Phenobarbital blockade of the preovulatory luteinizing hormone surge: association with phase-advanced circadian clock and altered suprachiasmatic nucleus *Period1* gene expression

Sandra J. Legan,1 Kathleen M. Donoghue,2 Kathleen M. Franklin,2 and Marilyn J. Duncan2

Departments of 1Physiology and 2Anatomy and Neurobiology, University of Kentucky Medical Center, Lexington, Kentucky

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Legan SJ, Donoghue KM, Franklin KM, Duncan MJ. Phenobarbital blockade of the preovulatory luteinizing hormone surge: association with phase-advanced circadian clock and altered suprachiasmatic nucleus *Period1* gene expression. Am J Physiol Regul Integr Comp Physiol 296: R1620–R1630, 2009. First published March 18, 2009; doi:10.1152/ajpregu.90914.2008.—The suprachiasmatic nucleus (SCN) controls the timing of the preovulatory luteinizing hormone (LH) surge in laboratory rodents. Barbbiturate administration during a critical period on proestrus delays the surge and prolongs the estrous cycle 1 day. Because a nonphotic timing signal (zeitgeber) during the critical period that phase advances activity rhythms can also induce the latter effect, we hypothesized that barbiturates delay the LH surge by phase-advancing its circadian timing signal beyond the critical period. In experiment 1, locomotor rhythms and estrous cycles were monitored in hamsters for 2–3 wk preinjection and postinjection of vehicle or phenobarbital and after transfer to darkness at zeitgeber time (ZT) 6 on proestrus. Phenobarbital delayed estrous cycles in five of seven hamsters, which exhibited phase shifts that averaged twofold greater than those exhibited by vehicle controls or phenobarbital-injected hamsters with normal cycles. Experiment 2 used a similar protocol, but injections were at ZT 5, and blood samples for LH determination were collected from 1200 to 1800 on proestrus and the next day via jugular cannulae inserted the day before proestrus. Phenobarbital delayed the LH surge 1 day in all six hamsters, but it occurred at an earlier circadian time, supporting the above hypothesis. Experiment 3 investigated whether phenobarbital, like other nonphotic zeitgebers, suppresses SCN *Period1* and *Period2* transcription. Two hours postinjection, phenobarbital decreased SCN expression of only *Period1* mRNA, as determined by in situ hybridization. These results suggest that phenobarbital advances the SCN pacemaker, governing activity rhythms and hormone release in part by decreasing its *Period1* gene expression.

IN LABORATORY RODENTS, THE timing of the preovulatory luteinizing hormone (LH) surge is controlled in part by a circadian neural signal that is entrained by the daily light-dark (L-D) cycle, or photoperiod. Under a standard laboratory photoperiod, such as 12:12-h or 14:10-h L-D cycle, the preovulatory LH surge occurs in regularly cycling rats and hamsters about once every 4 days (96 h) at the same time of day (4, 6, 8, 51). The daily occurrence of a neural signal for triggering the LH surge was established over 50 years ago by the finding that daily administration of barbiturates within a specific time period on the afternoon of proestrus for 1–3 days prevents ovulation or the LH surge for 1–3 days, respectively, thereby delaying the estrous cycle up to 3 days (16). On the basis of this finding, the “critical period” was defined as the time on proestrus when barbiturate administration blocks the preovulatory LH surge and ovulation, and it has been interpreted to indicate that interval during which the neural trigger for the LH surge occurs. The critical period begins about 2 or 3 h before the first detectable increase in circulating LH levels in proestrous Syrian hamsters (55) or rats (19), respectively, and lasts for about 2 h in both species (17, 40).

A variety of evidence demonstrates the existence of circadian regulation of the LH surge. For example, daily LH surges occur in anestrous (4, 7, 47) or untreated ovariectomized Syrian hamsters (57) and in estradiol-treated ovariectomized rats (5, 11, 23, 28) and hamsters (39). In addition, persistence of the LH surge with a free-running rhythm during exposure to constant light [rats (32)] or constant darkness (DD) [hamsters (54)] provides evidence that an endogenous circadian pacemaker controls the neural signal for the LH surge. Furthermore, lesions of the suprachiasmatic nucleus (SCN), site of the master circadian pacemaker in mammals, block the LH surge and estrous cycles (56, 61). Similar to the regulation of other circadian rhythms, the circadian rhythm in the neural signal for the LH surge is entrained by the light-dark cycle. During exposure to different photoperiods, LH surges occur at a consistent time relative to the midpoint of the dark phase, similar to activity onset (31, 36, 37) and can be phase advanced or delayed by corresponding shifts in the light-dark cycle (36).

