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Circadian organization of the estrous cycle of the golden hamster

(circadian rhythms/sexual differentiation/gonadotrophins/locomotor activity)

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ABSTRACT **In constant dim illumination the hamster estrous cycle free-runs with a period that is a quadruple multiple of the concurrently recorded rhythm of wheel-running activity; both activity and estrous cycles are generated by biological clocks. Maintenance of stable phase angle differences between heat onset and running onset before and after treatment with deuterium oxide suggests that a common circadian system generates periodicities in estrus and activity. An organization of the estrous cycle is proposed in which the stimulus for the ovulatory surge of luteinizing hormone is generated by a circadian system that includes the suprachiasmatic nuclei of the hypothalamus. Various possible interactions of estradiol and photoperiod with the neurogenic stimulus for the luteinizing hormone surge are described and implications of different types of circadian organization of the estrous cycle for theories of sexual differentiation are considered.**

Estrous and menstrual cycles are readily detectable in a wide variety of spontaneously ovulating polycyclic mammals (1, 2) and were among the first hormonally related rhythms described (3). Typically, the interval between successive recurrences of ovulation, behavioral receptivity (estrus), or release of pituitary ovulating hormone is relatively constant and species specific.

Female reproductive cycles depend upon rhythmic release of hormones from the anterior pituitary. The underlying basis for this phenomenon "remains one of the more poorly understood phenomena in regulatory biology" (ref. 4, p. 607). One exciting possibility is that these cycles are manifestations of biological clocks, defined as endogenous self-sustained oscillators specialized for time measurement (5, 6). If one were to establish the clock-like nature of the mechanism underlying the mammalian estrous cycle, then studies of reproductive phenomena could benefit from substantial theoretical and empirical generalizations of the discipline of biochronometry (6).

To establish that an observed rhythm is the manifestation of a biological clock it is adequate to demonstrate that the rhythm persists under constant environmental conditions with a period that differs significantly from that under entrained conditions. This has been accomplished for the estrous rhythm of one mammal. In a landmark study, Alleva *et al.* (7) observed that the period of the hamster estrous cycle was 96 hr when the animals were exposed to a 16 hr light:8 hr dark (LD 16:8) cycle; in constant illumination (LL), the estrous cycle persisted, with 18 of the 20 animals generating periods that differed significantly from 96 hr (range of 95.35–97.54 hr). This approximately 4-day cycle (a circaquadridian rhythm) established the endogenous nature of the hamster estrous cycle. Alleva *et al.* (7) speculated that the clock for the estrous cycle functioned with a circadian rather than a circaquadridian frequency. This hypothesis is supported by several lines of evidence; e.g., spontaneous or experimentally induced changes in the estrous cycle most commonly extend it by a single day and seldom by an integral multiple of 4 days (7–9). Subsequently a circadian mechanism was also implied by daily luteinizing hormone (LH) surges recorded in estrogen-primed ovariectomized hamsters (10) and in acyclic female hamsters maintained in nonstimulatory photoperiods as well as in lactating hamsters (11, 12).

The circadian organization of the hamster estrous cycle may be assessed more directly by comparing the circaquadridian estrous cycle with a known circadian rhythm (e.g., wheel running). Under appropriate constant conditions, one would expect two rhythms generated by separate oscillators with different frequencies to dissociate; i.e., over the course of some weeks, the phase angle difference (ψ) between the end-points used as indices of the underlying oscillators would progressively change. This phenomenon occurs in human circadian temperature and activity rhythms that free-run with quite different periods under constant conditions (13). If, on the other hand, the two rhythms were phase-locked to a single oscillator or to a system of hierarchically or mutually coupled oscillators, they would free-run with identical periods and maintain a constant ψ between the endpoints used to define these rhythms. Several aspects of these propositions were examined by concurrently monitoring the behavioral estrous and wheel-running cycles of hamsters, with a view to establishing the relation between their free-running periods.

