TIME-DEPENDENT DECREASE
OF PITUITARY RESPONSE TO LH-RH
AFTER CHRONIC TREATMENT OF INTACT FEMALE RATS
WITH ETHINYLOESTRADIOL AND NORETHINDRONE

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ABSTRACT
The effect of daily injections of 50 μg ethinyloestradiol and 1 mg norethindrone upon basal and LH-RH-stimulated LH release was investigated in intact adult female rats during a period of 30 days. Basal plasma LH was depressed by about 30% during the whole time of steroid treatment, and returned to the level of the control values two weeks after discontinuation. The injection of 30 ng and 150 ng LH-RH into untreated rats resulted in a significant increase of plasma LH which was, however, not dose-dependent. After the treatment with ethinyloestradiol and norethindrone for 5 days, the pituitary response to 150 ng was approximately four times higher than that to 30 ng. The LH release after injection of 150 ng LH-RH decreased significantly with the duration of steroid treatment, and was totally abolished by 30 days; whereas two weeks after the termination of steroid application the pituitary responded to LH-RH in the same manner as in control rats. When 30 ng LH-RH were injected into oestrogen/norethindrone treated rats, no decrease in LH release was found until day 20 of the experiment. By 30 days of treatment no rise in plasma LH could be elicited.

The activity of the LH-RH-degrading enzyme L-cystine arylyamidase was stimulated during the treatment with ethinyloestradiol and norethindrone in the pituitary by approximately 100%, whereas almost no effect on this enzyme was seen in the hypothalamus.
It is concluded that the chronic treatment of intact female rats with ethinyloestradiol and norethindrone causes time-dependent and reversible alterations in the storage of LH in the pituitary. The elevated activity of the LH-RH-degrading enzyme in the pituitary is possibly involved in these processes and/or in the mechanism responsible for the depression of basal LH.

The capacity of oestrogens and progestogens to augment or inhibit the release of gonadotrophins under certain physiological and pharmacological conditions depends on the amount of hormone secreted or administered, on the duration of its effect, and on the state of responsiveness of the hypothalamo-pituitary unit (Saunders 1964; Schally et al. 1968, 1970; Arimura & Schally 1970; Swerdlaff et al. 1972; Cooper et al. 1974). Although these positive or negative effects of sex steroids are believed to be mediated mainly by the hypothalamus, they also seem to act directly upon the pituitary by affecting the basal and LH-RH-stimulatable release of LH and FSH (Hilliard et al. 1966; Debeljuk et al. 1972; Negro-Vilar et al. 1973; Blake et al. 1974; Vilchez-Martinez et al. 1974; Steinberger & Chowdhury 1974; Apfelbaum & Taleisnik 1976). By inference, ovulation-inhibiting steroids may be assumed to inhibit the release of gonadotrophins not only by reducing the output of hypothalamic LH-RH but also by interfering with the action of LH-RH upon the pituitary gonadotroph.

Relatively little is known on the nature of the processes involved in the conveyance of feedback effects. The existence of a specific protein has been postulated which mediates the blocking effects of sex steroids upon gonadotrophin release, possibly by inactivating LH-RH or by inhibiting its release or synthesis, as actinomycin D interferes with the effect of oestrogens upon castration-induced rise in plasma LH (Schally et al. 1969). This protein could be an enzyme or a group of enzymes which inactivate LH-RH or inhibit its release or synthesis.

A hypothalamic and pituitary LH-RH-degrading enzyme, L-cystine arylamidase, appears to be integrated into the feedback mechanisms of gonadotrophin release in that its activity can be stimulated by injecting rats with LH or sex steroids (Kuhl & Taubert 1975a,b; Kuhl et al. 1976, 1977, 1978).

The inhibiting effect of oral contraceptives upon LH-RH-stimulated LH and FSH release in women was shown to be dose-dependent, and the restoration of pituitary responsiveness to be time-dependent. This suggests that the diminution of pituitary reactivity towards the action of LH-RH does not only depend on the dose of hormone administration but also on the duration of treatment (Dericks-Tan et al. 1976).

