text{I}}\). To do this, the Na\(^{125\text{I}}\) is oxidised to NaCl, with the concomitant reduction of Chloramine T. An electrophilic attack on the active iodine molecule, by the carbon, ortho to the hydroxyl
group on the tyrosine side chain, results in the labelled tyrosine side chain, and a hydronium ion.

The separation procedure is carried out in a Sephadex-G-25 fine column of about 10 cm$^3$ with a one cm diameter, and a 10 cm length. In a Sephadex column, molecules enter the polysaccharide derived beads to different degrees depending on their size. Larger molecules tend to be unable to penetrate the beads, and are therefore excluded, passing more rapidly through the column. This differential penetration allows for the desired separation. In the iodination of LH-RH, 0.5ml of 1% Albumin is added to the column, and 0.01M Acetic Acid is used for the elution at a rate of eight drops/min. or 0.5ml/min. Following the reaction, free iodine, damaged and undamaged labelled hormone, unlabelled hormone, and salts from the reactants are found in the eluting mixture. The pattern of the eluted one ml fractions is found in figure 1. The first low peak is probably due predominantly to damaged iodinated peptides, and the second is a result of the free iodine still remaining after the reaction, and perhaps some more damaged peptides. These products tend to elute first due to their now expanded size, in contrast to the more compact iodinated LH-RH. The remaining three peaks all predominantly contain the iodinated LH-RH.

However, the peaks may contain other impurities, and
therefore, a few fractions from the best peak, (usually showing the best binding ability) are pooled together and repurified. The elution pattern of the repurified fractions is shown in figure 2. These fractions can then be tested by a simple radioimmunoassay for binding, by comparing the values for total binding, nonspecific binding, and the total number of counts for the given aliquot of radioactive tracer. Table 1 shows some of the binding capabilities for a selected group of fractions from the first reactions and the pooled repurification. From the repurified fractions then, the best binding fractions are pooled and frozen to be used for future RIA.

B. Radioimmunoassay.

The radioimmunoassay, introduced by Yalow and Berson (14) in their study of detecting insulin levels, has since been used widely for a number of hormones. The principles behind the assay follow the general principles of antibody-antigen binding assays. Labelled hormone (e.g. ¹²⁵I-LH-RH) of a given aliquot is mixed with the corresponding antibody-hormone antibody (e.g. anti-LH-RH) of a given aliquot, and an unknown amount of unlabelled hormone. An antibody-hormone complex results in which the labelled and unlabelled hormone will compete to form the complex. The more unlabelled hormone that is present, the greater the chance
for the labelled complex to be displaced by an unlabelled complex. The complex is then precipitated and its radioactivity measured. Since the amount of radioactivity is inversely proportional to the amount of unlabelled hormone present, a standard curve of known unlabelled hormone quantities allows the amount of hormone in the unknown samples to be determined.

A representative standard curve is shown in figure 3. A calculation of the nonspecific reaction must also be made, and subtracted from the standard curve values for accurate determination. Finally, the total possible binding must also be calculated for a determination of the limiting sensitivity of the assay.

0. Perifusion System.

Perifusion systems have been used extensively to study the synthesis and release of hormones from the pituitary, and other endocrine glands. Recently, a perifusion system to study the release of hypothalamic hormones was introduced by Gallardo and Ra¡rez (15) and has the advantage of allowing for the study of dynamic hormone release.

Generally, the tissue in question (in this case, medial basal hypothalami) is placed in a chamber which is incubated in a 37°C water bath (fig. 4). The tissue is placed in the chamber such that it is sandwiched between a few fi-
bers of glass wool to assure a limited amount of movement. The chamber is enclosed at one end by a rubber stopper with three holes. Each of these openings serve one of three functions: as an injection port, for the oxygenation of the system, and as an outlet for the sampled medium to be collected. At the other end of the chamber, one opening allows the pumped Krebs-Ringer Phosphate solution to enter the system with the incubated tissue.

This in vitro perifusion system has been shown to accurately delineate the dynamic secretion patterns of LH-RH and other hormones, and therefore, provides a powerful method to study and manipulate hormone release from brain tissue.