MATERIALS AND METHODS

Female hamsters (*Mesocricetus auratus*) were maintained from birth on a photoperiod of 14 hr of light per day (LD 14:10). Each female selected for further study was at least 90 days old and was undergoing normal estrous cycles, as manifested by the post-ovulatory vaginal discharge every fourth day at 0900 hr, Pacific Daylight Time (14) and by the recurrence, at 4-day intervals, of behavioral estrus.

Upon satisfying these criteria each female was placed individually in a cage that provided free access to an activity wheel; wheel revolutions were monitored separately for each animal on an Esterline-Angus event recorder in continuous operation throughout the experiment at a chart speed of 18 inches per 24 hr. The activity cages were housed on a single rack and exposed to constant dim illumination provided by four 7.5 W incandescent bulbs. The light intensity at the level of the cage tops was between 100 and 200 lumens/m^2. Simonsen rat food (pelleted maintenance diet) and tap water were available ad libitum. Supplies were replenished and the pans beneath the cages were cleaned at weekly intervals; animals did not appear to be disturbed by these procedures. Heat onset (HO), defined by the female's assumption of the lordosis posture, was determined by placing a male in the female's cage and then stroking her hindquarters with a brush at 15-min intervals. The combination of these procedures maximized the reliability of HO and the time at which lordosis was first assumed was recorded.

Abbreviations: LH, luteinizing hormone (lutropin); GnRH, gonadotrophin releasing hormone (gonadoliberin); LD, light dark cycle; LL, constant light; D_2O, deuterium oxide; HO, heat onset; RO, running onset; ψ, phase angle difference; SCN, suprachiasmatic nuclei.

