tion of the sides and floor of the fourth ventricle, forming the dorsal landmark known as the obex (*14*). The highly vascular area postrema lacks a blood-brain barrier (*14*), and has been shown by Borison and Wang (*13*) to mediate the emetic effects of digitalis. Preliminary studies demonstrate that the area postrema may affect circulatory hemodynamics, presumably through the autonomic nervous system (*14*). Activation of this area by digitalis glycosides would be consistent with the effects observed in the lesioning experiments and also with the apparent neural facilitation of arrhythmias by ASI-222, a polar cardiac glycoside that does not cross the blood-brain barrier in detectable amounts.

The experiments described here confirm the appreciable influence exerted by neural factors in the facilitation of digitalis-induced cardiac arrhythmias. Further, these studies define an area in the medulla within 2 mm of the obex as the locus for the neurally mediated arrhythmogenic properties of digitalis.

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Delay of Constant Light–Induced Persistent Vaginal Estrus by 24-Hour Time Cues in Rats

Abstract. *The normal ovarian cycle of female rats is typically replaced by persistent estrus when these animals are housed under constant light. Evidence presented here shows that the maintenance of periodicity in the environment can at least delay (if not prevent) the photic induction of persistent vaginal estrus. Female rats in constant light were exposed to vaginal smearing at random times or at the same time every day. In another experiment, female rats were exposed to either constant bright light, constant dim light, or a 24-hour photic cycle of bright and dim light. The onset of persistent vaginal estrus was delayed in rats exposed to 24-hour time cues even though the light intensities were the same as or greater than those for the aperiodic control groups. The results suggest that the absence of 24-hour time cues in constant light contributes to the induction of persistent estrus.*

When female albino rats are placed in constant light, their 4-day estrous cycle of sexual receptivity, ovulation, and vaginal cornification is replaced over several weeks by continuous sexual receptivity, chronic vaginal cornification, and cessation of ovulation (*1-3*). This persistent estrus could be attributed either to the increase in the amount of light as such, or to the absence of strong 24-hour time cues, or to both. An explanation solely in terms of amount of light has been favored for two reasons. First, rats do not develop persistent estrus in constant darkness despite the fact that strong 24-hour time cues are absent (*1*, *4*). Second, an increase in the intensity of constant light speeds the onset of persistent estrus (*5*).

Despite the importance of light intensity, the absence of 24-hour time cues in constant light could interact with light to induce persistent estrus. Chen and Besch (*6*) have provided some support for this hypothesis. Rats in a cycle of 2 hours of light alternating with 2 hours of darkness (LD 2:2), a schedule that does not synchronize the rat's circadian activity cycle (*7*), showed lengthened estrous cycles with increased vaginal cornification compared to rats in a LD 12:12 cycle even though the total amount of light was the same in both conditions.

If the absence of 24-hour time cues contributes to the induction of persistent estrus in constant light, then adding such time cues should delay the onset of persistent vaginal estrus. In the present study 24-hour time cues were added in two ways: by taking vaginal smears at the same time each day in experiment 1, and by superimposing 12 hours of bright light on constant dim light every 24 hours in experiment 2. Onset of persistent estrus was defined as the first day of eight or more consecutive smears showing cornified or nucleated vaginal epithelial cells (*8*).

In experiment 1, 18 female rats were exposed to constant light (35 lux) for 53 days. Vaginal smears were taken from half the animals at the same time (1100) 6 days a week and from the other half at random times 6 days a week (*9*). Because periodic noise can be an effective time cue for synchronizing the activity cycle of some rats (*10*), the two groups were housed in separate rooms that were entered only to take vaginal smears.

As shown in Fig. 1, rats subjected to 24-hour periodic vaginal smearing developed persistent estrus more slowly (median onset, 44 days) than rats subjected to random vaginal smearing (28 days) ($P < .05$, Mann-Whitney U test, one-tailed). Although both groups were exposed to the same amount of light and vaginal smearing, the group with the 24-hour nonphotic time cue maintained estrous cyclicity longer.

