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Effects of Phenobarbital on Hypothalamic LHRH and Catecholamine Turnover Rates in Proestrous Rats (41085)

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Abstract. In previous studies we observed that LHRH concentrations increase in the preoptic–suprachiasmatic tuberoinfundibular system (PSTS) during the morning hours of proestrus when norepinephrine (NE) and dopamine (DA) turnover rates are low. Concomitant with the preovulatory surge in gonadotropins, NE turnover rates increase, DA turnovers decrease, and LHRH declines in PSTS. The present studies examine the effects of phenobarbital (PB) on these proestrous events. PB, when given at 0900 hr proestrus, does not prevent the rise in median eminence (ME) LHRH which occurs between 0900 and 1200 hr. When the barbiturate is administered at 1200 hr, LH, FSH, and prolactin proestrous surges are blocked. While PB (given at 1200 hr) does not affect the increase in turnover rates of NE and DA which occur between 1200 and 1400 hr proestrus it markedly suppressed NE turnover rates in ME and suprachiasmatic nuclei but not in the medial preoptic nuclei between 1500 and 1700 hr. The decline in ME-DA which occurs between 1500 and 1700 hr proestrus is not affected by PB treatment at 1200 hr. Pituitary responsiveness to two pulse injections of LHRH (60 min apart) is not reduced by PB treatment. We conclude that PB blockade of preovulatory gonadotropin surges may be via its suppression of hypothalamic NE release during the afternoon of proestrus.

In a previous study we described an important temporal sequence of hypothalamic events which occur during proestrus in rats exhibiting 4-day estrous cycles. These consist of: (a) 0900–1200 hr—an increased accumulation of LHRH in the preoptic–suprachiasmatic–tuberoinfundibular system (PSTS) including median eminence (ME) (1); (b) 1200–1400 hr—increased turnover rates of ME norepinephrine (NE) and dopamine (DA); (c) 1500–1700 hr—increased NE turnovers not only are maintained in ME but also are now evident in the medial preoptic nucleus (MPN), suprachiasmatic nucleus (SCN) and arcuate nucleus (AN). A reciprocal decline in ME-DA turnover rates also occurs at this time. In these same animals, ME-LHRH declines between 1200 and 1500 hr and LH, FSH, and prolactin (PRL) concentrations increase in serum (2). We interpret the increase in hypothalamic NE turnover rates, coupled with a decrease in ME-LHRH and increase in serum gonadotropins, to mean that NE could initiate the preovulatory surge of gonadotropins by evoking the release of newly accumulated LHRH in ME axon terminals. In the present study we attempted to block part of this sequence of events to determine which of these hypothalamic components is essential for proestrous gonadotropin surges to occur.

One class of drugs which effectively blocks LH release is the barbiturates. These drugs, when administered prior to the proestrous "critical period," prevent gonadotropin release for 24 hr (3). While the mechanisms and sites of action of the barbiturates are unknown, it is recognized that they depress excitability of the reticular activating system (RAS) (4, 5) and of hypothalamic "islands" (6) but have no effect on pituitary responsiveness to LHRH (7). Since the cell bodies of the hypothalamic noradrenergic system reside, in part, within the RAS (8), it is possible that the barbiturates could affect the physiological activation of this catecholamine (CA) system.

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In the present study we examine the effects of an ovulation blocking dose of phenobarbital on three component parts of the proestrous LH surge system: (a) the ability of LHRH to induce LH release from the pituitary gland; (b) the accumulation of LHRH in the ME on proestrous morning, and (c) CA turnover rates in the MPN, SCN, and ME during proestrus.

Materials and Methods. Female Sprague–Dawley rats (175–200 g) were purchased from Zivic–Miller (Allison Park, Pa.) and housed in a temperature- (22–24°) and light-controlled room (lights on 0400–1800 hr). Food and water were supplied *ad libitum*. Daily vaginal lavages were taken and only rats which had two consecutive 4-day cycles were used in these studies.

Drugs. Phenobarbital (PB) (100 mg/kg body wt) was injected (ip) in saline, and control rats received 0.5 ml of saline ip. α-Methyl-*p*-tyrosine (α-MPT) (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 0.1 *N* HCl and the pH was adjusted to 5.5–6.0 with 5 *N* NaOH. Rats received either 400 mg/kg of α-MPT or vehicle ip.

Radioimmunoassays. Serum LH, FSH, PRL, estradiol (E_2) progesterone, (P) and ME-LHRH were assayed by methods previously described (9–11). NIAMDD-rat LH-RP-1 was used as the standard in the LH assay which has a biological potency equivalent to 0.03 × NIH-LH-S1. NIAMDD-rat FSH-RP-1 was used as the FSH standard with a potency of 2.1 × NIH-FHS-S1 standard. NIAMDD-rat PRL-RP-1 was used as the prolactin standard and it has a potency of ~11 IU/μg. Single radioimmunoassays were made for each hormone measured to avoid problems of intraassay variability. A two-tailed *t* test was used for statistical comparisons of hormone concentrations at different time intervals on proestrus.