It has long been known that whenever the LH surge is temporarily blocked or delayed, there is a delay in the estrous cycle. Thus, in addition to the barbiturate-induced delay of estrous cycles, the delay in LH surges that is caused by exposure to constant light (32) and the delay in LH surges occurring in middle-aged rats (62, 63) are also associated with lengthened estrous cycles. More recently, a phase advance induced by a nonphotic stimulus was also shown to lengthen estrous cycles (25). Thus, transferring hamsters to a clean home cage or placing them in a novel running wheel near the beginning of the critical period on proestrous afternoon can delay estrous cycles by one day, but only in those animals whose circadian activity rhythm is phase advanced more than −1.5 h (25). In conjunction with the finding that pentobarbital administration to some strains of male mice phase can advance the locomotor activity rhythm (14), the latter finding suggests that phenobarbital-induced delay of the estrous cycle in female hamsters is also mediated by a phase advance. Therefore, we hypothesized that administration of phenobarbital during the critical period on proestrus afternoon delays the LH surge and the estrous cycle by advancing the phase of the circadian pacemaker past the critical period.

Address for reprint requests and other correspondence: S. J. Legan, Dept. of Physiology, Univ. of Kentucky, Lexington, KY 40536-0298 (e-mail: sjlegan@uky.edu).
With regard to the mechanism by which phenobarbital advances the circadian pacemaker, other nonphotic stimuli (e.g., transfer to a novel wheel, dark exposure, or injection of triazolam or 8-OH-DPAT) that advance the phase of the circadian pacemaker during the midsleep period appear to reset the clock by decreasing SCN expression of Period1 (Per1) and Period2 (Per2) mRNAs (13, 24, 30, 33). The finding that SCN administration of Per1 antisense oligonucleotides induces nonphotic-like phase advances in the hamster locomotor activity rhythm demonstrates the mechanistic importance of this decrease in Per1 expression (22). On the basis of these findings in male rodents, we also tested the hypothesis that phenobarbital administration during the critical period on proestrus suppresses Per1 mRNA expression in the female hamster SCN. Finally, many extra-SCN brain regions exhibit circadian expression of Per1 and Per2 (3, 21, 65), but whether nonphotic phase-resetting signals affect expression of these genes in extra-SCN regions has not been reported. Therefore, we investigated the effect of phenobarbital on expression of Per1 and Per2 mRNAs in several other brain regions.

MATERIALS AND METHODS

All procedures were performed according to the American Association for Accreditation of Laboratory Animal Care Guide and were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animals. Adult female Syrian hamsters were obtained from Harlan (Indianapolis, IN) and were maintained between 20°C and 22°C under a 14:10-h light-dark photoperiod (lights on at 0600). Light intensity ranged from 100 to 500 lux. The hamsters were housed individually in cages equipped with running wheels that were electronically interfaced with a computer. Wheel revolutions were recorded using hardware and software from the Chronobiology Kit (Stanford, CA). The cages were enclosed in light-tight, sound-attenuated, ventilated compartments that contained samples of soiled bedding from male hamsters. Food and water were available ad libitum. Estrous cyclicity was determined by daily examination of vaginal discharge for viscosity, color, and odor. On the day of estrus, the vaginal discharge is stretchy, white, and pungent (42).

Experiment 1. Effect of phenobarbital administration during the critical period on estrous cycles and phase of the locomotor activity rhythm. To determine whether administration of phenobarbital during the critical period on proestrus afternoon delays the estrous cycle by causing a phase advance in the circadian pacemaker, estrous cycles and activity rhythms were monitored daily. Hamsters exhibiting at least two consecutive estrous cycles were injected subcutaneously (sc) with vehicle (1% ethanol in 0.9% NaCl) or phenobarbital (100 mg/kg in vehicle) on proestrus at 1400, 6 h before lights off (zeitgeber time [ZT] 6), which is near the onset of the critical period. ZT 12 is conventionally defined as the time of lights off. This dose of phenobarbital induces drowsiness but not unconsciousness, as assessed by responses to toe pinches or observations of changes in the animal’s position or location in her cage every 30–40 min for the next 3 h. Immediately after injection, all hamsters were exposed to DD, and wheel-running rhythms were recorded continuously and vaginal discharge was checked daily using dim red light (<10 lux) for the next 2–3 wk.