In experiment 2, eight rats were ex-

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posed for 67 days to constant bright (700 lux) light, eight to constant dim (10 lux) light, and eight to 12 hours of bright light alternating with 12 hours of dim light. Vaginal smears were taken randomly 6 days a week (*11*).

If the amount of light per se is the predominant factor in the induction of persistent estrus, then the onset of persistent estrus should be fastest for the group in constant bright light, slowest for the group in constant dim light, and intermediate for the group in cycling light. On the other hand, if the lack of 24-hour time cues is an important factor, then the onset of persistent estrus for the cycling light group should be delayed compared to that in both constant light groups.

As shown in Fig. 2, the absence of photic periodicity in constant dim light led to an earlier, and higher, incidence of persistent estrus than did a light cycle in which the average light intensity was greater (10 lux compared to an average of 355 lux in cycling light). Animals in the cycling light group developed persistent estrus more slowly than either group in constant light ($P < .01$, Mann-Whitney U test, one-tailed, for both comparisons). There was a nonsignificant trend ($P < .10$, Mann-Whitney U test, one-tailed) toward an earlier onset of persistent estrus when constant light intensity was increased (median onset: 17 days at 700 lux and 38 days at 10 lux), as reported by others (*5*).

These experiments demonstrate that the addition of 24-hour time cues by two different methods (photic and nonphotic) delays the onset of persistent vaginal estrus induced by constant light. The results support the hypothesis that the absence of 24-hour cues in constant light contributes to the induction of persistent estrus.

The fact that persistent estrus is induced in constant light whereas estrous cyclicity with a period of about 4 days is maintained in constant darkness (*12*) is similar to the response of many circadian rhythms to light. In general, these rhythms fade out more quickly in constant light than in constant darkness (*13, 14*). For example, the circadian activity rhythms of house sparrows (*15*) and hamsters (*16*) persist in constant darkness (and constant dim light) but are disrupted in bright constant light. Likewise, circadian rhythms of pineal *N*-acetyltransferase, serotonin, and melatonin in the rat and chicken persist in constant darkness but fade in constant light (*17*).

The maintenance of rhythms in constant light by 24-hour time cues has been demonstrated in a few nonmammalian species. Hillman (*18*) showed that a 24-hour temperature cycle prevents the damage to tomato plants that occurs in constant light and temperature. He suggested that the damage may be due to the disruption of an endogenous circadian rhythm in constant conditions. Indeed, Bünning (*19*) showed that in constant light and temperature the circadian rhythm of leaf movement in *Phaseolus multiflorus* is disrupted but can be reinitiated by a sudden temperature change. The circadian rhythm of eclosion in drosophila also damps out in constant light and temperature but is maintained by a 24-hour temperature cycle (*13*). Our data are the first to our knowledge to demonstrate that 24-hour time cues can maintain rhythmicity in constant light in a mammal.

Two different mechanisms could account for the delay of persistent vaginal estrus in constant light by 24-hour time cues. A single biological oscillator whose rhythmic activity is suppressed by light but maintained by 24-hour time cues may regulate the estrous cycle. The suprachiasmatic nuclei, whose destruction results in persistent estrus, locomotor and drinking arrhythmias, and chronically low levels of pineal *N*-acetyltransferase (*16, 20*) may be the site of such an endogenous oscillator (*21*). Alternatively, the estrous cycle may consist of multiple endogenous oscillators that are normally synchronized with each other. Constant light may allow these oscillators to free-run independently, and the resultant "uncoupling" of the biological oscillators could lead to persistent estrus. Lawton and Schwartz (*3*) have found that vaginal, uterine, ovarian, and hypophyseal components of the estrous cycle undergo dysphasia during the first five cycles in constant light. A 24-hour time cue may delay the internal desynchronization of rhythmic components by providing an external synchronizer to which they can entrain (*22*).