We therefore examined the effect of 50 µg ethinyloestradiol and 1 mg norethindrone given daily sc for periods ranging from 5 to 30 days upon LH-RH stimulated LH release in intact female rats, and whether or not changes in
gonadotrophin release would be reflected in alterations in hypothalamic and pituitary enzyme activity. The doses chosen for the study were based on the findings of Neumann & Domenico (1965) for ovulation inhibition by sex steroids in the rat.

**MATERIAL AND METHODS**

The following hormones were utilized in this study: norethindrone (17α-ethynyl-17β-hydroxy-4-oestren-3-one) was obtained from Schering AG, Berlin, FRG; ethinyl-oestradiol (17α-ethynyl-1,3,5(10)-oestratriene-3,17β-diol) from Merck, Darmstadt, FRG, and LH-RH from Hoechst AG, Frankfurt am Main, FRG. Rat LH and anti-rat LH were kindly provided by NIAMDD, Bethesda, Md. USA.

The experiments were performed with intact female SIV rats (45 to 50 days of age at the beginning of the study) which had been kept under standard conditions of 12 h of light alternating with 12 h of darkness. Food and water were given *ad libitum*. At the day of sacrifice only those animals were used as controls which had been shown by vaginal smears to be in the dioestrous or metoestrous stage, respectively. The animals were injected sc daily with 50 µg ethinyl-oestradiol and 1 mg norethindrone in 0.2 ml arachis oil/benzyl benzoate (6:4) or the solvent only. Groups of 6 animals were decapitated between 09.00 and 11.00 h on day 0, 5, 10, 20, and 30 of treatment, and 2 weeks after its cessation.

The hypothalamus, comprising the area between the optic chiasm and the mamillary bodies, and the pituitaries were removed immediately after decapitation according to the method of Bickel et al. (1972), and homogenized in a Potter-Elvehjem homogenizer at 0°C in Ringer’s. The activity of L-cystine arylamidase was measured in homogenates according to the method of Kuhl et al. (1974). The enzyme activity was expressed as µg p-nitroaniline · mg protein⁻¹ (µg p-NA/mg protein) which was split off the substrate L-cystine-bis-(4-nitroanilide) during 30 min of incubation at 37°C.

The protein content was determined by means of a modification of the method described by Lowry et al. (1951).

In experiments on the effect of LH-RH, 2 ml of blood was obtained from each rat from the retro-orbital plexus by means of a heparinized Pasteur pipette. The rats were anaesthetized 30 min prior to blood-sampling by the sc injection of 2 ml of a 10% urethane solution. Immediately after blood sampling the rats received 30 or 150 ng LH-RH in 0.5 ml Ringer’s into a tail vein. Fifteen min later the animals were decapitated, and the blood was collected. Each rat served as its own control for the determination of the LH-RH effect. Plasma LH was measured by a double-antibody solid-phase method described by Dericks-Tan & Taubert (1975). LH values (ng/ml) are expressed in terms of the NIAMDD rat LH-RP-1 reference preparation.

The paired *t*-test was used to evaluate the significance of increases of plasma LH which occurred in each group of rats after the injection of LH-RH. Comparisons between values in two groups were made with Student’s *t*-test.

**RESULTS**

**Plasma LH**

The effect of daily treatment of intact adult female rats with 50 µg ethinyl-oestradiol and 1 mg norethindrone upon the plasma concentration of LH is
Effect of daily injections of ethinyloestradiol (EE) and norethindrone (N) into intact adult female rats upon basal plasma LH. Black columns indicate steroid-treated rats, white columns untreated dioestrous and metoestrous rats (mean ± sn), n = 12.

depicted in Fig. 1. Within 5 days of treatment plasma LH was significantly \((P < 0.01)\) decreased by approximately 30 % as compared to solvent-treated controls. This degree of suppression was maintained during the remaining 25 days of daily injections with the ovulation inhibitor. Two weeks after the discontinuation of steroid administration, plasma LH was found to have returned to the level of the control rats. Normal cycles were found to have been restored in most of these rats within 2 weeks.

**Pituitary response to LH-RH**

When intact female rats of dioestrous or metoestrous stage were injected iv with 30 ng or 150 ng LH-RH, a significant rise in plasma LH could be observed in all animals after 15 min (Figs. 2 and 3). There was, however, no significant difference in the extent of the pituitary response where the two doses of LH-RH were compared. After the injection of 30 ng, in all control groups (Fig. 2) there was an increase in plasma LH by 126.5 ± 121.8 ng/ml \((n = 35)\) as compared to 137.2 ± 89.6 ng/ml \((n = 33)\) in the control rats receiving 150 ng LH-RH at the days 0, 5, 10, 20, 30, and 45 of the study (Fig. 3).