D. Statistics

The peak responses of KCl to preinjection levels were analyzed by a paired t-test. All statistical analysis across groups (i.e., different age groups in Part A, and the different intact and OVX groups in part B) use an analysis of variance followed by a least significant difference examination between pairs of groups, when the F values in the ANOVA were found to be significant. The analysis of the possible significant differences between each group's morning and afternoon values was studied by using either
the one-tailed or two-tailed t-test as indicated in the results.
III. PROCEDURE AND RESULTS
Part A
Procedure I.

To study the developing of LH-RH secretion in vitro, immature female rats (Moltzmann) ranging from 21 to 32 days of age at the time of sacrifice, with free access to food and water, were used. The animals were sacrificed by decapitation and the medial basal hypothalam (MBH) were surgically dissected. The MBH from each group of six animals were then halved and these twelve halves were placed in the perfusion system described above. After a half hour preincubation period, the samples of perfusate were collected at four minute intervals for 72 to 116 minutes, with approximately one ml of perfusate in each collection. At the end of the perfusion, 50 μl of 600 mM KCl was injected into the chamber, to test for tissue viability, and samples were collected for an additional 24 minutes. The samples were then acidified to a 0.1N HCl concentration, and frozen until the time of the assay.

For the assay, these samples were neutralized to a pH of 6.7-7.4 with 2N NaOH and 0.5X Phosphate Buffer, and then centrifuged at 5000 rpm for twenty minutes. Finally, 300 μl duplicate samples were tested for LH-RH levels with the radioimmunoassay.
Procedure-II.

After the perifusion, the LH-RH concentration in the KBH tissue is determined. To this end, the KBH fragments were removed from the perifusion chamber, weighed, homogenized in 0.1ml of 0.1N HCl, and finally completed to a total 1 ml volume with additional 0.1N HCl. The sample is then frozen until the assay, at which time it is centrifuged at 4000 rpm for one half hour. Five duplicate radioimmunoassay determinations were used, and the final concentration is expressed in pg/mg.
Results I.

Figure 5 shows a representative graph demonstrating the pattern of LH-RH release from MBH tissue, and its response to KCl. The injection of KCl causes depolarization of the neurons by upsetting the ionic concentrations of the environment. The resulting stimulations of hormone levels is dramatically shown here. In all the perifusions performed, KCl was able to cause a one and a half to ten times increase in LH-RH release levels, when values are compared to levels directly preceding the injection.

Figure 6 shows the combined data of all the perifusions. In each of the cases, the mean peak KCl response is significantly higher than the mean LH-RH levels (p<0.05 using the paired t-test).

Figure 7 shows representative graphs of the LH-RH release from a single perifusion from rats at 21, 25, 30, and 32 days of age. Along with figure 6, the results seem to show that the LH-RH levels rise in the immature female rat from 21 to 30 days of age, after which the levels drop again to low levels. However, the actual differences between the release levels are not found to be significant by analysis of variance at the 0.05 level.

Figure 7 also seems to suggest some interesting questions regarding pulsatile secretion. By defining a pulse
as two times the preceding or following value, the incidence of pulses also seems to increase to the critical day of 30 days. However, problems regarding the definition, varying lengths of the perifusion, and the amount of time per collection, make it difficult to form more detailed conclusions concerning pulsatile secretion at this time.

Results-II.

The results from the LH-RH concentration determinations in the MBH of developing female rats is shown in Table 2. Both the 35 and 38 day old rats were sacrificed after vaginal opening, generally regarded in most studies as the onset of puberty. The apparent trend, therefore, indicates that there is a gradual increase in LH-RH concentrations up to the point of puberty, after which levels drop again to earlier concentration levels. However, as with the LH-RH release rates, the differences are not found to be significantly different from an analysis of variance.
Part II

Procedure-I.

In this second set of experiments, the question of the effect of ovarian hormones on the in vitro LH-RH release rates and hypothalamic concentration levels of this decapeptide was further probed. Immature Holtzman female rats of 30 days at the time of sacrifice, with free access to food and water, were used. The earlier experiments in Part A indicated that by using thirty-day-old rats, we might be working with a critical turning point in the rat's development. Also, at thirty days, we would be working with generally higher LH-RH release rates (see earlier Fig. 6), and could therefore assay release rate levels of LH-RH more accurately.