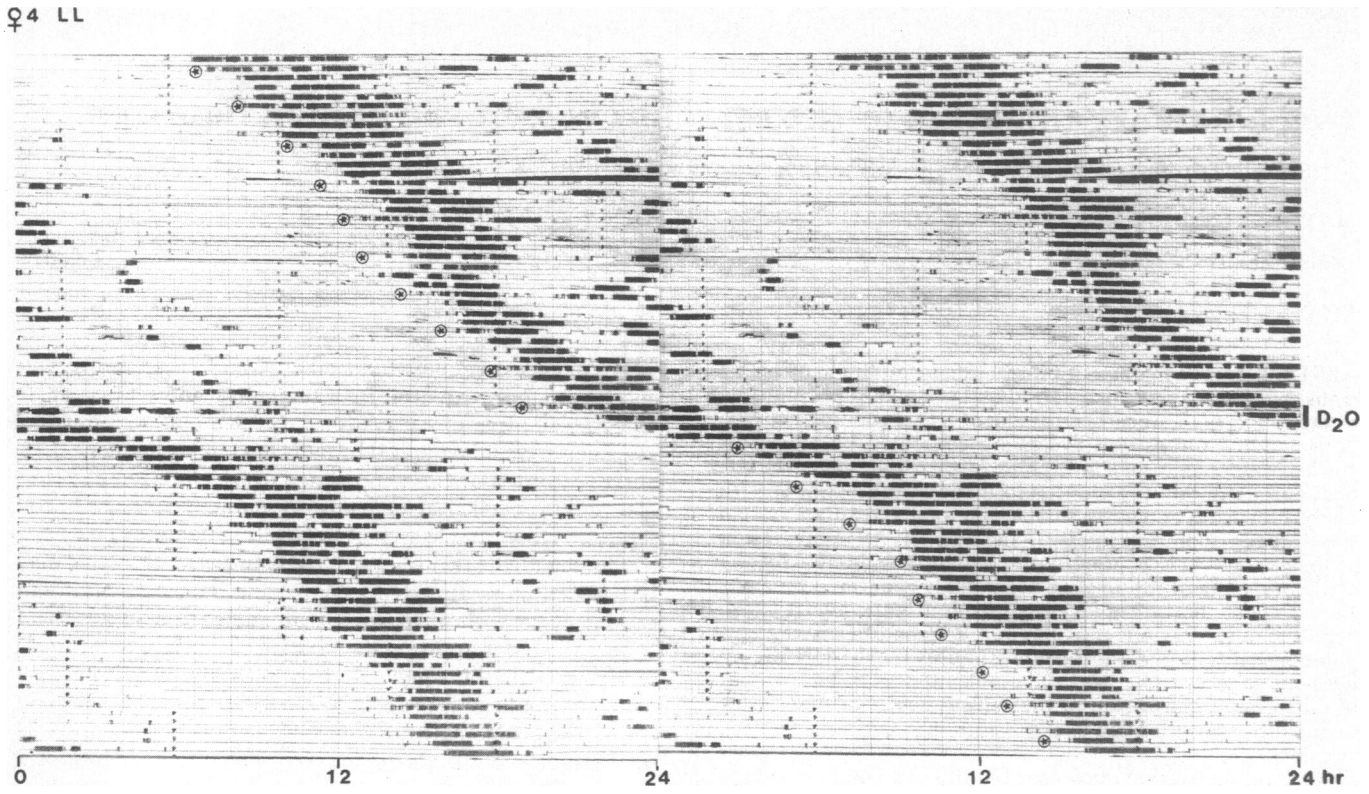

FIG. 1. Free-running heat onset and wheel-running behavior of a hamster maintained in constant dim illumination. The record has been double plotted in the standard way to permit visualization of the continuity of the free-running rhythms. The days during which the animal was drinking 50% D_2O are indicated by a vertical bar to the right of the activity record. Heat onsets are designated by the circled stars. The abscissa indicates Pacific Standard Time.

Copulation was prevented in every instance by appropriately timed removal of the male. Testing had to begin at least 45 min prior to HO for the given onset point to qualify for use in the data analysis. Since preliminary experimentation showed that HO was displayed only on days of a proestrus vaginal smear record, testing was subsequently limited to every fourth day, beginning with the day on which the initial HO was established. The daily vaginal smear procedure was discontinued while the periodicity of the estrous and activity cycles was being assessed, except as noted below.

Running onset (RO) in the activity wheel was often erratic on the day that a given hamster was tested for HO; running data obtained on those days were therefore not included in the analyses. Separate regression analyses were performed to determine the straight lines ($Y = a + bX$) that best fitted HO and RO. The coefficients a and b were calculated by the method of least squares. For a given hamster the Y values corresponding to HO or RO were expressed in hours from the noon or midnight nearest the first analyzed data point; X was the day of measurement. These procedures were adopted from those used by Alleva *et al.* (7). The period (τ) of the estrous cycle could thus be determined by the formula $\tau_E = 96 + 4\,b_{HO}$ hr and the τ for running by $\tau_R = 24 + b_{RO}$ hr. The difference between the Y intercept values for a given set of running and heat onsets ($a_{RO} - a_{HO}$) represents the phase angle difference (ψ) between these events. After at least four heat onsets had been obtained the tap water of four females was replaced with 50% deuterium oxide (D_2O) dissolved in a 3% dextrose–0.125% saccharin vehicle; the animals were each allowed to consume between 30 and 40 ml of the D_2O solution, at which point they were returned to drinking tap water.

Animals were tested as long as HO and RO could be obtained. After an animal had ceased to display HO at the expected times and before it was discarded from the testing pool, the daily vaginal smear procedure was re-instated.

RESULTS

The free-running nature of HO and RO is readily apparent from Fig. 1 and is confirmed by the different slopes associated with the different animals (Table 1); this attests to the lack of synchronization of these rhythms by environmental cues.

HO and RO were well described by the regression analyses; 21 of the 22 data sets analyzed had correlation coefficients significant at $P < 0.05$, or better. The most significant finding is that the slope (b) of the line describing HO for any given animal was not significantly different from the slope for the corresponding series of RO (Table 1); in some instances the slopes were identical or nearly so (hamster 3, days 40–54). This confirms and extends findings reported in abstract form by Alleva *et al.* (15). The period of the hamster estrous cycle is thus a multiple (×4) of the circadian running rhythm, both under free-running conditions and in the entrained state (7).