Experiment I. To determine the effects of phenobarbital on pituitary responsiveness to LHRH in rats having similar plasma E_2 levels, a group of rats (n = 14) were ovariectomized. One week later Silastic capsules (25 mm in length) which contained 150 μg/ml of E_2 in sesame oil were implanted sc. Two days later the right external jugular veins were cannulated at 0600 hr under ether anesthesia. At 0700 hr, either phenobarbital or saline was injected into 14 rats (n = 7/group). At 1000 and again at 1100 hr both groups received 50 ng/100 g body wt LHRH iv (Beckman, Lot No. D0430). Serial blood samples were taken via the indwelling cannulae at 0, 20, 60, 80, 120, and 180 min after the first injection of LHRH. The blood was centrifuged and the plasma was stored at −20° until it was assayed for LH concentrations.

Experiment II. Sixteen proestrous rats were injected with either saline or phenobarbital at 0900 hr, they were decapitated at 1200 hr and the ME's were removed as described below for measurements of LHRH concentrations. Trunk blood was collected, centrifuged, and the serum was stored at −20° until it was assayed for hormone concentrations.

Experiment III. NE concentrations, rate constants, and turnover rates in the MPN, SCN, and ME were examined at the proestrous time periods of 1200–1400 and 1500–1700 hr in rats injected with either phenobarbital or saline at 1200 hr. DA values were obtained using the same protocol but only in the ME. Rats receiving α-MPT at 1200 or 1500 hr were sacrificed 120 min later. Other rats received only α-MPT vehicle at 1200 or 1500 hr and they were sacrificed immediately.

After decapitation, brains were rapidly removed, frozen on dry ice, and hypothalamic nuclei were dissected by the method of Palkovits (12), as described previously (11). Both dorsal and ventral parts of MPN and medial and lateral portions of ME were removed. Tissue punches were homogenized in 0.1 *N* HCl, and centrifuged (15,600*g*), and the supernate was removed and divided into 5-μl aliquots for catecholamine and LHRH assays. The tissue pellet was redissolved in 1.0 *N* NaOH and stored until protein determinations were made by the Bradford dye binding technique (13). Tissue NE and DA concentrations were determined by a micromodification of the method of Coyle and Henry (14), and turnover rates and rate constants were calculated (15). The standard errors of turnover rates were calculated according to

the formula presented in Hohn and Wuttke (16). Significance of difference between rate constants was tested by the *t* test. For statistical treatment of turnover rate data, the difference in turnover rates, divided by a combination of their standard errors, was compared to a standard normal distribution. Data reported for 0900 to 1100-hr plasma hormones and CA turnover rates are from a previous study (2). Peripheral serum hormone levels and ME-LHRH concentrations were measured from the rats sacrificed for determinations of initial CA concentrations (1200 and 1500 hr).

Results. *Experiment I.* Silastic capsules containing E_2 produced plasma E_2 concentrations of 21.1 ± 3.2 pg/ml within 6 hr of being implanted into 7-day ovariectomized rats. These plasma E_2 concentrations did not change significantly over the next 4 days.

When the first pulse of LHRH was injected into saline or PB-treated, ovariectomized E_2-treated rats, similar rises in plasma LH had occurred 20 min later. When a second pulse injection of LHRH was given at 60 min, peak LH responses were obtained by 80 min in both groups and they were significantly greater ($P < 0.01$) than those observed after the first LHRH injection. Further, plasma LH concentrations in PB-treated rats were significantly greater at 120 and 180 min after the second LHRH pulse injection than in saline-treated control rats (Fig. 1).

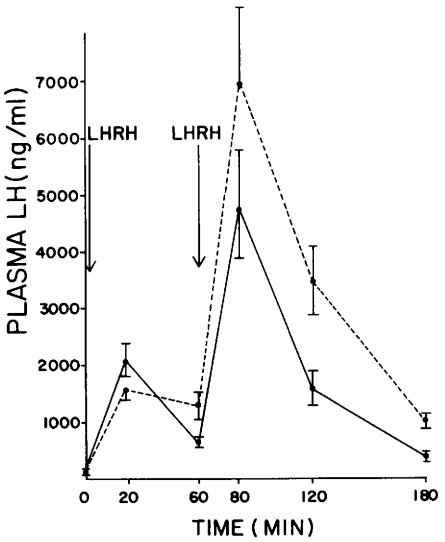

FIG. 1. Plasma LH release in saline (solid line) or phenobarbital-injected (dashed line) E_2-treated ovariectomized rats after two iv injections of LHRH spaced 60 min apart. Phenobarbital-treated rats show higher plasma LH concentrations at 120 and 180 min than control rats. Vertical lines represent ±SEM.