Experiment 2. Effect of phenobarbital administration on estrous cycles, phase of the locomotor activity rhythm, and timing of the LH surge. On the basis of the results of experiment 1, we proceeded to determine whether administration of phenobarbital during the critical period on proestrus afternoon delays the estrous cycle by rapidly phase advancing the timing signal for the LH surge past the critical period, such that the LH surge in DD occurs 1 day later but at an earlier time. Hamsters were treated similarly as in experiment 1, with the following two differences. On diestrus, hamsters that had exhibited at least three consecutive estrous cycles were anesthetized with isoflurane and fitted with right atrial cannulae for serial blood sampling. On proestrus (day 1), vehicle (1% ethanol in sterile 0.9% NaCl) or phenobarbital (100 mg/kg in vehicle) was injected subcutaneously 1 h earlier than in experiment 1, i.e., at 1300, 7 h before lights off (ZT 5). Immediately after the injections, all hamsters were exposed to DD, and characteristics of vaginal discharge were recorded daily using dim red light. Activity rhythms were recorded continuously for the next 2–3 wk. Blood samples (0.2–0.3 ml) were obtained for determination of circulating LH concentrations at 1200, and hourly from 1400 to 1800 h on the day of the expected LH surge, and at 1400, 1600, and 1800 on the other day. In DD conditions, samples were obtained using dim red light. To avoid possible effects of handling on the phase of the circadian clock, in some animals in each group, samples were collected via an extended cannula. After each blood sample was collected, it was centrifuged in a microfuge at ~2,000 rpm (~2,000 g) for 10 min, and the plasma was drawn off and stored at −20°C until analyzed.

Radioimmunoassay. Plasma LH concentrations were determined in 2.5- to 75-μl aliquots of plasma by means of an RIA described previously (27). The first antibody was CSU 120 (kindly provided by Dr. Terry Nett, Colorado State University, Fort Collins, CO), used at a working dilution of 1:10,000 in 1:100 normal rabbit serum (Millipore, St. Charles, MO; formerly Linco Research) in 0.05 M PBS.
containing 0.1% gelatin (gel-PBS). The tubes were incubated at 4°C for 48 h following the addition of 100 μl first antibody and after adding radiolabeled LH (~22–32,000 cpm/100 μl diluent, MP Biomedicals LLC, Santa Ana, CA), and for 72 h after the addition of 200 μl second antibody (anti-rabbit gamma globulin, 1:50 in gel-PBS; Millipore, St. Charles, MO). Plasma LH concentrations are reported in terms of ng RP-3 per milliliter (National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases, and Dr. Parlow). All samples were analyzed in 2 assays for which the sensitivity (100% - 2 SD of maximum binding) averaged 0.17 ng/tube, and the interassay coefficient of variation was 8.8%.

LH data analysis. Mean peaks of LH surges, i.e., the maximum levels attained, and the times at which the peak levels occurred were compared between treatment groups by Student’s t-tests.

In situ hybridization. In situ hybridizations for Per1 and Per2 mRNAs were conducted, as described previously (13). Riboprobes were produced using partial cDNA sequences of hamster Per1 (nucleotides 726-1367) in pBluescript SK(+) (Stratagene, La Jolla, CA) and hamster Per2 (nucleotides 822-1601) in pGEM-T Easy Vector (Promega, Madison, WI), which were generously provided by Drs. H. Okimura (Kobe University, Kobe, Japan) and S. Shibata (Waseda University, Saitama, Japan), respectively. Bacteria were transformed with these plasmids, and vector DNA was purified for c-RNA synthesis. [The characteristics of these clones and their utility for in situ hybridization have been published previously (24)].

