STIMULATION OF LH SECRETION IN CONSTANT ESTROUS RATS UNDER CONSTANT ILLUMINATION BY RAT HYPOTHALAMIC EXTRACT

TAKASHI KOBAYASHI, TAKURO KOBAYASHI, TOMONORI KIGAWA AND HISASHI ICHIKAWA

Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo

SYNOPSIS

Adult female rats were rendered anovulatory by continuous illumination. Pituitary LH of these constant estrous rats decreased with the time length of illumination. Acidic extract from the rat hypothalamus was injected into these rats through the carotid artery for testing LH-RF activity. The extract, if a dose more than 2 hypothalamic fragments per rat was given, induced ovulation. Ovulatory response was determined by observation of tubal ova and formation of new corpora lutea. Cerebral cortical extract, lysine vasopressin and oxytocin were not active in stimulation of ovulation. Pituitary LH and plasma LH shortly after intracarotid injection of the hypothalamic extract were estimated by the OAAD method. A single injection produced a transient rise in pituitary LH, and the maximum occurred 20 mins. after injection. Sixty mins. after injection pituitary LH showed a significant decrease. Corresponding to this fall in pituitary LH, plasma LH showed a significant rise. Both pituitary LH and plasma LH returned to near-initial levels 4 hrs. after injection. These results suggest that hypothalamic extract may stimulate the synsntesis of LH by the anterior pituitary of constant estrous rat as well as the release of LH.

A number of different experiments have demonstrated that the release of luteinizing hormone (LH) by the anterior pituitary is controlled by a hypothalamic releasing factor, namely, luteinizing hormone releasing factor (LH-RF).

In the rat, treatments such as exposure to continuous illumination (Browman, 1937), placement of appropriate hypothalamic lesion (Hillarp, 1949), or injection of testosterone soon after birth (Barraclough, 1961), produce persistent vaginal cornification associated with small follicular ovaries devoid of corpora lutea. Since it has been suggested that each of these treatments results in interference with cyclic release of LH by affecting hypothalamic structure responsible for the secretion of LH (Bradshow and Critchlow, 1966), in constant estrous rats made anovulatory by these treatments exogenous hypothalamic extracts seem induce ovulation.

Johnson (1963) has demonstrated that in androgen-sterilized rats oovulations were induced by intravenous injection of extracts from the sheep hypothalamus. And also, Shiavi et al. (1963) have reported that partially purified sheep LH-RF preparation induced oovulations in hypothalamic lesioned rats. These findings indicate that hypothalamic extracts stimulate the anterior pituitary of constant estrous rats to release LH in a sufficient amount to induce ovulation. How-
ever, pituitary and plasma LH were not determined in these experiments. This paper will analyse LH contents in both the pituitaries and the plasmas in constant estrous rats following the treatment with acidic extracts from the rat hypothalamus. At the same time the possibilities of inducing ovulation by such treatment was also examined.

MATERIALS AND METHODS

Preparation of constant estrous rats

Adult female rats of the Wistar strain, weighing 180 to 230 g, were used in the experiments. They were kept initially under conditions of rhythmic lighting (14 hrs. light daily) in a constant temperature room (25°C). After being checked for the normality of vaginal cycles for 2 weeks, they were exposed to a constant illumination (approximately 180-200 watts fluorescent illumination), and their vaginal smears were taken daily except Sunday until the day of autopsy for confirmation of vaginal cornification.

Preparation of acidic hypothalamic extract from rats

Crude acidic extracts from the rat hypothalamus and the cerebral cortex were prepared by the same procedures as described previously (Kobayashi et al., 1966).

Procedures of administration of hypothalamic extract

Hypothalamic extract, cerebral cortical extract, lysine vasopressin (Sandoz, Co. Ltd) or oxytocin (Sandoz, Co. Ltd) was dissolved in saline solution and injected into the carotid artery or the jugular vein of constant estrous rats after 30 days of the illumination under the anesthesia of Nembutal (0.5 mg/Kg). The injection was finished within 20 seconds from its start. Hypothalamic extract and cerebral cortical extract were boiled for 30 mins. at 80°C prior to injection.

Determination of ovulation

Twenty four hrs. after injection recipient animals were killed by decapitation, and ovaries and oviducts were removed en bloc with surrounding fatty tissues. Then, the oviducts were carefully separated from the ovaries, compressed between a slide and a cover slip, and examined for tubal ova at 100 X magnification. The ovaries were fixed in Formalin solution and stained with hematoxyline-eosin for histological examination.

