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20. The assays of Tables 1 and 2 were carried out while C.R.H.R. was a guest worker in the Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases. This investigation was supported in part by grants CA 15898, CA 13943, and CA 15909 awarded by the National Cancer Institute, and by a grant from the University of Wisconsin Graduate School to C.R.H.R. Address reprint requests to J.G.T.

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Estradiol Shortens the Period of Hamster Circadian Rhythms

Abstract. *Continuous administration of estradiol benzoate by means of subcutaneously implanted capsules shortened the free-running circadian period of locomotor activity of blind hamsters (*Mesocricetus auratus*) that had had their ovaries removed. Estradiol also advanced the phase of the wheel running of sighted female hamsters without ovaries that were entrained to a photoperiod with 12 hours of light and 12 of darkness. These results, and findings from hamsters undergoing natural estrous cycles, indicate that endogenous estradiol is involved in the regulation of circadian periodicity.*

The period (τ) of circadian oscillations remains remarkably stable under all but a very few chemical or pharmacological challenges (*1*, *2*). Recent work indicates hitherto unsuspected and important regulatory actions of hormones on vertebrate circadian rhythms (*3*). The experiments described here confirm and extend this relationship; they demonstrate that estradiol shortens the period of female hamster circadian activity rhythms and suggest that endogenously secreted estradiol exerts a significant effect in the regulation of the circadian system.

Female hamsters were housed in standard activity apparatuses with continuous free access to food, water, and an activity wheel. Hamster locomotor rhythms were recorded in the traditional way (*2*). We initially observed that the phase angle difference (ψ), defined as the interval between lights off and the beginning of the active period (*4*), was less negative on days 3 and 4 than on days 1 and 2 of the females' estrous cycles. We have termed this pattern of cyclically earlier activity on days 3 and 4 "scalloping" (*5*). We have since observed scalloping in hamsters housed in 12 hours of light and 12 of darkness (LD 12 : 12) or constant illumination as well as in blind animals (Fig. 1) (*6*); in each case the less negative ψ's occurred on those days of the cycle during which estradiol secretion is greatest (*7*).

We next tested the influence of exogenously administered steroids (*8*) on the phase angle differences of hamsters from which the ovaries had been removed and which were entrained to the LD 12 : 12 cycle (*9*). Estradiol benzoate (EB) implanted in a capsule produced a significant phase advance in wheel running. Implantation of an empty capsule delayed the onset of running (Table 1) (*10*).

To test whether estradiol could affect τ independent of its effects on the perception of light intensity, ovariectomized hamsters were blinded by orbital enucleation and then allowed to assume their free-running periods. Subsequent implantation of empty Silastic capsules did not change the mean circadian period of the six animals tested; progesterone-filled capsules were also without consistent effect. However, each of 11 animals implanted with an EB-filled capsule shortened the period of its circadian activity rhythm (Table 2) (*11*, *12*). Furthermore, five of the animals in which the empty Silastic capsules were originally implanted were subsequently given capsules containing EB, and each of these animals responded by shortening the τ of its activity rhythm (τ = 24.10 ± .06 hours for the control period and 23.95 ± .06 hours during stimulation with EB).

Figure 2 illustrates the record of one animal with an average response to the hormone (τ changed from 24.08 to 23.87 hours). The change in τ was manifested within the first 2 days after the EB implant was in place. For 10 of the 16 animals, τ shortened within 3 days, although the latency to a stable new τ was longer ($\bar{X}$ = 9.2 ± 1.3 days). The magnitude of the change in τ induced by EB decreased with the interval elapsing between blinding and hormone treatment (r = .57, $P < .02$), and reached an asymptote about 60 to 70 days after blinding. This may reflect a decreased