In situ hybridization was conducted using slide-mounted tissue sections that were pretreated as follows: equilibrated to room temperature, fixed for 15 min in 4% paraformaldehyde in 0.1 M PBS (pH 7.4), and washed 2 times for 5 min each in PBS. The sections were acetylated in triethanolamine (0.1 M, pH 8.0) and 0.25% acetic anhydride, dehydrated and delipidated, and air dried. The sections were hybridized overnight at 55°C with a saturating concentration (determined from preliminary studies) of 35S-labeled riboprobes that were diluted in hybridization cocktail containing salmon sperm DNA (250 μg/ml), yeast total RNA (625 μg/ml), Tris-HCl (20 mM, pH 7.4), EDTA (1 mM), NaCl (300 mM), dithiothreitol (100 mM), SDS (0.1% wt/vol), and Na-thiosulfate (0.1% wt/vol). After 18 h, sections were rinsed twice for 10 min each in 2× SSC/10 mM DTT (1× SSC 0.15 M sodium citrate, pH 7.2) at 22°C, treated with RNase (40 mg/ml) for 30 min at 37°C, washed for 15 min in 1× SSC and incubated for 1 h at 63°C in 0.1× SSC. The sections were rinsed in 0.1× SSC at 22°C, quickly dehydrated, and air dried. All solutions, except Tris buffer, were prepared with diethyl pyrocarbonate-treated water (0.1%) and autoclaved. Slides and radioactive standards (14C-microscales; Amersham Life Sciences, Piscataway, NJ) were exposed to X-ray film (Biomax MR, Kodak, Rochester, NY) for times determined in preliminary experiments. Autoradiograms were analyzed by microdensitometry, using an M4 image analysis system (Imaging, St. Catherines, Ontario, Canada), as previously described.

For Per1 and Per2 mRNAs, the effect of phenobarbital in each brain region was assessed by Student’s t-test.

Fig. 1. Phenobarbital at zeitgeber time (ZT) 6 blocks estrous cycles in most hamsters. Daily incidence of estrous (E) or nonestrous (non-E) vaginal discharge is depicted during the 2 wk before and after injection (arrows) of vehicle (V, top) or phenobarbital (Ph, middle and bottom) at ZT 6 on proestrus afternoon. Phenobarbital treatment blocked the next estrous discharge in most animals (middle), but not in two of the hamsters (bottom). The expected days of estrous discharge every 4th day after treatment are indicated by the vertical lines, either solid (first estrus after treatment) or broken (subsequent cycles). Phase shifts (h) in the circadian activity rhythm of each animal are indicated in the upper right of each panel. The shaded area represents the time during which animals were housed in constant darkness (DD).
phenobarbital treatment at ZT 6 blocked the next expected estrous discharge in 4 of 6 hamsters (Fig. 1, middle and bottom; missing data for animal F14). Unexpectedly, subsequent cycles in 5 of these 7 pentobarbital-treated hamsters were blocked for variable periods of time (middle). However, when estrous discharges reappeared, they were delayed by 1 day more than a multiple of the expected 4-day length of a normal estrous cycle. In the other two hamsters, phenobarbital did not delay the next estrous discharge (bottom). Although the timing of ovulation was not determined in these studies, ovulation is dependent on the LH surge and occurs on the morning after an LH surge, which usually, but not always, coincides with an estrous vaginal discharge. Phenobarbital delay of LH surges also delays ovulation until the day after the LH surge for up to 3 days (55). Therefore, it is highly likely that ovulation was delayed until the day after the LH surge, whenever it next occurred. Actograms from a representative animal in each group are depicted in Fig. 2. Delay of the estrous cycle in response to phenobarbital treatment was associated with the magnitude of the resulting phase advances in the circadian activity rhythms. In animals in which phenobarbital delayed the estrous cycle, the average phase advance (2.9 ± 0.3 h) was approximately twofold greater than that observed in either vehicle-treated hamsters (1.4 ± 0.3 h) or those in which phenobarbital had no effect on estrous cycles (1.5 ± 0.1 h) (Fig. 3). In general, phase advances ≥2.3 h were associated with delays in the estrous cycle and phase advances <2.3 h were not, suggesting that the threshold phase advance for delaying estrous cycles is about 2.3 h. Thus, one vehicle-injected hamster exhibited a phase advance of 2.3 h in the absence of any change in estrous cyclicity, whereas one phenobarbital injected hamster in which estrous cycles were delayed had a 2.35 h phase advance (Fig. 1).