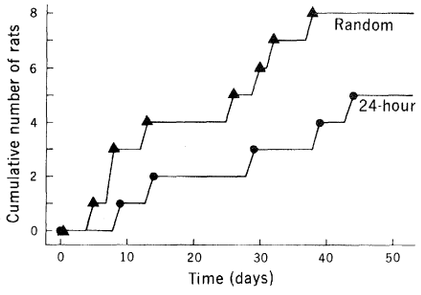

Fig. 1. Cumulative onset of persistent vaginal estrus for rats exposed to random or 24-hour periodic vaginal smearing in constant light. Onset of persistent estrus is the first day of eight or more consecutive smears showing cornified or nucleated vaginal epithelial cells.

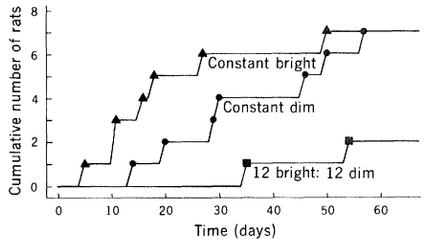

Fig. 2. Cumulative onset of persistent vaginal estrus for rats exposed to constant bright light, constant dim light, or 12 hours of bright alternating with 12 hours of dim light.

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9. Sprague-Dawley (Cox) rats were received at about 97 days of age from Laboratory Supply (Indianapolis, Ind.) where they had been raised in LD 12:12 (lights 0630 to 1830). The rats were maintained in the same light cycle for 2 weeks before the start of the experiment. On arrival in the laboratory, vaginal smears were taken by saline lavage with a dropper and examined under 100× magnification without staining. Smears were taken from all the rats in the random group at either 0800, 1100, 1400, 1700, or 2000 depending on the day of the week. Each rat was housed individually in a cage 20.3 by 26.7 by 22.9 cm (Unifab Corp., Kalamazoo, Mich.). Each group was housed in a light-tight ventilated metal cabinet 2.0 by 0.9 by 0.5 m equipped with fluorescent lights (20-W cool white) partially masked with black electrical tape. All cages were equidistant from the light source. The luminance was measured at the bottom of the cage with a MacBeth illuminometer. Purina rat chow and water were freely available and were replenished when vaginal smears were taken.
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Inhibition of Gonadotropin by Δ^9-Tetrahydrocannabinol: Mediation by Steroid Receptors?

Abstract. *Competition assays for estradiol receptors in cytosol preparations of uteri from rhesus monkeys and humans showed that Δ^9-tetrahydrocannabinol (THC) does not compete with estradiol for intracellular estrogen receptors. Although isotopically labeled THC bound to macromolecules in uterine cytosol from the rhesus monkey, the binding was not displaced by unlabeled THC, diethylstilbestrol, estradiol, progesterone, cortisol, or 5α-dihydrotestosterone. Scatchard analyses indicated that high-affinity saturable binding of THC to cytosol did not occur. Thus the inhibitory effect of THC on gonadotropin and steroid secretion in primates is not mediated by the interaction of THC with intracellular steroid hormone receptors.*

Long-term use of large amounts of marihuana may adversely affect sexual and reproductive functions (*1*). The mechanism by which marihuana use produces these effects is not known. We showed previously that Δ^9-tetrahydrocannabinol (THC), the major psychoactive component of marihuana, can cause significant decreases in the sex hormones of the adult male and female rhesus monkey (*2*). This inhibitory effect is thought to be produced by a reversible pituitary-hypothalamic action, since both gonadotropins and sex steroids are decreased.