Four groups of animals were used in this experimental design, and the four groups were identified by the terms, Intact, OVX, OVX+E, and OBX+E+Prog.

Intact: The first group consisted of intact animals, sacrificed at thirty days of age by decapitation. The medial basal hypothalamic suprachiasmatic preoptic (MBH-sch-POA) region which was studied, consisted of a piece of tissue dissected out from the brain by cutting rostral to the chiasma (after removal of the optic nerves), laterally at the level of the hypothalamic sulci, caudally at the level of the mamillary body, and dorsally at a level one to two mm from the surface, with fine surgical scissors. The whole
piece was then lifted and placed into cold medium. Later, each piece was divided into two halves with a sharp razor blade at the level of the third ventricle, and placed into the perfusion chamber and studied, as described in both the Materials and Methods, and in Part A. The samples of perfusate were, however, collected at ten minute intervals (1 ml volume/collection) in this experiment. After 140 minutes, 50 μl of 600mM KCl was injected to test for tissue responsiveness, and samples were collected for an additional forty minutes after the injection. Each perfusion contained the equivalent of six hypothalami.

OVX: The second group of animals were ovariectomized at 28 days of age, and then sacrificed at thirty days. The MBH-som-FOA fragments were perfused in the same way as the intact animals.

OVX+E₂: The third group of animals were similarly ovariectomized at 28 days of age. At the time of the ovariectomy, however, a 235μg estradiol benzoate capsule, which had been previously incubated for 24 hours, was also implanted in the back of the neck of each rat. The MBH-som-FOA from the animals were dissected out, and studied two days following ovariectomy, using the same procedure as for the intact and OVX groups.

OVX+E₂+Prog.: The fourth group of animals were also ovariectomized at 28 days of age and implanted with the
estradiol benzoate capsules (235μg). Four to six hours before the time of sacrifice at thirty days of age, however, 1 mg progesterone was given to the animals by injection. This time interval has been estimated as the approximate length of time it takes for progesterone to activate the release of LH in OVX+E₂ animals. The MBH-sch-POA from these animals were similarly studied for LH-RH release as with the above groups.

Finally, to study any possible circadian rhythms involved with the hormone release in these variously treated animals, groups were sacrificed either in the morning between 9:30 and 10:45 A.M., or in the afternoon between 3:30 and 4:30 P.M.

Procedure-II.

As in part A, after the perfusion, the LH-RH concentration in the MBH-sch-POA was determined through radioimmunoassay, using 54μl duplicate samples. A more detailed description of the procedure can be found in the procedure Part A-II section.

Procedure-III.

To obtain values for LH, blood samples were taken from the trunk after decapitation, and the radioimmuno-
assay for LH was used to determine plasma LH levels, according to the general guidelines of the NIH kit for gonadotropins RIA (radioimmunoassay).
Results-I.

Figure 8 shows two graphs depicting the LH-RH secretion of an intact and OVX group in the morning. Notice that due to the increased time collection intervals, the dynamic pulsatile secretion of these thirty-day-old rats is minimized here in comparison to the graphs in part A. The resulting increase in time collection tends to "wash out" the smaller dynamic fluctuations. Figure 8 also demonstrates the tissue responsiveness by the strong stimulatory response to KCl. In every perfusion, a one and a half to three times increase in LH-RH levels over preinjection levels were found, when considering the peak response to KCl.

The combined results from the study of LH-RH release rates in the intact and ovariectomized rats are shown in figure 9. The upper graphs (a and b) give the dynamic picture of the release process, and the lower graphs (c and d) combine the mean value of LH-RH release of each perfusion, for easier statistical analysis. In the morning groups, higher levels are found in the intact group. By using an analysis of variance, (p<0.05), the OVX and OVX + E2 groups are found to be significantly lower than the intact group. Apparently, the absence of ovarian hormones due to ovariectomy has reduced the endogenous re-
lease of LH-RH for these MBH-sch-POA fragments. However, with the addition of progesterone, the levels of LH-RH reached values even beyond intact levels. Apparently, the combined action of these two hormones is necessary to restore the normal levels of LH-RH release. In this experimental case, however, the levels of E2 and progesterone are probably in excess to those normally seen by the intact immature female rat, and therefore, this can explain the release of LH-RH above normal levels.