**Experiment 2. Effect of phenobarbital administration on estrous cycles, phase of the locomotor activity rhythm, and timing of the LH surge.** Vehicle administration at ZT 5 had no effect on estrous cycles in 4 of 6 hamsters based on recorded estrous discharges, the timing of subsequent estrous discharges relative to the expected 4-day intervals (Fig. 4, left), and on the occurrence of the LH surge on proestrus (day 1) (Fig. 6, left). In the remaining two hamsters (F75 and F87), there was a 1-day delay in estrous cycles. In contrast, on the basis of the same three criteria, phenobarbital administration at ZT 5 blocked the next expected estrous discharge in 6 of 6 hamsters (Fig. 4, right). In five of the six phenobarbital-treated hamsters, estrous cycles were blocked for 1 day more than a multiple of the expected 4-day estrous cycle. In the sixth animal (F80), estrous discharges appear to have been delayed 3 days more than a multiple of the 4-day estrous cycle. The phase shifts within each group were not different whether animals were transferred to a small container for collection of each blood sample or remained in their cages and were sampled remotely (P = 0.16, vehicle and P = 0.31, phenobarbital); therefore, data from all animals within a group were combined. Just as in experiment 1, the delay in estrous cycles after phenobarbital treatment was associated with magnitude of the resulting phase shifts (Fig. 5). Generally, a phase advance of about 2 h or longer resulted in a 1-day delay of the estrous cycle; a shorter phase advance had no effect on estrous cyclicity. This relationship was observed in 5 of the 6 animals in both groups. In addition, in all 8 animals in which estrous cycles were delayed by 1 day (two vehicle-treated and six phenobarbital-treated), the LH surge was also delayed by 1 day and occurred 1.6 h earlier on the average than that in vehicle-treated animals, with no difference in magnitude (Fig. 6).

**Experiment 3. Effect of phenobarbital administration on Per1 and Per2 expression in select brain areas.** Phenobarbital administration selectively suppressed SCN Per1 mRNA expression. Namely, Per1 mRNA expression in the SCN was...
50% lower in phenobarbital-treated hamsters than in vehicle-treated hamsters \((P < 0.01)\), while \(\text{Per2}\) mRNA expression in the SCN was not affected by phenobarbital \((P = 0.31)\) (Figs. 7 and 8). In contrast to the SCN, phenobarbital treatment attenuated \(\text{Per2}\) mRNA but not \(\text{Per1}\) mRNA in the cingulate cortex but had no effect on the expression of either \(\text{Per1}\) or \(\text{Per2}\) mRNA in the piriform cortex, CA3, or dentate gyrus (Fig. 8). In the midline thalamus, \(\text{Per1}\) mRNA expression was higher in phenobarbital-injected hamsters vs. vehicle-injected hamsters \((P < 0.005;\) Fig. 8), while \(\text{Per2}\) mRNA was undetectable in this region in both treatment groups.

**DISCUSSION**

These findings support the hypothesis that phenobarbital administration to female hamsters near the beginning of the critical period causes a robust, rapid phase advance of the circadian pacemaker, which entrains both the activity rhythm and the neural circadian signal for the LH surge. Although several previous studies have investigated the effects of barbiturates on circadian locomotor activity rhythms in male rodents, the effects were inconsistent and appear to depend on species or strain. Thus, pentobarbital (50 mg/kg ip) induces...
circadian phase advances during the midsubjective day and small phase delays during the subjective night when administered to male mice of the DBA/2 or SK strains but not when given to C57BL mice or male rats or hamsters (14, 46). In contrast, the present results clearly show that in female hamsters, phenobarbital (100 mg/kg sc) administered at ZT 5 and 6 induces a phase advance in both circadian locomotor activity rhythms and the timing signal for the LH surge. Although vehicle-treated hamsters also exhibited phase advances in their wheel-running rhythms, these phase advances were about half as large as those seen in animals treated with phenobarbital, and the small phase advances were not sufficient to delay the LH surge. These smaller phase advances may have resulted at least in part from the transfer to DD because exposure to a dark “pulse” of at least 3 h duration, or to DD, during the midsubjective day following exposure to constant light can induce a 2–3 h phase shift (2, 9). In addition, it is possible that the volume of vehicle injected (10 ml), which was much larger

Fig. 6. Phenobarbital at ZT 5 delays the day and phase advances the time of the LH surge. Left: Line drawing depicting means ± SE plasma LH concentrations from 1200 to 1800 on the day of (day 1, left) and the day after (day 2, right) injection (Inj, arrows) of either vehicle (Veh, top) or phenobarbital (Phen, bottom) on proestrus at ZT 5. The vertical lines depict the time of the LH peak in the vehicle-treated hamsters. Right: bar graphs depicting means ± SE peak level of plasma LH concentrations (left) and time of the LH peak (right) in hamsters treated with either vehicle (Veh) or phenobarbital (Phen) at ZT 5 on proestrus afternoon. Numbers in parentheses indicate sample size. Although all six phenobarbital-treated animals had an LH surge on day 2, results are only depicted for 5 of them due to missing samples in the sixth hamster (F78).