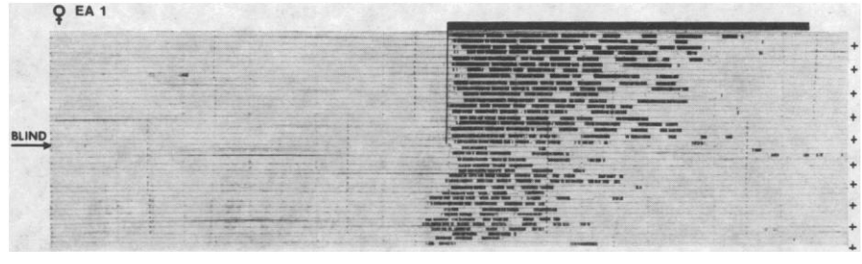

Fig. 1. "Scalloping" of wheel-running activity by a female hamster during exposure to an LD 14 : 10 photoperiod before and after orbital enucleation (arrow). A phase advance occurs on days 3 and 4 of the estrous cycle and is seen particularly clearly in the three estrous cycles that immediately preceded blinding; after blinding, the scalloping continues while the circadian rhythm is free-running. Each + at the right margin indicates detection of the postovulatory vaginal discharge. Each horizontal strip of the record represents 24 hours, with successive days pasted day below preceding day. The 10-hour daily dark period is indicated by the heavy horizontal line at the top of the figure.

Table 1. Influence of hormones on phase angle difference (ψ) of wheel running in hamsters entrained to an LD 12 : 12 cycle. The differences (Δ) between ψ's measured before and during treatment with the hormones are positive to signify a phase advance or earlier activity onset and negative to signify a later onset. The mean phase angles were calculated from the medians of 8 days of data collected before treatment began and the medians of 20 days of treatment data for each animal. Results are expressed as means ± the standard errors of the means.

Treatment	Number of animals	ψ (minutes)	Δ* (minutes)
Estradiol benzoate	14	33.2 ± 2.0	+7.1 ± 2.4
Empty capsule	11	28.9 ± 3.8	−2.8 ± 1.2
Progesterone	8	32.1 ± 4.2	−5.2 ± 2.5

*Within-group changes in Δ were significant for the EB ($P < .02$) and empty-capsule ($P < .05$) treatments, (two-tailed t-test) and were not significant ($P < .10$) for the progesterone treatment. In a between-groups comparison, EB changes differed significantly from those of the other two treatments ($P < .02$, each comparison); differences between progesterone and empty-capsule treatments were not significant.

sensitivity to estradiol as a function of time since blinding [compare with (*13*)].

There was virtually no change in the mean circadian period of eight animals observed for 20 days after the EB implant was removed, although minor changes did occur in individuals (Fig. 2). The shorter τ first induced by EB persisted as an aftereffect in each of four animals observed for many weeks after the hormone capsule was withdrawn (*14*). To the best of our knowledge, this result is the first demonstration of a persistent aftereffect of a chemically induced alteration in the circadian clock. The lengthening of τ began about 65 days after the hormone was removed (Fig. 2).

Estradiol consolidated activity bouts within the early hours of each subjective night; this was observed in 10 of 16 EB-implanted females (four showed no change and two others showed a broader distribution of activity) (*15*). There was no evidence of rhythm splitting or disintegration during the hormone treatments [compare with (*16*)].

Our results establish estradiol as one of a small number of substances that affect the period of circadian rhythms. This fact assumes added importance because endogenous estrogens almost certainly influence the phasing of physiological and behavioral rhythms; this may well be of significance for maintaining appropriate synchronization of the internal milieu as well as coordination of the organism with the external environment. Of particular note is the short latency with which estradiol can influence the circadian system. For example, the phase advance in wheel running first evident on day 3 of the estrous cycle occurs only 7 to 10 hours after the initial increase in the concentration of estradiol 17-β in blood plasma (*7*).

The circadian system is thought to be designed for the long-term maintenance of a constant relationship between biological rhythms and entraining agents (*17*); estradiol may be unique in its ability to change ψ on a daily basis. Our findings suggest that estradiol has direct access to the circadian system. The suprachiasmatic nuclei (SCN) are a critical part of the neural substrate for the generation or integration of circadian periodicity (or both) (*2*, *13*, *18*). Estradiol may change τ by directly influencing the SCN. However, the SCN do not appear to be among neural target tissues that selectively concentrate estradiol (*19*). We therefore propose that either (i) τ is affected by estradiol-binding systems (for example, in the medial preoptic region) that provide direct neural input to the SCN (*20*), or (ii) estradiol acts on the neural substrate for circadian rhythmicity through nonclassical binding systems, perhaps by influencing membrane-related events (*21*).