Preparation of pituitary and plasma samples for LH assay

There were 6 groups of animals; (1) the first group was sacrificed by the withdrawal of blood from the abdominal aorta under laparotomy prior to injection, (2) the 2nd 10 mins., (3) the 3rd 20 mins., (4) the 4th 30 mins., (5) the 5th 60 mins., (6) the 6th 240 mins. following injection of hypothalamic extracts. Another 6 groups with cortical extracts served as the controls. The bloods drown were pooled at every group and centrifuged. After the withdrawal of blood, pituitaries were removed, pooled at every group, homogenized in cold saline, and centrifuged. Plasma and supernatants of pituitary homogenates were stored in the frozen state until the day of bioassay.

Bioassay of LH

A modification of the ovarian ascorbic acid depletion method of Parlow (1961) was used. Female rats of the Wistar-Imamichi strain, 25 days old, were made pseudopregnant by injection of 100 IU of PMS, followed by 30 IU of HCG 60 hrs. later. On the sixth day after injection of HCG, they were intravenously injected with either LH standard (NIH-LH-S 11) or pituitary or plasma samples. Four hrs. later bilateral ovaries were removed, and the concentration of ascorbic acid was determined by the method of Schaffert and Kingsley (1955). For the assay of pituitary extracts, four point analysis (5 animals/point) which compared two dosages of the pituitary extracts (1/2, 1/8 pituitary/rat) to two dosages of the LH standard (1, 4 μg/rat) was applied. For plasma samples each assay included one dosage of plasma (2 ml/rat) and two dosages of the LH standard (1, 4 μg/rat), with 5 animals at each point. LH potencies were expressed in terms of the LH standard.
Individual assays were analysed by the statistical methods of Bliss (1956) and the significance of difference between groups was determined by Student’s t test.

RESULTS

Development of constant estrus
By 21 days after exposure to constant illumination, approximately 85% of animals ceased to have vaginal cyclicity, showed either proestrous or estrous type of smears and remained in this state as long as the exposure was continued.

Pituitary LH contents of constant estrous rats
As shown in Table 1, pituitary LH content decreased with the time length of illumination. There was a significant difference between the content of normal control rat and that of 42 days-illuminated rat or that of 90 days-illuminated rat. Normal control group consisted of 4 rats at proestrous stage and 4 at estrous stage.

Induction of ovulation by hypothalamic extract in constant estrous rats
Twenty four constant estrous rats were intravenously (via jugular vein) injected with various doses of hypothalamic extract but ovulatory reaction was negative in all treated animals (Table 2). The dose of hypothalamic extract ranged from one HE (extract equivalent to one hypothalamic fragment) to 8 HE per rat. On the other hand, intracarotid injection of hypothalamic extract induced ovulation 14 out of 31 rats, as shown in Table 2. All of 16 animals treated with one HE per rat failed to ovulate, but 3 out of 12 with 2 HE, 6 out of 11 with 4 HE and 5 out of 8 with 8 HE ovulated. The incidence of ovulatory rat and the number of ova detected in oviducts increased as the dose of hypothalamic extract increased. Cerebral cortical extract, lysine vasopressin and oxytocin were not active in stimulation of ovulation (Table 2).

Histological findings of ovaries
Figure 1 shows the histological appearance of an ovary treated with 4 cerebral cortical extracts (equivalent in weight to 4 hypothalamic extracts), which contains a number of large follicles but no new corpora lutea. In contrast with this, in an ovary of a rat treated with 4 HE there were a number of new corpora lutea (Fig. 2).

Effects of hypothalamic extract on pituitary LH and plasma LH
Changes of pituitary LH and plasma LH after intracarotid injection of 4 HE per rat

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Avg. body weight (mg)</th>
<th>Avg. pit. weight (mg)</th>
<th>Avg. ovar. weight (mg)</th>
<th>Avg. uterine weight (mg)</th>
<th>pit. LH content* (μg/gland)</th>
<th>λ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (estrous, proestrous)</td>
<td>8</td>
<td>216 ± 8.0</td>
<td>8.7 ± 0.4</td>
<td>47.6 ± 6.4</td>
<td>340 ± 42.1</td>
<td>16.3 (11.7—20.5)**</td>
<td>0.274</td>
</tr>
<tr>
<td>Constant light, 21 days</td>
<td>8</td>
<td>207 ± 9.8</td>
<td>8.4 ± 0.3</td>
<td>38.7 ± 8.3</td>
<td>280 ± 53.7</td>
<td>12.1 (8.3—17.2)</td>
<td>0.282</td>
</tr>
<tr>
<td>Constant light, 42 days</td>
<td>8</td>
<td>211 ± 12.3</td>
<td>9.1 ± 0.6</td>
<td>37.8 ± 2.8</td>
<td>317 ± 38.0</td>
<td>9.2 (6.4—13.5)13</td>
<td>0.304</td>
</tr>
<tr>
<td>Constant light, 90 days</td>
<td>8</td>
<td>192 ± 20.5</td>
<td>7.8 ± 0.4</td>
<td>34.3 ± 5.2</td>
<td>298 ± 45.0</td>
<td>7.8 (5.8—11.7)22</td>
<td>0.245</td>
</tr>
</tbody>
</table>

* Expressed as μg NIH-LH-S11 equivalents per gland.
** 95% confidence limits.
13 The value is significant (P<0.05), as compared with the control value.
22 The value is significant (P<0.025), as compared with the control value.