It had been shown by Seyler & Reichlin (1973) and by Döhler et al. (1977) that changes in plasma LH can be induced by various techniques of anaesthesia and blood sampling in the rat. This could possibly be responsible for these large variations. Therefore, the effect of blood sampling (2 ml) from the retro-orbital plexus upon plasma LH was additionally investigated in 8 rats of dioestrous
Effect of LH-RH upon plasma LH 15 min after iv injection of 30 ng into oil-treated and ethinyloestradiol(EE)/norethindrone(N)-treated intact adult female rats (mean ± sd).

**Fig. 2.**

Effect of LH-RH upon plasma LH 15 min after iv injection of 150 ng into oil-treated and ethinyloestradiol(EE)/norethindrone(N)-treated intact adult female rats (mean ± sd).

**Fig. 3.**
and metoestrous stage. There was a slight increase in plasma LH from 68.8 ± 11.0 ng/ml in the blood taken immediately before the injection of 0.2 ml Ringer's to 101.0 ± 59.8 ng/ml 15 min thereafter. The difference was, however, not significant.

Contrary to the untreated control rats, there was a significant difference between the pituitary response to the two doses of LH-RH when the animals had been pre-treated with ethinyloestradiol and norethindrone for a period of 5 and 10 days (Figs. 2 and 3). After 20 days of treatment the injection of 150 ng LH-RH still caused a higher release of LH than that of 30 ng. The difference was, however, not significant. The pituitary response to LH-RH was completely abolished when the animals had received the ovulation inhibitor for a period of 30 days.

It is shown in Fig. 4 that there was a highly significant \( P < 0.001 \) negative correlation between the pituitary response to 150 ng LH-RH and the duration of treatment with ethinyloestradiol and norethindrone. This clearly demonstrates that the amount of pituitary LH capable of being released decreases

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\text{Fig. 4.}
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Time-dependent decrease of pituitary LH release to 150 ng LR-RH during chronic treatment with ethinyloestradiol (EE) and norethindrone (N) as compared to oil-treated control groups.
Regression line of steroid-treated groups:
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y = 358 - 11.67 x \quad (P < 0.001).
\]
Regression line of control groups:
\[
y = 129 + 0.52 x \quad (N.S.).
\]
Effect of daily injections of ethinyloestradiol and norethindrone into intact adult female rats upon L-cystine arylamidase activity in the hypothalamus. Black columns indicate steroid-treated rats, white columns control groups. Enzyme activity is expressed as $\mu g$ p-nitroaniline $\cdot$ mg protein$^{-1}$ (mean $\pm$ sd).

Effect of daily injections of ethinyloestradiol and norethindrone into intact adult female rats upon L-cystine arylamidase activity in the pituitary. Black columns indicate steroid-treated rats, white columns control groups. Enzyme activity is expressed as $\mu g$ p-nitroaniline $\cdot$ mg protein$^{-1}$ (mean $\pm$ sd).

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with the time of treatment. Two weeks after the termination of treatment, the pituitary responded to the injection of 30 ng and 150 ng LH-RH in the same manner as the control animals.

L-cystine arylamidase activity in the hypothalamus and the pituitary

The chronic treatment of intact female rats with the ovulation inhibitors caused only a slight change in L-cystine arylamidase activity in the hypothalamus. The increase in enzyme activity was found to be significant ($P < 0.01$) only in the group receiving the steroids for a period of 10 days (Fig. 5).

Contrary to this, there was a remarkable stimulation of enzyme activity in the pituitary by about 45%, when the animals had been injected with the steroids for 5 days (Fig. 6). This effect became even more pronounced when the rats had been treated for 10 days; the arylamidase activity rose from $6.41 \pm 1.52 \mu g \cdot p-NA \cdot mg \cdot protein^{-1}$ in the control group to $11.50 \pm 3.01 \mu g \cdot p-NA \cdot mg \cdot protein^{-1} (P < 0.01)$. During the subsequent time of treatment with norethindrone and ethinyloestradiol, the pituitary enzyme activity remained at this high level, and returned to control values 2 weeks after the termination of the daily injections (Fig. 6).