With the afternoon groups, similar conclusions can be drawn, after statistical study by an analysis of variance and LSD test (with sign considered). Once again, the intact levels are higher than both the OVX and OVX + E2 groups, and the latter two groups are not significantly different from each other. Also similar to the morning groups, the OVX + E2 + Prog. groups show significantly higher levels of LH-RH release than that of the OVX + E2 group. The combined treatment of estrogen and progesterone restores the LH-RH release to intact levels, but does not exceed them. A possible explanation for this difference from the morning group is offered later. Also, the OVX groups and the OVX + E2 + Prog. groups were significantly different only at the 0.1 level. However, the large standard error in the OVX groups reflects the level of significance, plus the fact of having only six total groups involved, implies stricter
statistical limits.

These experiments demonstrate that ovarian hormones can manipulate the LH-RH release levels in vitro for up to three hours using the perifusion system.

The question of possible circadian rhythm release patterns can also be answered by reviewing the results shown in figure 9. By using the t-test between each group's morning and afternoon data, the results reflect significantly (p<0.025) lower LH-RH release levels in the afternoon in the intact animals. This phenomena is lost in the OVX and OVX+E2 groups, but is found again (p<0.025) in the OVX+E2+Prog. groups. Once again, the combined estrogen and progesterone treatment was necessary to restore the phenomena of circadian rhythm release of LH-RH in the ovariectomized immature female rat. This apparent cue that the rat takes from the light-dark cycle may also reflect the lack of a significant difference between the intact and OVX+E2+Prog. groups in the afternoon, despite excessively high estrogen and progesterone levels. Perhaps, the suppression of LH-RH levels due to the circadian rhythm also suppresses full stimulatory possibilities in the afternoon, even though the ovarian hormones are present.

Finally, a review of the more dynamic patterns in figure 9 (a and b), raise interesting questions. It
might be noted, for instance, that the fluctuations appear more dramatic in the morning intact and OVX+E₂+Prog. groups than in their afternoon counterparts. The intact morning groups also show a gradual overall decline in release levels, while the OVX+E₂+Prog. morning group shows an overall rise in release levels. Another interesting pattern is the smooth decline of release levels in the OVX afternoon group over an eighty minute period, before leveling off. All of the perifusions responded markedly to KCl after 140 minutes, and so these declines are not a result of tissue damage. These intriguing patterns of LH-RH release found in vitro will probably warrant more intensive study in the future.

Results-II.

The results obtained by the radioimmunoassay determinations of LH-RH concentration, expressed in pg/mg, are found in figure 10. Some interesting comparisons are found in the LH-RH concentration values in the MBH-sch-POA, that both mirror and contrast LH-RH release patterns found through the perifusion study (fig. 9). A comparison of the intact, OVX, OVX+E₂, and OVX+E₂+Prog. groups in the morning, by analysis of variance and LSD (p<0.05, one-tailed) reveal significantly lower levels in
the OVX and OVX+E₂ groups from that of the intact and OVX +E₂+Prog. groups. Similarly, each of these pairs are not significantly different from each other. This seems to indicate that LH-RH concentration levels have decreased because of the loss of some ovarian hormones after ovariectomy. It is only after the combined treatment of estrogen and progesterone, that LH-RH levels are restored to intact values.

The results from the afternoon groups are slightly more complex. Using an analysis of variance and LSD (one-tailed, p<0.05) the only significantly different comparisons show that the OVX+E₂+Prog. treatment results in higher LH-RH concentration levels than the intact and OVX+E₂ groups. In contrast to the morning, the intact levels are not significantly different than that of the OVX and OVX+E₂ treatments. Intriguingly, the estrogen and progesterone combined treatment resulted in higher LH-RH levels in the ovX of these animals as compared to those found in the intact and the OVX+E₂ groups. The lack of significance statistically at the 0.05 level for the OVX+E₂+Prog. vs the OVX group can be accounted for by the large standard error, and the resulting stricter statistical limits due to the small number of groups involved.