Brief deuteration markedly increased the τs of the hamster locomotor and estrous rhythms (Fig. 1, Table 1). The maintenance of a relatively stable ψ between HO and RO before and after deuteration is not inconsistent with the hypothesis that a single system underlies the cyclic recurrence of RO and HO. The extreme period lengthening during deuteration neither split the activity rhythm (cf. 6) nor produced any aberrant ψ during the post-deuteration interval assessed in this study.

The ψs assessed across different animals, and including only the first determination for each hamster, were positively correlated with the τs of the free-running circadian rhythms. The regression line describing this relation ($P < 0.05$) was also sig-

Table 1. Regression analyses ($Y = bX + a$) of heat onset (HO) and running onset (RO) for female hamsters maintained in constant dim illumination

Female no.	Days analyzed	Endpoint*	b†	a	ψ (hr)‡	Period (hr)	D_2O consumed on days	50% D_2O consumed (ml)
4	53–90	HO (10)	0.320	6.44	1.00	97.28	90–92	36
		RO (28)	0.336	7.44		24.34		
	96–132	HO (9)	0.307	5.27	1.38	97.23		
		RO (28)	0.301	6.65		24.30		
5	58–68	HO (3)	0.845	0.72	5.50	99.42		
		RO (8)	0.883	6.22		24.88		
	83–101	HO (4)	0.811	8.42	3.76	99.24	101–103	36
		RO (13)	0.903	12.18		24.90		
	108–119	HO (3)	0.759	9.64	4.20	99.04		
		RO (9)	1.103	13.84		25.10		
3	14–37	HO (4)	0.419	7.81	2.56	97.68	37–38	37
		RO (19)	0.400	10.37		24.40		
	40–54	HO (2)	0.646	0.96	1.59	98.58		
		RO (12)	0.646	2.55		24.65		
2	27–53	HO (4)	−0.106	8.34	1.65	95.58		
		RO (20)	−0.116	9.99		23.88		
	52–68	HO (5)	0.058	6.19	1.09	96.23	68–69	31
		RO (12)	0.089	7.28		24.09		
8	55–88	HO (6)	0.239	7.53	1.97	96.96		
		RO (25)	0.222	9.50		24.22		
9	13–34	HO (5)	0.135	6.18	1.97	96.54		
		RO (17)	0.112	8.15		24.11		

For each endpoint, Y refers to time of day and X to day analyzed. Groups of endpoints were analyzed separately before and after deuteration (females 4, 5, and 3), after substantial changes in the free-running period (female 2), and after lengthy intervals without testing (female 5).

* The number of onsets of the behavior used in the regression analysis is shown in parentheses.

† The correlation coefficient for each set of points was significant at $P < 0.05$. The single exception was the b_{HO} for female 2 on days 52–68. No b_{HO} was significantly different from its corresponding b_{RO}.

‡ The correlation coefficient between ψ and free-running circadian period was significant at $P < 0.05$ for the first determination per animal and at $P < 0.01$ for all determinations.

nificant if all points, including multiple determinations on some animals, were included in the analysis ($P < 0.01$).

Irregularities developed in the estrous cycles of some animals maintained in LL; this was expected from earlier reports (16, 17) and presented a further opportunity to assess the relation of the circadian system to estrous cyclicity. In no instance was a normal 4-day estrous cycle observed in hamsters with disrupted circadian activity rhythms ($n = 6$), although it must be noted that a rigorous analysis of the disrupted estrous rhythm was not attempted because daily tests for lordosis influence wheel-running activity. Hamster 8B (Fig. 2) shows a loss of integrated circadian rhythmicity and coincidentally there was a failure on our part to detect 4-day behavioral estrous cycles. In other hamsters failure to detect HO at the expected time was associated either with a spontaneous phase shift in the wheel-running rhythm or in disorganization of circadian wheel-running activity. On the other hand, loss of normal 4-day estrous cycles was not sufficient to change circadian organization. A number of hamsters who displayed lordosis at other than 4-day intervals or whose vaginal smear records indicated complete absence of estrous cyclicity generated normally integrated circadian wheel-running rhythms.