Fig. 7. Pseudocolored autoradiograms showing expression of Per1 mRNA and Per2 mRNA. Darker, brighter colors (black and red) indicate higher levels of expression than lighter colors (yellow, green, blue).
than that typically used in phase-shifting studies, contributed to the phase shift magnitude. [This volume was chosen to mimic the volume used previously to block the LH surge in hamsters (55).] The repeated arousal for hourly blood sampling was most likely not a contributory factor because there was no difference in the phase shifts between animals that were sampled and those that were not (compare vehicle-treated animals in Figs. 3 and 5). Finally, it is unlikely that the 1% ethanol in the vehicle contributed to the phase advance because it has been shown that ethanol attenuates phase advances induced either by light or a nonphotic stimulus, triazolam (48).

It is interesting to note that in contrast to most other nonphotic stimuli that phase advance the circadian pacemaker, such as dark pulses and triazolam (59), phenobarbital strongly suppresses rather than stimulates activity and alertness. Physiological activity appears to be necessary for the phase-shifting effect of some nonphotic stimuli (35, 38, 59), perhaps through increased arousal. In support of this possibility, sleep deprivation is sufficient to induce a phase shift (35). The present findings suggest that neither increased arousal state nor activity is required for a phenobarbital-induced phase advance in female hamsters.

It has long been known that pentobarbital administration in rats can block the LH surge and ovulation, thereby delaying the estrous cycle by 1 day. However, it is essential to recognize that there are important functional differences between the two barbiturate anesthetics that block LH surges and ovulation in rodents, which are revealed when comparing their actions between rats and hamsters. Thus, although administration of either pentobarbital or phenobarbital can block ovulation in rats (16), only phenobarbital blocks LH surges in hamsters (40). Both drugs are completely effective only if administered at the beginning of the 2-h critical period. If either drug is administered 2 h later, neither of them blocks the LH surge. In both species, the dose of phenobarbital that is sufficient to block the LH surge does not induce surgical anesthesia, as does the surge-blocking dose of pentobarbital, indicating that these two drugs may be acting by different mechanisms to block the LH surge (16, 40). In this regard, administration of pentobarbital to proestrous hamsters at the beginning of the critical period delays the time when the neural signal for the LH surge occurs, as indicated by a 1-h delay in the LH surge and a delay in the critical period, i.e., the time when phenobarbital administration can block the LH surge (40). These findings suggest that in hamsters, pentobarbital may act by delaying the critical period, whereas our findings suggest that phenobarbital acts via a phase advance in the circadian clock.

It should also be noted that although phenobarbital administration advanced the timing of the LH surge on the next day, it remains to be determined whether this phase shift is permanent (stable) or only transient. The former possibility receives strong support from our finding that phenobarbital administration induces a permanent phase shift in the circadian pacemaker regulating the onset of activity and from previous findings that the onset of activity and timing of the LH surge are strongly linked in hamsters (36, 37, 58). Addressing the issue of a permanent phase shift in the timing of the LH surge would be more feasible in hamsters that are exhibiting daily LH surges, such as anestrous hamsters exposed to short days (6, 7, 47) or ovarietomized hamsters in the presence or absence of estradiol (39, 57), than in intact, long day-exposed hamsters, such as those used in the present study.

When the phenobarbital-induced phase advance is greater than about 2 h, the next estrous discharge is blocked. On the basis of previous findings that the duration of the critical period is about 2 h and on the finding herein that after phenobarbital, the LH surge occurs between 1 and 2 h earlier, 1 day later, these results suggest that phenobarbital delays the hamster estrous cycle 1 day, in part, by advancing the phase of the circadian clock past the critical period for the neural signal that initiates the LH surge. Although phenobarbital phase-advances the circadian clock, it remains to be demonstrated that this is sufficient to block the LH surge and whether there are other noncircadian mechanisms whereby barbiturates block the LH surge. For example, in ewes, pentobarbital suppresses LH pulse frequency (20), which is a possible mechanism for blockade of the LH surge by phenobarbital in rodents. In addition, in induced ovulators like rabbits, in which the LH surge is not controlled by the circadian clock, pentobarbital only reliably blocks the LH surge when administered such that it blocks the coitus-induced neural signal for LH release (34). Therefore, in species that lack circadian control of the preovulatory LH surge, barbiturate blockade of the LH surge must occur via a different mechanism than in spontaneous ovulators (e.g., rodents). We are currently using a nonphotic zeitgeber to...