It is notable that the estradiol-induced change in τ persisted long after the hormone-containing capsule was removed. It is unlikely that residual systemic hormonal stimulation is responsible for this effect (*22*); instead, the effect may reflect a resetting of circadian oscillators by supranormal hormonal stimulation that exceeds the maximal estrogenic stimulus available during the estrous cycle. In ovariectomized hamsters entrained to the LD 12 : 12 cycle, the phase-angle differences that existed before hormone treatment reappeared relatively rapidly (5.4 ± 1.5 days) after the EB implants were removed; aftereffects of the hormone may be counteracted by light influencing the SCN through the direct retinohypothalamic projection (*23*).

Table 2. Effect of estradiol benzoate on the period (τ) of circadian wheel-running activity in blind and ovariectomized hamsters. Periods are given as means ± standard errors of the means.

Treatment	Number of animals	Period (hours)	
		Pre-treatment	During treatment
Estradiol benzoate	11	24.18 ± .04	23.91 ± .02*
Empty capsule	6	24.10 ± .03	24.09 ± .05
Progesterone	4	24.24 ± .07	24.20 ± .05

*The change in τ for animals in this group was significant at $P < .001$, two-tailed t-test.

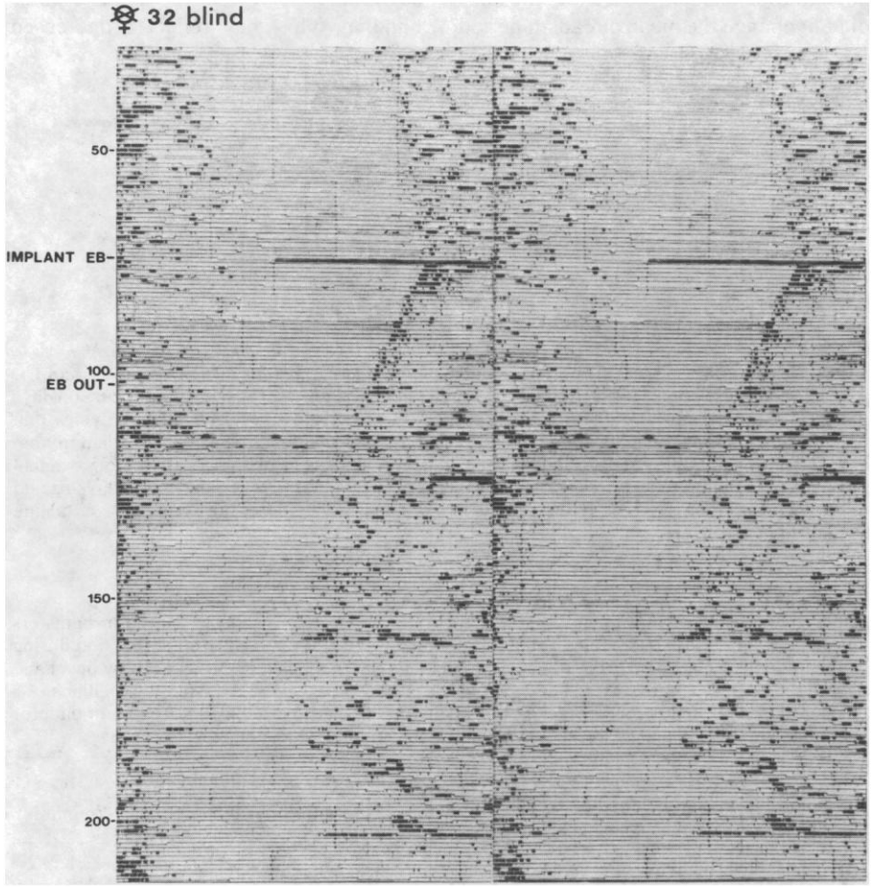

Fig. 2. Continuous record of wheel-running activity of an ovariectomized and blind hamster in which a capsule containing estradiol benzoate (*EB*) was implanted and subsequently removed. The record has been photographically duplicated and double-plotted on a 48-hour time base to aid visual inspection of the free-running rhythm. The numbers on the left indicate the time in days since this animal was blinded.