The marked difference in LH-RH levels between intact animals killed in the morning vs. the afternoon, might be due to lower circulating levels of estrogen and progesterone.
in the afternoon, or a change in feedback mechanisms depending on a light cue. The added estrogen and progesterone in excess to normally seen values by the immature female rat are then able to override this suppression of LH-RH levels, as seen by the levels of LH-RH found in the afternoon groups (see OVX+E₂+Prog. morning vs afternoon values).

A look at the comparison of morning vs. afternoon values for each treatment then becomes very interesting. Indeed, in the intact group, the morning and afternoon differences show a significant difference at p<0.005 (t-test, two-tailed). In contrast, the OVX groups shows no circadian rhythm. However, in the OVX animals treated with estrogen, because the LH-RH values are even lower than in the OVX group alone, a significant difference between the morning and afternoon levels was found (p<0.05, t-test, one-tailed). Similarly, the OVX+E₂+Prog. groups show a significant difference in levels (p<0.05, t-test, one-tailed).

Therefore, both the treatment of estrogen and estrogen with progesterone are able to restore the circadian rhythm. On hypothalamic LH-RH levels found in intact animals, however, only the combined estrogen and progesterone treatment can restore morning LH-RH concentration levels of ovariectomized animals to intact levels.
Results-III.

The results of the LH determinations are presented in figure 11. Analysis of this data shows that across the morning groups, the OVX groups have much higher LH values than in any of the other three treatments (ANOVA, and LSD, p<0.05). Apparently, the lack of ovarian hormones due to ovariectomy somehow causes LH levels to rise. Possibly the lack of estrogen and the resulting absence of the negative feedback action on the pituitary allows for higher LH levels. Another possibility is an increase in the pituitary sensitivity to stimulatory actions for LH release. The addition of estrogen apparently restores the levels of LH detected in intact rats.

Across the afternoon groups, the OVX group shows a higher level of LH than that found in the intact group, but they are not statistically significant (ANOVA, LSD, p<0.05, one-tailed). This in part can be explained by the small number of animals considered in the afternoon—j in the morning, there are 42 values, in comparison to only 13 in the afternoon. The OVX+E2+Prog. groups are found to have levels of LH significantly higher than all the other treatments (ANOVA, LSD, p<0.001). However, the OVX+E2 group shows levels of LH which are significantly higher than those for the intact group (ANOVA, LSD, p<0.05, one-tailed), but
not significantly different from those for the OVX group. It appears that in the afternoon, the high levels of estrogen allow the immature female rat to respond to a light cue, causing a dramatic rise in LH levels. Furthermore, the addition of progesterone, appears to facilitate the action of estrogen to an even greater extent. In studying the circadian rhythm of plasma LH levels, all but the intact group shows significantly different levels of LH between the morning and afternoon. Curiously, however, the LH levels in the OVX group are significantly higher in the morning, whereas for the estrogen and estrogen with progesterone groups, the reverse is true—the afternoon levels are higher (t-test, p < 0.001).
IV. DISCUSSION
The mechanism behind the release of LH-RH in immature female rats appears to be highly complex. In general, we have found that the hypothalamic LH-RH concentration and release rate appear to change during the course of development from 21 to 32 days of age, although the values are not significantly different after statistical analysis. A rise does occur in the LH-RH release rate to a peak at 30 days of age, and a similar rise occurs in the concentration of LH-RH in the hypothalamus to a peak at 32 days. This corroborates with previous findings (7), using a bioassay, of a rise in LH-RH levels shortly before vaginal opening, and a precipitous drop after the onset of puberty. Such a rise in LH-RH levels might trigger some action in either the pituitary responsiveness or the "gonadostat" sensitivity, thereby activating mechanisms important in the onset of puberty.