Control group includes 4 rats at proestrous stage and 4 at estrous stage.
Table 2. Stimulation of ovulation in constant estrous rats by rat hypothalamic extract

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>No. of rats ovulated</th>
<th>No. of ova*</th>
<th>Incidence of ovulatory rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 HE, iv</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 HE, iv</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 HE, iv</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 HE, iv</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 HE, ic</td>
<td>16</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 HE, ic</td>
<td>12</td>
<td>3</td>
<td>2.5</td>
<td>25.0%</td>
</tr>
<tr>
<td>4 HE, ic</td>
<td>11</td>
<td>6</td>
<td>3.8</td>
<td>54.5%</td>
</tr>
<tr>
<td>8 HE, ic</td>
<td>8</td>
<td>5</td>
<td>6.4</td>
<td>62.5%</td>
</tr>
<tr>
<td>4 CE, ic</td>
<td>8</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lysine
vasopressin, ic
I U        4          0
100 mU     4          0
Oxytocin, ic
I U        4          0
100 mU     4          0

* Mean of the number of ova per rat ovulated.
1 HE: extract equivalent to one hypothalamic fragment, 4 CE: cerebral cortical extracts equivalent in weight to 4 HE, iv: intrajugular injection, ic: intracarotid injection.

are shown in Table 3, and diagrammatically represented in Figure 3. A single injection initiated an increase of pituitary LH content. The maximum occurred 20 mins. after injection. Over 20 mins. after injection pituitary LH showed a decrease, 60 mins. after injection reached the lowest level, and 240 mins. after injection returned to near-initial level. The maximum level (14.7 µg/pit.) was statistically significant (P<0.05) and the lowest level (3.9 µg/pit.) highly significant (P<0.01), as compared with initial level (9.7 µg/pit.). On the other hand, plasma LH was undetectable 10 to 20 mins. after injection. Over 20 mins. after injection there was observed a rise in plasma LH. The maximum occurred 60 mins. after injection (1.6 µg/ml). Four hrs. after injection plasma LH was undetectable. On the contrary, intracarotid injection of 4 CE per rat had no significant effect on pituitary LH and plasma LH (Table 3). And, as shown in Table 4, intravenous injection of 4 HE (via jugular vein) failed to show significant effect on pituitary LH and plasma LH.

DISCUSSION

In the present experiments, approximately 85% of rats exposed to continuous illumination was rendered constant estrous by this treatment. These ovaries were exclusively occupied with a number of cystic follicles but no new corpora lutea, indicating that in these rats ovulatory release of LH is blocked.
Coincident with the reports of Marie et al. (1965), and Bradshow and Critchlow (1966), pituitary LH content of continuous illuminated rat was lower than that of normal cyclic rat, and showed a tendency to decrease with the time length of constant illumination.

In 30 days-illuminated rats a single intracarotid injection of acidic extract from the rat hypothalamus actually induced ovulation, as demonstrated by the presence of tubal ova and the formation of new corpora lutea. Probability that this ovulation was due to LH contaminated in the extract was denied by following evidence: a) Hypothalamic extract was boiled prior to injection. McCann (1962) reported that boiling of stalk-median eminence extract inactivated contaminated LH but not releasing factor activity. b) Systemic (iv) injection of the extract was not active in stimulation of ovulation, even if a dose as large as active on intracarotid injection was given. c) While intracarotid injection of the extract produced rises in both pituitary LH and plasma LH, intravenous injection of the extract was ineffective. Intracarotid injection of cerebral cortical extract, lysine vasopressin or oxytocin failed to induce ovulation. Thus, it was indicated that intracarotid injection

![Fig. 3. Effect of hypothalamic extract on pituitary LH and plasma LH of constant estrous rats.](image)

**Table 3.** Effects of hypothalamic extract and cerebral cortical extract on pituitary LH and plasma LH of constant illuminated rats by intracarotid injection