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9. Illumination intensity during the light phase was about 130 lux at the cage level.
10. Differences in the amount of wheel running were not significant among the groups during the pretreatment period; each treatment induced an approximately 20 percent decrease in the number of wheel revolutions. In each of seven hamsters tested, removal of the EB capsules delayed the phase of wheel running by delaying its onset. Similar delays occurred in only 3 of the 12 animals upon removal of the empty or progesterone capsules ($P < .005$, Fisher exact probability test).
11. The interval (mean ± standard error of the mean) between blinding and the insertion of the capsules was 61.7 ± 15.8 days for the empty-capsule group and 52.4 ± 6.3 days for the group given EB capsules.
12. The activity records were pasted day below preceding day. The circadian period was measured by fitting a straight line by eye through the onset of activity. Two of us (L.P.M. and K.M.F.) independently calculated τ during the 20 days that preceded each manipulation. The τ for the treatment interval was calculated for a block of 20 consecutive days after the period of the activity rhythm had stabilized. In six instances, shorter intervals (between 9 and 18 days) were used to estimate τ. Estimates were made without reference to prior or subsequent activity patterns. The two estimators were in agreement to within .04 hour on 39 of the 42 estimates; the remaining three estimates were reanalyzed independently. The mean of the two independent estimates was used in all analyses.
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Stress-Induced Modulation of the Immune Response

Abstract. *After mice were exposed to a daily auditory stressor for varying lengths of time, the responses of their splenic lymphoid cells in vitro were assessed. Both the blastogenic activity of concanavalin A or lipopolysaccharide and the ability of immune lymphocytes to lyse P815 target cells showed the same patterns of immunosuppression and enhancement.*

While the immunosuppressive properties of short-term exposures to various stressors have been well established (*1*), the effects of long-term stress on the immune response are less clear (*2*). To further elucidate the latter interaction, we have assessed in vitro the responses of lymphoid cells obtained from mice subjected to various periods of environmental stress. The immunoresponsiveness of splenic lymphocytes was evaluated by determining their blastogenic activity following mitogen stimulation (*3*), and by the ability of splenic lymphocytes obtained from mice immunized in vivo to kill P815 target cells (*4*).

Male mice (7 to 12 weeks old) of the AKR or $C57/Bl_6$ strains were used. The mice (four per cage) were subjected to a broad band noise at about 100 db daily for 5 seconds every minute during a 1- or 3-hour period around midnight, at the height of the diurnal activity cycle. Unstimulated controls were exposed only to the normal activity of the animal room. The controls and experimental mice were killed at the same time. Their spleens were removed, and suspensions of splenocytes were prepared. Erythrocytes were lysed with 0.83 percent NH_4Cl buffered to *p*H 7.5 with tris for 10 minutes and then washed in Hanks balanced salt solution. Cells were resuspended and maintained in RPMI 1640 and 10 percent heat-inactivated fetal calf serum.

Both assays of immunologic reactivity, one nonspecific and the other specific, showed the same temporal pattern of hypo- and hyperresponsiveness (Fig. 1), the data having been confirmed in further replications. The activities of both B- and T-cells (derived from bone marrow and thymus, respectively) appeared to be affected similarly: B-cell function as reflected by stimulation with lipopolysaccharide (LPS), and T-cell function as reflected both by stimulation with concanavalin A (Con A) and by the specific lysis of P815 target cells.

The clearest demonstration of the stress-induced modulation of immune function is found in Fib. 1B. Short-term exposure of the animals to the sound stressor (initiated during the week preceding or following immunization with a T-dependent antigen) clearly depressed the lymphocyte-mediated cytotoxic response, while enhancement occurred with longer exposures to sound stress.

Assays of plasma cortisol (*5*) in similarly stressed $C57/Bl_6$ mice (Fig. 2A) show that there is an increase in the circulating levels of this adrenal corticosteroid corresponding to the depression of the immunologic function, and not apparently associated with the enhancement. Daily exposure to the sound stress for more than 10 days produces an adaptation which brings cortisol to base-line levels. However, acute stress increases the blood cortisol to concentrations that are able to suppress the immunologic response to antigens introduced during this period. Coincident with the heightened steroid level is a decrease in the number of viable nucleated cells recovered per spleen from the mice subjected to short-term stress compared to the number recovered from the nonstressed controls (Fig. 2B). This result corresponds to similar decreases in lymphocyte cytotoxicity and spleen index after the administration of exogenous hydrocortisone, as reported by Fernandes *et al.* (*6*). Such depletion of lymphoid cells could occur as a result of either cortisone-induced toxicity or changes in lymphocyte migration patterns (*7*). It is interesting that there is a comparable de-