Fig. 8. Effect of phenobarbital administration on *Per1* mRNA and *Per2* mRNA expression. Values shown are the means ± SE. SCN, suprachiasmatic nucleus; Thal, midline thalamus; Cg, cingulate cortex; Pir, pyriform cortex; CA3, CA3 hippocampal subfield; DG, dentate gyrus, ND, not detectable. *P < 0.05 vs. vehicle in the same region; #P < 0.005 vs. vehicle in the same region.
address whether a phase advance alone is sufficient to block the LH surge and advance the circadian phase of its peak in hamsters.

The difference in effectiveness of phenobarbital to delay estrous cycles between experiments 1 and 2 may be related to the time of phenobarbital administration in relation to the critical period. In previous studies, phenobarbital administration to proestrous hamsters at ZT 5 blocked LH surges in 100% of animals (55); therefore, in experiment 2, the time of injection was advanced 1 h to ZT 5 and succeeded in delaying estrous cycles in all animals. These results suggest that the critical period in hamsters begins between ZT 5 and ZT 6, about 2 h before onset of the LH surge.

Our observation that phenobarbital administration blocks the vaginal estrous discharge, often for a number of days, suggests that this drug may exert reproductive effects on other tissues (e.g., vaginal epithelium) besides the SCN circadian pacemaker. The estrous discharge reflects mucus secretion by the vaginal epithelial cells (26), and although it is often used to monitor estrous cyclicity, it is not always an accurate indicator of LH release or ovulation. It remains to be determined whether phenobarbital chronically decreases vaginal mucus production, thereby disrupting the estrous vaginal discharge selectively, while regular 4-day estrous cycles, including LH surges and ovulation, continue to occur delayed by 1 day. Alternatively, it is possible that the cessation of vaginal estrous discharges is caused by the disruption of normal circulating levels of estrogen and/or progesterone occurring as a result of the phenobarbital administration. In this case, estrous cycles would also be disrupted during the absence of vaginal estrous discharges.

Phase resetting of the master circadian pacemaker is associated with changes in expression of the core circadian clock genes, Per1 and Per2, as demonstrated by many studies in male rodents (13, 24, 30, 33). Thus, in hamsters, induction of phase shifts by light at night is associated with increased SCN expression of Per1 and Per2 (24). In contrast, induction of phase shifts during the daytime by a variety of nonphotic stimuli, such as systemic injections of either triazolam or 8-OH-DPAT, is associated with decreased SCN expression of Per1 and Per2 (13, 24, 30, 33). Similarly, our results show that phenobarbital administration to proestrous hamsters in the early afternoon (ZT 5) decreased SCN expression of Per1 mRNA 2 h later. On the basis of the observation that acute reduction of SCN Per1 mRNA by local administration of antisense oligonucleotides induces phase advances in the circadian locomotor activity rhythm in male hamsters (22), our finding that phenobarbital decreases SCN Per1 mRNA elucidates the mechanism by which phenobarbital advances the circadian rhythm of wheel running in proestrous hamsters. This finding also supports the hypothesis that phenobarbital blockade of the LH surge is mediated at least in part by an advance in the timing of the master circadian pacemaker.

In contrast to its effect on SCN Per1 mRNA expression, phenobarbital administration to female hamsters in the present study did not alter SCN Per2 mRNA expression at 2 h postinjection. The differential effect of phenobarbital on these two Per genes at this timepoint distinguishes it from most other nonphotic stimuli, which suppress expression of both Per1 and Per2 when presented in the middle of the subjective day (13, 24, 30, 33). However, the present finding parallels that of another study in which the GABA_\alpha receptor agonist, muscimol, was microinjected at circadian time 6 into the SCN region of hamsters exposed to either 14 days or 42 h of DD (15). This treatment suppressed SCN Per1 mRNA at either 2 or 3 h postinjection, but suppression of Per2 mRNA was only observed 3 h, not 2 h, postinjection in hamsters that were exposed to 42 h of darkness (15).

The differential suppressive effect of muscimol on Per1 vs. Per2 mRNA at 2 h postinjection might be related to the different temporal patterns of SCN expression of these genes. The circadian rhythm of Per2 mRNA expression in the male hamster SCN, after exposure to darkness for 3 days, peaks at circadian time 8 (CT 8), 4 h later than that of SCN Per1 mRNA expression (24). In male hamsters exposed to a light-dark cycle (14:10-h L-D), SCN Per2 mRNA expression exhibited a broad “peak” from ZT 4 to ZT 15, in contrast to the sharp peak in SCN Per1 mRNA expression at ZT 4 (30). Thus, it may be easier to induce suppression of Per1 mRNA than Per2 mRNA in the midafternoon (either CT or ZT 4–8) because expression of the former, but not the latter, is normally decreasing during this interval. Also, muscimol might only induce a decrease in SCN Per2 mRNA at 3 h but not 2 h postinjection (15) because the former timepoint but not the latter occurs during the falling phase of Per2 mRNA expression during exposure to darkness (24).