In understanding the various phenomena seen in the manipulation of hormone release through ov-riectomy and estrogen and progesterone treatments (refer to table 3), the following model is proposed. It is known that LH-RH acts on the pituitary to cause the release of LH, and the negative and positive feedback mechanisms of estrogen can modify both the function of the pituitary and the hypothalamus. In this model, we propose that an inhibitory substance which is controlled
by a light cue, is released from the brain. The release of this light inhibitory substance (LIS) seems particularly prominent in the afternoon. The existence of such a substance is supported by the fact that in the intact animal, both LH-RH release in vitro, and concentration levels of the perifused hypothalamic fragments are lower in the afternoon than in the morning. The lack of a circadian rhythm phenomena in the LH plasma levels can be explained by the presence of other various hormones in the intact animal which either suppress the expression of LIS, or, more probably act to increase LH levels so that the end result balances out both effects. In the OVX animals, the LIS no longer is effective in inhibiting LH release (particularly in the morning) because the ovariectomy results in the loss of ovarian hormones, thereby releasing the gonadotroph from the inhibitory feedback action of estrogen, at that level. It also appears that the lack of ovarian hormones produces a marked decrease in concentrations levels of LH-RH in the hypothalamus, along with a clear decrease in the in vitro release rate of LH-RH in both the morning and afternoon measurements.

Corresponding to this hypothesis, we find significantly lower LH levels in the afternoon, from those in the morning. Estrogen is unable to restore the circadian rhythm for LH-RH concentration or release levels, as seen in
the ovariecromized and estrogen treated group. However, alongside estrogen's negative feedback action on the release of LH from the pituitary, estrogen also appears to be effective in blocking the action of LIS. This suggestion is supported by the high plasma LH levels in the afternoon group of OVX and estrogen treated animals. Alternatively, estrogen may act in another mechanism that depends on the light-dark cycle, which then stimulates the release of LH, instead of blocking the LIS action. For instance, estrogen may sensitize the pituitary responsiveness to LH-RH, but the expression may occur only under the proper light cue.

Finally, the OVX +E+Progesterone treatment allows for and increase in the LH-RH metabolism, as well as provides for the proper conditions for significantly higher release of LH in the afternoon. This phenomenon can be explained by hypothesizing that progesterone facilitates the action of estrogen as seen in the OVX+E group, as well as in acting on the brain, to stimulate the metabolism of LH-RH. This possible facilitatory action of progesterone on estrogen has been hypothesized in other instances (3,10). In the recent study suggesting an inhibitory effect by progesterone on LH-RH release (8), the sampling was taken over 24 hours after progesterone injection as compared to the four to six hour time interval between injection and sampling used in this procedure.
In the developing female rat, then, several competing mechanisms appear to be in operation: LH-RH release from the hypothalamus to the pituitary, changes in pituitary responsiveness, the activation by light of an inhibitory substance, and its blockage by estrogen, and the facilitatory action of progesterone on estrogen actions, and its own effect on LH-RH metabolism. The exact nature of these mechanisms remains unknown at this time, but should provide for interesting revelations into the mechanisms of hormone release in the immature female rat, when they become better understood.
V. Figures and Tables
Figure 1:

Elution pattern through a Sephadex-G-25 Fine column of LH-RH iodinated by the Chloramine T method. The one ml fractions show labeled peptides in the first peak, free iodine in the second and active $^{125}$I LH-RH in the remaining three peaks.
Iodination of LH-RH on a Sephadex-G-25 Fine Column

fraction # (one ml each)
Figure 2
Figure 2:

Elution pattern (one ml fractions) of repurified LH-RH fractions taken from the third peak in figure 1, using a Sephadex-G-25-Fine column. Fractions 14-19 were pooled and frozen to be used in the radioimmune assay.
Fig. 2.

Repurification of $^{125}$I-LH-RH
on a Sephadex G-25 Fine Column

10,000
1000
100

Fraction# (1ml/each)
Figure 3:

A representative standard binding curve used to determine the LH-RH values in the samples taken. Higher levels of LH-RH tend to displace the $^{125}$I-LH-RH-anti-LH-RH complex, resulting in a lower radioactive count.
Figure 3.
Standard Binding Curve for LH-RH
Figure 4.
Figure 4:

A schematic diagram of the perfusion system indicating at points C, D, and E, the tube through which the oxygen bubbles in, the sampling outlet, and the injection port, respectively. Krebs-Phosphate medium enters through B, perfuses through the brain tissue (A), and is sampled through the sampling port (D). Diagram from Gallardo and Ramirez (15).
Figure 4.
A Schematic Diagram of the Porifusion System

- TEMPERATURE REGULATED WATER BATH
- PIECES OF HYPOTHALAMUS
- RUBBER STOPPER
- RUBBER STOPPER
- C
- D
- E
Figure 5.
Figure 5:

A representative graph showing the dynamic release of LH-RH as seen by the in vitro perfusion system. There is a dramatic rise in LH-RH release levels following an injection of 50μl of 600mM KCl.
Fig. 5

The Dynamic Release of LH-RH and its Response to KCl in the 30-day-old female rat

LH-RH release rate (pg/min)

Time (4 min. intervals)
Figure 6.
Figure 6:

LH-RH release in the developing female rat of 21 to 37 days of age, along with the peak response to KOl. All of the KOl responses were significant at the 0.05 level (paired t-test). LH-RH mean levels of release appear to rise to a peak at 30 days, but the difference in levels are not statistically significant, using the ANOVA test.
Fig. 6

LH-RH Release in the Developing FEMALE RAT

Mean LH-RH release
Peak Response to KCl

Mean LH-RH Release 3
(pg/min)

Age of Rats (Days)
Figure 7.
Figure 7:

Representative graphs showing the dynamic release of LH-RH as seen by the in vitro perifusion system in the 21, 25, 30, and 32-day-old female rat. Levels appear to generally rise to a peak at 30 days of age. Concomitant with this rise is a tendency towards greater and more dramatic fluctuations in the release levels. Times of sacrifice: 21-day-old: 9:30A.M., 25-day-old: 2:30p.m., 30-day-old: 9:45A.M. and 32-day-old: 9:30A.M.
The dynamic release of LH-RH in a 21, 25, 30, and 32-day-old immature female rat.

**21-day old**

**25-day old**

**30-day-old**

**32-day-old**

Time (Minutes)

LH-RH release measured in pg/min in four min. intervals.
Figure 8.
Figure 6:

Two representative graphs showing the dynamic response of LH-RH in vitro, and its dramatic response to KCl, in an intact and OVX 30-day-old female rat sacrificed in the morning.
The Dynamic Release in the Morning of LH-RH and its Response to KCl in and intact and OVX 30-day-old Female Rat.
Figure 9.
Figure 9:

*In vitro* LH-RH release levels in 30-day-old intact or OVX female rats. Figures a and b show the dynamic pattern at ten minute intervals using the perifusion system. Figures c and d combine the means of the above values. In figure c, (OVX+E2+Prog. > Intact > OVX, OVX+E2 (p < 0.05, analysis of variance and LSD). In figure d), Intact > OVX+E2, OVX, and OVX+E2+Prog. > OVX+E2 (p < 0.05). In the circadian rhythm study, between groups Intact a.m. > Intact p.m. and OVX+E2+Prog. a.m. > OVX+E2+Prog. p.m. (p < 0.025, t-test). The bars represent the mean ± S.E., and the numbers within the bars, indicate the number of perifusions.
The diagram shows the in vitro release of LH-RH in intact and OVX 30-day-old female rats. The graphs depict the LH-RH release over time (10 min intervals) with different conditions indicated by various symbols and lines: Intact, OVX+E+Prog., OVX, and OVX+E.

The bar graphs at the bottom show the mean LH-RH release (pg/min) for each condition: INTACT, OVX, OVX+E, and OVX+E+Prog., with bars indicating the range of values.
Figure 10:

LH-RH Concentration levels (20-day-old-female rat) in the Khb-sch-POA following perfusion. Open bars show the A.M. groups Intact, OVX+E₂+Prog > OVX, OVX+E₂ (p < 0.05, two-tailed, ANOVA). Hatched bars show the P.M. groups, OVX+E₂+Prog > OVX+E, Intact (p < 0.05, one tailed, ANOVA).

In comparing each A.M. vs. P.M. treatment, Intact a.m. > Intact p.m. (p < 0.005, t-test, two-tailed), OVX+E₂ a.m. > OVX+E₂ P.M., and OVX+E₂+Prog a.m. > OVX+E₂+Prog p.m. (p < 0.05 t-test, one-tailed) Mean ± S.E. is expressed (numbers within the bars indicate the number of groups).
Fig. 10

Concentration of LH-RH in the MBH-sch-POA of 30-day-old immature intact and OVX female rats following perfusion.