DISCUSSION

The hamster estrous cycle is dependent upon a biological clock and has a period that is a quadruple multiple of the concurrently recorded locomotor activity rhythm. The organization of the hamster estrous cycle outlined in Fig. 3 stipulates that the stimulus for the ovulatory surge of gonadotrophins is generated by a circadian system that includes the suprachiasmatic nuclei (SCN). The SCN signal is transmitted via unspecified neural pathways and possibly also via bloodborne chemicals to neural elements that store gonadotrophin releasing hormone (GnRH). These neurons can release GnRH into the hypophyseal portal vessels; the surge of gonadotrophins, of which LH is one species, occurs upon appropriate stimulation of the anterior pituitary by GnRH.

Because of the constant rate of LH release by isolated pituitary tissue (18) and failure of sex differentiation within the pituitary (19), we stipulate that *in vivo* plasma LH rhythms reflect rhythmic release or transport of GnRH and not clock-like properties of pituitary secretory elements (4). We specify the SCN as part of the relevant system because destruction of these nuclei is unique in disrupting the circadian activity rhythm (20–22) as well as the estrous cycle (23–25).

Is the relevant neurogenic signal generated on a circadian basis and if so why does the LH surge occur only every fourth day? Resolution of this question requires consideration of the role of ovarian estrogens in the aforementioned processes. LH surges are detectable only on the day of proestrus, lagging peak values of serum estradiol by several hr (26). In photostimulated hamsters LH surges are eliminated by ovariectomy, except that removal of the ovaries late on diestrus II or early on the day of proestrus does not preclude a surge on that day (27). LH surges also can be restored in ovariectomized hamsters treated with estradiol benzoate; a single injection of 5 μg of estradiol benzoate to ovariectomized hamsters results in repeated LH surges at 24-hr intervals and at times of day indistinguishable from that of the proestrous surge (10). The amount of estradiol secreted prior to proestrus (26) appears to be insufficient to permit the