Muscimol shares a common neurochemical mechanism of action with phenobarbital; both drugs have a high affinity for GABA_\alpha receptor complexes that form ligand-gated chloride channels (12). Activation of GABA_\alpha receptors stimulates opening of chloride channels, allowing an influx of chloride ions and thereby inducing hyperpolarization of the plasma membrane. Binding of phenobarbital to the barbiturate binding sites on the GABA_\alpha receptor complex increases the opening time of the channel, as well as the affinity of the receptor for GABA binding (12, 50). GABA_\alpha receptors are expressed in many brain regions, including the SCN (41, 49). Furthermore, in male hamsters, activation of SCN GABA_\alpha receptors by microinjections of muscimol during the midsubjective day not only reduces SCN Per1 and Per2 mRNA expression, as discussed above, but also induces circadian phase advances (15, 52, 53). These findings are very similar to the effects that were observed following phenobarbital administration in female hamsters and suggest that activation of SCN GABA_\alpha receptors may constitute a common mechanism for induction of phase advances.

In the present study, phenobarbital’s effects on Per1 and Per2 expression differed in a brain region-dependent manner. For example, although phenobarbital induced a large decrease in Per1 mRNA in the SCN, no phenobarbital-induced decreases in Per2 mRNA were observed in any other region examined, and surprisingly, an increase was observed in the midline thalamus. Further, although Per2 mRNA expression was unaffected by phenobarbital in most regions investigated, it was suppressed in the cingulate cortex. These regional differences in the effects of phenobarbital may be due to differences among brain regions in the temporal patterns of Per1 and Per2 expressions and/or in the time required for the drug to exert its effects. The former possibility is supported by a number of previous findings demonstrating that the temporal expression and mechanisms of regulation of Per in the cortex differ from those in the SCN. Thus, Per1 and Per2 gene
expressions in the SCN both peak in the daytime (24, 30). In contrast Per gene expression in the cerebral cortex peaks at night in both female rats (29) and male mice (1, 60) exposed to a light-dark cycle. In the parietal cortex of female rats, Perl and Per2 mRNAs exhibit 24-h rhythms, but neither gene shows a 24-h expression rhythm in the cingulate cortex (60). Cortical Per expression is also affected differently from the SCN by various stimuli that alter the time of activity and arousal. For example, chronic treatment of rats with methamphetamine reverses the day-night phases of the locomotor rhythm concomitant with a large shift in the peak of Perl mRNA in the cortex but not the SCN. In mice, restricted diurnal feeding selectively shifts the Perl mRNA peak in the cortex but not the SCN (60). Also, sleep deprivation of mice increases cortical but not SCN expression of Perl and Per2 mRNAs (18, 64). In view of these observations, one caveat of our results is that only one injection time and one time of brain collection postinjection were investigated.

In conclusion, the results herein confirm that administration of phenobarbital during the critical period on proestrus delays the LH surge and the estrous cycle by 1 day, as shown previously. In addition, they demonstrate for the first time that phenobarbital administration during the critical period phase advances the wheel-running activity rhythm, as well as the time of the peak of the LH surge that occurs on the following day. Because phenobarbital advances both of these circadian rhythms, it appears to reset the circadian pacemaker in the SCN, thereby supporting the hypothesis that phenobarbital advances the time of the peak of the LH surge that occurs on the following day. This phenomenon and perhaps also in the regulation of other circadian endocrine rhythms.

The present studies add to a growing body of evidence revealing interactions between the circadian timing system and pharmaceutical agents. Although circadian influences on the efficiency, duration, and toxicity of barbiturates and other drugs are well known (10), the current findings demonstrate that barbiturates can robustly reset the circadian pacemaker and thus suggest that time of drug therapy, including the use of phenobarbital for epilepsy, may influence many circadian rhythms and the processes that they modulate. Also, the demonstrated association between alterations in circadian rhythms and temporary disruption of a reproductive hormone rhythm may have clinical relevance and also provide some insight into the alterations in endocrine rhythms characteristic of depression in pregnant, postpartum, and postmenopausal women (43–45).

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