It has also been suggested that marihuana disrupts the reproductive system by acting directly at the cellular level on the reproductive target organs. Studies by Shoemaker and Harmon (*3*) indicate that THC may compete with sex steroids for their receptor proteins in the target organ cells. The binding of THC to these receptors would either antagonize the trophic effects of steroids in these tissues or produce the same trophic effect as the sex steroid. Solomon *et al.* (*4*) demonstrated a positive trophic effect of THC on reproductive tissues in ovariectomized rats injected intraperitoneally with THC. However, their work was criticized by Okey and Bondy (*5*) who claimed that experiments in which THC is administered intraperitoneally give unreliable results and cause inflammation of abdominal organs. Further, Okey and Bondy found that unlike estradiol, THC administration did not elicit a dose-dependent increase in uterine weight and did not compete in vitro for estrogen receptor sites in rodents. In an attempt to settle the dispute, we have studied the binding of THC to estrogen receptors in primates rather than rodents, because Smith *et al.* (*6*) demonstrated that steroid receptors from different animals do not necessarily have the same ligand binding specificity.

We prepared cytosols from the uteri of rhesus monkeys that had been ovariectomized 4 days prior to hysterectomy and from the uteri of humans undergoing voluntary therapeutic hysterectomy. The tissues were minced and homogenized at 4°C in four volumes of buffer containing 10 m*M* tris-HCl (*p*H 7.4), 1.5 m*M* EDTA, 12 m*M* thioglycerol, and 10 percent glycerol. The homogenate was centrifuged at 1000*g* for 15 minutes to remove nuclei, and the resulting supernatant was centrifuged at 105,000*g* for 90 minutes. A fixed concentration of the supernatant was incubated with increasing concentrations of [^{3}H]estradiol (specific activity, 96 Ci/mmole; 0.1 to 4.0 n*M*) for 18 hours at 4°C in the presence and absence of a 250-fold excess of diethylstilbestrol (DES). We used DES to measure the nonspecific binding of [^{3}H]estradiol in cytosol because at these concentrations DES displaces estradiol from its receptor sites rather than from any contaminating serum binding proteins such as sex-steroid binding globulin. After the incubation period, the [^{3}H]estradiol not bound to macromolecules was removed by charcoal adsorption (*7*). Scatchard analyses (*8*) were performed on these data. The specifically bound estradiol was computed by subtracting nonspecific binding from total binding; the specifically bound compound was plotted against the ratio of bound to free estradiol.

Figure 1 shows a typical curve for the rhesus monkey estrogen receptor. The curve is biphasic, similar to that observed previously in Scatchard analyses of data from human uterine cytosol and chick oviduct cytosol (*9*). By using the method described by Rosenthal (*10*) to resolve the two binding components, we found that the receptor having the highest affinity for estradiol had an equilibrium dissociation constant (K_D) of 0.17 ± 0.07 n*M* ($N = 4$). This K_D is similar to that measured for the human uterine cytoplasmic estrogen receptor (*11*). The concentration of binding sites for estradiol in the monkey uterine cytosol was 22 ± 4 pmole per milligram of protein ($N = 4$). From the Scatchard analyses we selected a concentration of [^{3}H]estradiol which preferentially bound to the high-affinity estrogen receptor, and we used this concentration in the subsequent competitive binding studies to confirm that the [^{3}H]estradiol binding was specific for estradiol and DES.

To determine whether THC would compete for estrogen receptor sites, we performed competitive binding assays on cytosol using a fixed concentration of [^{3}H]estradiol (2 n*M*) with increasing concentrations of THC (0 to 3.8 μ*M*); for comparison, we performed similar assays with increasing concentrations of DES (0 to 1.2 μ*M*). The mixtures were incubated for 18 hours at 4°C and the amount of [^{3}H]estradiol bound at each concentration of competitor was determined by charcoal adsorption assay. The amount of [^{3}H]estradiol bound was then plotted against the log of the concentration of the competitor.

Figure 2 shows that whereas DES competes significantly for estrogen receptor sites at a concentration as low as 1.4 n*M* (0.2 ng), THC does not compete even at a concentration of 3.8 μ*M* (600 ng). Table 1 shows results of studies with three additional uterine specimens, together with the results for cytosols from human uteri. The slopes of the DES competition curves from the data in

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