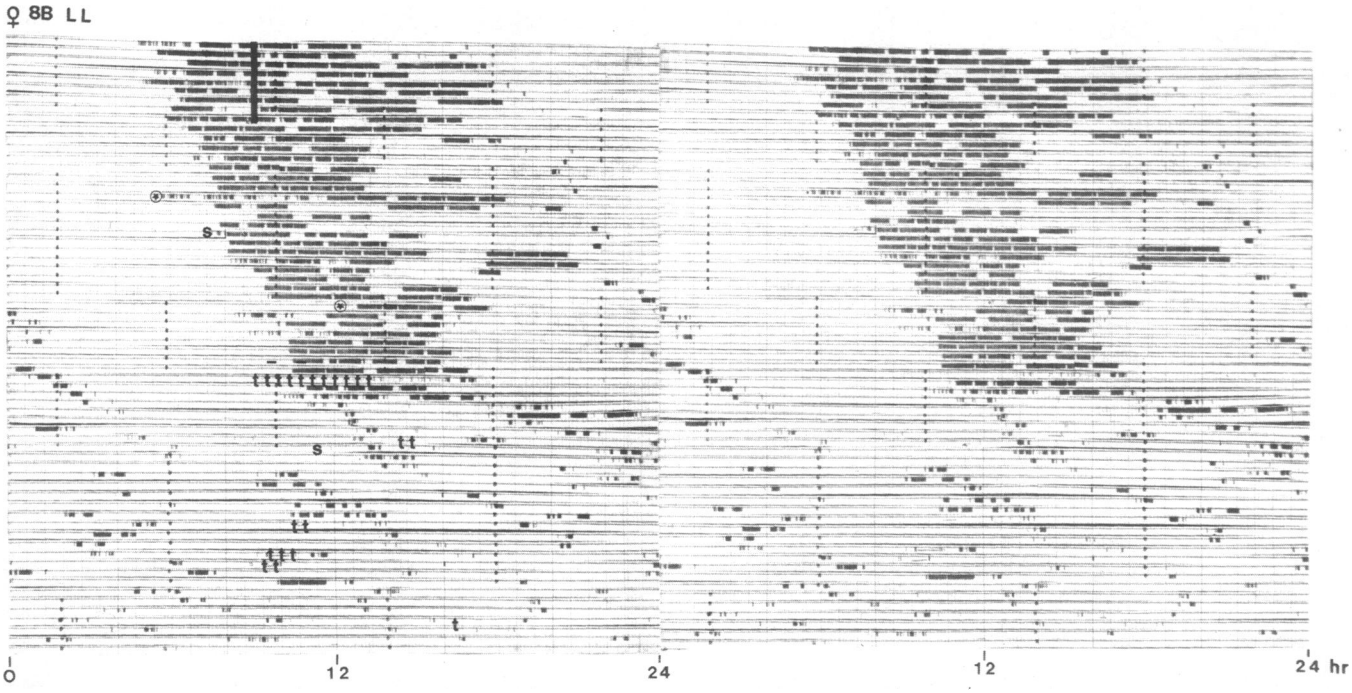

FIG. 2. Activity and heat behavior of a hamster maintained in constant dim illumination. Prior to and during the first 9 days of this portion of the activity record the animal's vaginal discharges were assessed daily at the time indicated by the vertical band. Normal 4-day cycles were recorded. During the next 2 weeks heat onset (designated by the circled stars) and presence of lordosis (s) were in keeping with our experience with other cycling free-running females. In the subsequent 2-week interval the 4-day estrous cycle was no longer detected and testing (t) at various times did not establish its re-emergence. Note the deterioration of the running pattern during this time. The animal's physical appearance was normal. Other symbols and conventions are as in Fig. 1.

normal LH surge. We have considered four ways in which estradiol could influence the occurrence and timing of the LH surge. Estradiol may: (*i*) activate coupling mechanisms between the circadian system and the GnRH release system, (*ii*) sensitize GnRH-containing neurons to the circadian signal, (*iii*) increase pituitary sensitivity to GnRH (28) and thereby permissively regulate timing of the LH surge by the circadian system, or (*iv*) actually control *generation* of the neurogenic stimulus for LH release (see Fig. 3).

Daily LH surges occur in ovariectomized as well as intact acyclic hamsters maintained in a nonstimulatory photoperiod (LD 10:14) and in hamsters that for unknown reasons become acyclic or are lactating in the LD 14:10 cycle (11, 12). The timing and magnitude of LH peaks in these animals is also suggestive of the proestrous surge. Seegal and Goldman speculate that changes in photoperiod and in the secretion of estradiol each induce rhythmic LH secretion via an unspecified common mechanism (11). We suggest that the principal relevant change effected by photoperiod and by estradiol may be within the circadian system. In this scheme occurrence of the neurogenic stimulus essential for the LH surge depends on particular phase relations among constituent oscillators within this system. The signal is generated if and only if a critical internal coincidence of phases exists (cf. 6), regardless of the means used to establish this condition. These speculations are consistent with the known effectiveness of both photoperiod (29) and estradiol (20) in altering the period and phase relations of hamster circadian rhythms, and with the changes in phase that occur during the normal estrous cycle (20). It will require additional experimentation to establish whether estrogens in-