- A.M.
- P.M.
Figure 11.
Figure 11:

LH blood levels in the female immature rat of thirty days revealing in the morning, higher levels, in the OVX group (ANOVA, LSD p < 0.05, two-tailed) and in the afternoon, OVX + E₂ + Prog > OVX + E₂, OVX, Intact, and OVX + E₂ > Intact (p < 0.05, ANOVA, LSD, one-tailed). Within a single treatment, OVX a.m. > OVX p.m., OVX + E₂ p.m. > OVX + E₂ a.m., and OVX + E₂ + Prog. p.m. > OVX + E₂ + Prog a.m. (t-test, p < 0.001) mean ± S.E. is expressed. The number of subjects is indicated by the numbers within the bars.
Fig 11.

LH plasma Levels in the 30-day-old immature Intact and OVX Female Rat.

A.M. P.M.

605 5529

LH Levels (mg/ml)

90 110 130

50 75 100

30 10

10

Intact OVX OVX+E2 OVX+E2+Prog. Intact OVX OVX+E2 OVX+E2+Prog.
Table 1:

<table>
<thead>
<tr>
<th>Fraction #</th>
<th>Non-Specific Binding</th>
<th>Total Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>9.3%</td>
<td>12.77%</td>
</tr>
<tr>
<td>11</td>
<td>1.64%</td>
<td>4.62%</td>
</tr>
<tr>
<td>15</td>
<td>1.33%</td>
<td>29.43%</td>
</tr>
<tr>
<td>24</td>
<td>1.44%</td>
<td>66.54%</td>
</tr>
<tr>
<td>25</td>
<td>1.38%</td>
<td>61.01%</td>
</tr>
<tr>
<td>30</td>
<td>1.68%</td>
<td>60.31%</td>
</tr>
<tr>
<td>33</td>
<td>1.76%</td>
<td>55.44%</td>
</tr>
<tr>
<td>34</td>
<td>1.93%</td>
<td>51.69%</td>
</tr>
<tr>
<td>Repurified pooled fraction</td>
<td>1.91%</td>
<td>66.86%</td>
</tr>
</tbody>
</table>

Table 1:
Non-specific and total % binding of selected fractions of \(^{125}\text{I-LH-RH}\) from figures 1 and 2, showing the highest % binding in the pooled fraction from the repurified \(^{125}\text{I-LH-RH}\) fractions.
Table 2:

The concentration of LH-RH (pg/mg) in the MBH of the developing female rat, following perfusion of the tissue from both the morning and the afternoon. The values show a gradual rise in levels up to 32 days of age. After vaginal opening (35 and 38 days), the concentration levels drop again.

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th># of Perfusions</th>
<th>pg/mg</th>
<th>Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>4</td>
<td>4.42</td>
<td>± 0.62</td>
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<tr>
<td>25</td>
<td>8</td>
<td>7.37</td>
<td>± 2.33</td>
</tr>
<tr>
<td>30</td>
<td>7</td>
<td>8.69</td>
<td>± 1.81</td>
</tr>
<tr>
<td>32</td>
<td>3</td>
<td>17.19</td>
<td>± 5.43</td>
</tr>
<tr>
<td>35</td>
<td>1</td>
<td>5.13</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>1</td>
<td>10.36</td>
<td></td>
</tr>
</tbody>
</table>
Table 3:

Summary of the Relative LH-RH Release, Concentration, and LH Plasma Levels in the Intact and OVX immature 30-day-old Female Rat

<table>
<thead>
<tr>
<th>Groups</th>
<th>LH-RH Release</th>
<th>LH-RH Concentration Level</th>
<th>LH Plasma Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>OVX</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>OVX+E2</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>OVX+E2+Prog</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3:

The relative hormone values of LH-RH released, hypothalamic LH-RH concentrations, and plasma LH levels measured in the 30-day-old intact and OVX female rat. The '+'s and '-'s do not indicate any exact quantitative values, but rather, relative values, based on their statistical significance. The model suggests that an inhibitory substance that is activated by light, (LIS), is mainly released in the afternoon, that estrogen acts to block this inhibitory effect, and that progesterone acts on the brain to restore LH-RH metabolism and to facilitate the action of estrogen. See Discussion for details.
REFERENCES