**DISCUSSION**

Although oestrogens and progestogens have been shown to be capable of preventing the post-castration rise of plasma LH in rats (Schally et al. 1968, 1970; Negro-Vilar et al. 1973; Blake et al. 1974), there is relatively little known about their effects upon the basal LH secretion in intact rats during chronic treatment. Moreover, the inhibitory or augmentative effect of sex steroids upon LH release is a time- and dose-dependent phenomenon (Cooper et al. 1974; Swerdloff et al. 1972; Vilchez-Martinez et al. 1974). It remains, therefore, to be shown whether the results of single injections or short-term treatment could be corroborated when these compounds were given over longer periods of time.

Chronic treatment of intact female rats with 50 $\mu g$ ethinyloestradiol and 1 mg norethindrone per day caused a marked reduction in plasma LH, which remained more or less at the same low level from day 5 to 30 of the experiment but returned to the normal range within two weeks after the termination of the injections. It is not yet possible to state clearly whether this effect on LH secretion is due to a reduction of LH-RH release from the hypothalamus, or an impairment of pituitary response towards LH-RH.

Contraceptive steroids have been presumed to act predominantly by influencing the hypothalamus or higher centres as they did not suppress the
stimulatory effect of LH-RH upon LH release (Schally et al. 1968, 1970) when given to ovariectomized rats for 5 days.

The results presented here clearly demonstrate that the response of the pituitary to LH-RH decreased in high correlation with the duration of steroid treatment. In similarity to the results obtained by Schally et al. (1968), we observed a marked rise in plasma LH after the injection of 150 ng LH-RH into rats pre-treated with sex steroids for 5 days only, and the magnitude of the response was considerably greater than in controls. This appears to reflect the so-called positive effect of oestrogens and progestogens observed by other investigators (Colombo & Sawyer 1973; Arimura & Schally 1971; Kulkarni et al. 1974). The augmentative effect on LH-RH-stimulated LH release decreased, however, with the duration of treatment, and was completely abolished after 30 days. This indicates that the long-term treatment of intact female rats with ethinyloestradiol and norethindrone causes a gradual functional change in the LH-secreting cells of the pituitary. A similar effect has been reported by Yen et al. (1974). During the chronic administration of 1 μg/kg ethinyloestradiol per day to hypogonadal women the initial augmentation of pituitary LH-release to 150 ng LH-RH was followed by a progressive decrease resulting after 4 weeks in a response which was lower than in the control group.

In the present study the diminution of the pituitary responsiveness did not become apparent before day 30 of treatment when the dose of 30 ng LH-RH was used. The acutely releasable LH sufficed apparently for the stimulatory action of the low dose of LH-RH until day 20, and was blocked not before the injections were extended to 30 days. This pituitary suppression by ethinyloestradiol and norethindrone was found to be completely reversible within 2 weeks after discontinuation of steroid administration. Similarly, the pituitary block caused by the administration of high-dosed oral contraceptives to women persisted only for 2 weeks after the treatment was stopped (Dericks-Tan et al. 1976).

While the responsiveness of the pituitary towards the action of exogenous LH-RH became increasingly impaired during chronic treatment with ethinyloestradiol and norethindrone, the suppression of basal LH secretion remained unchanged at a level of –30 %/n as compared to controls without any further decrease. This constant suppression is possibly due to the action of the LH-RH-inactivating enzyme the activity of which rose by approximately 100 %/n in the pituitaries of these rats.

It is, nevertheless, unlikely that the enzyme could have prevented the effect of a sudden increment of LH-RH in plasma such as caused by the bolus injection given. This leads to the conclusion that the inhibition of pituitary responsiveness towards the action of 30 and 150 ng LH-RH injected after 30 days of steroid treatment does not seem to be caused directly by the activated enzyme but rather by alterations in the mechanism of synthesis and storage of LH.
During a longer period of steroid treatment, however, the permanently increased degradation of LH-RH in the pituitary may well have consequences in the regulation of the storage of LH which is probably controlled by the levels of LH-RH and oestradiol.

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