<table>
<thead>
<tr>
<th>Substance*</th>
<th>Min. after injection</th>
<th>No. of rats</th>
<th>Pituitary LH** (µg/gland)</th>
<th>Plasma LH** (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>95% confid. limits</td>
</tr>
</tbody>
</table>
| 4 HE       | 0                    | 6           | 9.7  | 6.8—13.4 | 0.247  | undetectable
|            | 10                   | 6           | 12.3 | 7.5—15.7 | 0.283  | "                     |
|            | 20                   | 6           | 14.7 | 11.3—21.0| 0.283  | "                     |
|            | 30                   | 6           | 7.8  | 5.2—11.4 | 0.247  | 0.84 | 0.34—1.2 | 0.314 |
|            | 60                   | 6           | 3.9  | 2.1—5.6  | 0.247  | 1.6  | 0.95—2.1   |
|            | 240                  | 6           | 9.8  | 5.9—14.2 | 0.247  | undetectable
| 4 CE       | 10                   | 6           | 10.7 | 4.2—17.3 | 0.263  | undetectable
|            | 20                   | 6           | 9.5  | 5.5—18.1 | 0.283  | "                     |
|            | 30                   | 6           | 10.3 | 6.4—14.5 | 0.283  | "                     |
|            | 60                   | 6           | 10.5 | 7.1—13.8 | 0.263  | "                     |
|            | 240                  | 6           | 9.4  | 5.8—13.6 | 0.263  | "                     |

* 4 HE : extracts equivalent to 4 hypothalamic fragments.
4 CE : cerebral-cortical extracts equivalent in weight to 4 HE

** µg equivalents of NIH-LH-SII

1) The value is significant (P<0.05), as compared with the initial value.
2) The value is significant (P<0.01), as compared with the initial value.
carotid injection of hypothalamic extract stimulates the release of endogenous LH to induce ovulation, presumably by directly acting on the anterior pituitary.

Since the storage of pituitary LH is lower in continuously illuminated rats than that in normal rats, in those rats intracarotid injection of hypothalamic extract may induce ovulation not only by the release of LH previously stored before injection but also by the release of LH newly synthetized after injection. It seems, therefore, interesting to examine changes of both pituitary LH and plasma LH shortly after intracarotid injection of the extract. A single intracarotid injection of the extract produced a decrease of pituitary LH with the maximum 60 mins. after injection. Corresponding to this fall in pituitary LH, a rise of plasma LH was observed. Both pituitary LH and plasma LH returned to near-initial levels 4 hrs. after injection. These results clearly indicate that the hypothalamic extract, LH-RF, stimulates the pituitary to release LH in continuously illuminated rats.

In the experiments a transient rise of pituitary LH was observed immediately after a single injection of the extract. The maximum increase occurred 20 mins. after injection, and it was followed by both a depletion of pituitary LH and a coincident rise of plasma LH. In our previous experiments (Kobayashi et al., 1967) the anterior pituitary glands were cultivated with addition of LH-RF preparation partially purified on a column of Sephadex. After 2 days cultivation with the LH-RF, LH activities of the culture media and the glands were separately estimated by the ovarian ascorbic acid depletion method. Significant increase of LH was observed in both the media and the glands. These results suggest that the hypothalamic extract, LH-RF, may stimulate the pituitary to produce LH in vivo and in vitro. But, it seems difficult to conclude that LH-RF is concerned with the synthesis of LH as well as the release. Since in our experiments crude hypothalamic extract of partially purified LH-RF was used, it could not be determined if hypothalamic extract contained two substance, that is, one responsible for the release of LH and the other responsible for the synthesis. Experiments in which highly purified LH-RF is used can give final conclusion to the problem whether the same factor is responsible for the synthesis of LH as well as the release.

Concerning FSH-RF, David et al. (1965) have reported that rat hypothalamic extract caused about 50% depletion of pituitary FSH content in intact adult male rats 20 mins. after intracarotid injection. Kuroshima et al. (1966) also have demonstrated that intracarotid injection of beef or pig stalk-median eminence extract depleted the pituitary FSH of ovariectomized, estrogen- and progesterone-treated rats. The depletion of FSH reached the maximum 20 to 30 mins. after injection and it was associated with a
corresponding rise in serum FSH. From these results it is indicated that FSH-RF is concerned with the release of FSH, just as LH-RF is concerned with the release of LH. However, these investigators have demonstrated that no increase of pituitary FSH was induced prior to a depletion of pituitary FSH by the intracarotid injection of hypothalamic extract. Discrepancies between these results about FSH-RF and ours about LH-RF now can not be explained.

ACKNOWLEDGEMENT

The authors wish to acknowledge the gifts of FSH and LH standard preparations by the Endocrinology Study Section of the National Institutes of Health, U.S.A.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education and a Grant from the Population Council, N. Y. (Grant No. M-67, 77).

REFERENCES