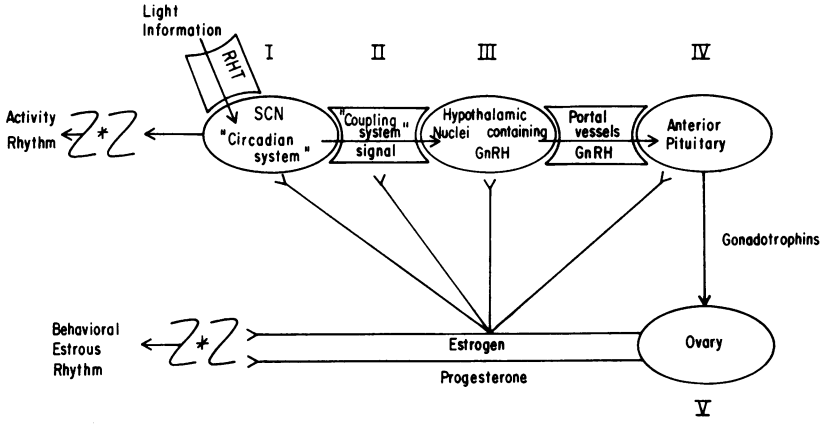

FIG. 3. Proposed features of the system required for the circadian timing of the hamster estrous cycle. Crucial estrogen effects occurring at I through IV (see discussion in *text*) may affect the period of the estrous cycle. The surge of gonadotrophins, including LH, can recur daily in the absence of the ovary (see *text*), which leaves I as the probable essential timer. * indicates additional neural systems not critical for the timing in question. RHT, retinohypothalamic tract; SCN, suprachiasmatic nuclei; GnRH, gonadotrophin releasing hormone.

fluence the generation of the signal as suggested here, or merely permit its expression (propositions *i*, *ii*, and *iii* above).

Specification of the nature of the underlying circadian system is important to our understanding of the process of sexual differentiation, as manifested in the cyclic female pattern of gonadotrophin release and in the tonic male secretory pattern (30, 31). According to the prevailing view, an inherently oscillatory neural region regulates cyclic release of gonadotrophins in female rodents (31). Cyclicity of this substrate is supposedly suppressed in males by androgens secreted during perinatal sexual differentiation. An alternative view is that the clock for cyclic LH release may be present in adults of both sexes and females differ from males according to the functional nature of the coupling between the circadian clock and the hypophysiotropic apparatus for gonadotrophin release (30).

If a single oscillator generates both RO and gonadotrophin release rhythms, then the persistence of circadian wheel running in adult male rodents implies that sexual differentiation does *not* suppress the relevant circadian clock; also, the suprachiasmatic nuclei are crucially involved in circadian wheel running and in estrous rhythms of rats and hamsters (20–22, 24, 25) and remain functional, at least with respect to the former rhythm, in adult animals of both sexes (21, 22, 32).

On the other hand, a coupled multi-oscillator system for running activity and gonadotrophin cycles permits the selective perinatal androgenic suppression in males of the clock responsible for cyclical gonadotrophin release. This can be accomplished without affecting a separate circadian activity substrate and represents a type of organization consistent with the classical view of sexual differentiation (cf. 31.)

According to the model (Fig. 3), the circadian substrate generates the signals necessary but not sufficient for the expression of the gonadotrophin surge. Loss of this surge and disruption of the estrous cycle, as measured by HO, ovulation, or vaginal discharge patterns, can evidently occur at a number of levels below the circadian oscillatory system (e.g., pituitary, median eminence) and need not affect unrelated rhythms regulated by the same circadian system. However, the model demands that any disturbance at the level of the basic circadian timekeeper will necessarily result in disruption of the estrous cycle. The data are consistent with this hypothesis; disturbances in the circadian system for wheel running invariably were accompanied by disrupted estrous cycles, whereas the converse relation did not obtain.

We speculate that circadian organization of the estrous cycle may be limited to a subset of mammals in whom environmental and physiological factors require a tight coordination between reproductive behavior and time of day. Although the spontaneously ovulating golden hamster can show behavioral estrus for as long as 18 hr (7), the deterioration of ova (33) and the time required for sperm potentiation (34) almost certainly limit the period of maximal fertility to a fraction of that interval. Furthermore, the female not in behavioral estrus can be intolerant of the male (35), and individuals may ordinarily be solitary in the field. Behavioral estrus and the fertile period must thus overlap with the period of nocturnal burrow emergence and activity to ensure the social interactions required for a successful mating. Precise timing may be less critical in species where one or another of these factors is altered (20).

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