Experiment II. The administration of phenobarbital at 0900 hr proestrous did not prevent the normal rise in ME-LHRH which occurs between 0900 and 1200 hr. In both control and phenobarbital-treated groups, ME-LHRH was significantly increased by noon proestrus when compared to 0900-hr values (Table I). While no changes in basal concentrations of serum LH, FSH, PRL, and P were observed,

TABLE I. EFFECT OF PHENOBARBITAL (PB) ADMINISTERED AT 0900 OR 1200 hr PROESTRUS ON ME-LHRH AND PLASMA HORMONES AT 1200 AND 1500 hr, RESPECTIVELY

Injection	Saline	Saline	PB	Saline	PB
Time of injection (hr)	0900	0900	0900	1200	1200
Time tissue collected (hr)	0900	1200	1200	1500	1500
ME-LHRH (pg/μg)	105.9 ± 8.3†	181.9 ± 16.5	177.9 ± 17.2	122.4 ± 12.1†	135.6 ± 13.2†
Plasma					
LH (ng/ml)	48.4 ± 5.1**	104.1 ± 11.1	85.8 ± 5.7	3986.0 ± 338.7††	43.8 ± 3.8**
FSH (ng/ml)	122.5 ± 3.5**	162.9 ± 21.8	177.9 ± 17.2	624.0 ± 108.0††	181.0 ± 16.6**
PRL (ng/ml)	58.5 ± 14.3**	107.5 ± 20.2	111.0 ± 24.5	505.7 ± 32.6††	183.9 ± 73.6**
E_2 (pg/ml)	43.1 ± 5.2	36.8 ± 2.9	23.2 ± 1.7††	47.0 ± 3.2	19.0 ± 2.2**
P (ng/ml)	3.6 ± 1.8**	2.5 ± 1.2	2.4 ± 0.7	22.5 ± 1.5††	3.6 ± 0.5**

Note. Mean ± SEM. n = 6–8 rats/group.
* $P < 0.05$ compared to saline-injected rats sacrificed at 1500 hr.
** $P < 0.01$ compared to saline-injected rats sacrificed at 1500 hr.
† $P < 0.01$ compared to saline-injected rats sacrificed at 1200 hr.
†† $P < 0.01$ compared to saline-injected rats sacrificed at 1200 hr.

phenobarbital produced a significant reduction in serum E_2 between 0900 and 1200 hr.

Experiment III. In control rats, serum LH, FSH, PRL, and P concentrations were significantly elevated at 1500 compared to 1200 hr proestrus. The administration of PB at 1200 hr effectively prevented these gonadotropin surges from occurring, and also significantly reduced plasma E_2 levels when compared to 1500-hr control values (Table I). In spite of the failure of gonadotropin surges to occur in PB-treated proestrous rats, ME-LHRH concentrations declined by the same order of magnitude as they did in proestrous control rats between 1200 and 1500 hr (Table I).

In a previous study (2), NE turnover rates were low in MPN and SCN between 0900 and 1100 hr and initial concentrations of SCN-NE were increased at 1200 when compared to 0900 hr. At 1200–1400 hr, ME-NE turnovers were significantly elevated when compared to earlier morning values (Table II). Between 1500 and 1700 hr proestrus, increased ME-NE turnover rates were still significantly elevated and during this time interval increased NE turnover rates also were observed in MPN and SCN. The administration of PB at 1200 hr proestrus did not alter the elevated ME-NE turnover rates which occurred between 1200 and 1400 hr. Further, the increased NE turnovers in MPN noted between 1500 and 1700 hr also were not affected by PB. In contrast, PB when given at 1200 hr, had a dramatic suppressive effect on NE turnover rates, rate constants, and initial concentrations in both SCN and ME between 1500 and 1700 hr (Table II, Fig. 2).

Control DA turnover rates in ME were significantly increased between 1200 and 1400 hr and then were significantly reduced between 1500 and 1700 hr. PB treatment of proestrous rats did not prevent the significant decline in ME-DA turnover rates from occurring but it significantly reduced initial concentrations of ME-DA at 1500 hr (Table III).

Discussion. Barbiturates are effective blockers of proestrous preovulatory gonadotropin surges in rats and the present studies confirm these findings (3). We studied the action of phenobarbital on various neuroendocrine systems as it is more effective in suppressing the proestrous afternoon rise in serum FSH than is pentobarbital (15). This barbiturate also appears to have direct effects on ovarian function as plasma E_2 declined in animals

TABLE II. NE ACTIVITY DURING PROESTRUS IN SALINE (CONTROL) AND PHENOBARBITAL-INJECTED (PB) RATS

	TIME (hr)				
	0900–1100 Control	1200–1400 Control	1200–1400 PB	1500–1700 Control	1500–1700 PB
MPN					
Turnover rate (pg/μg protein/hr)	10.9 ± 4.6	16.7 ± 5.9[a]	17.7 ± 6.4	34.7 ± 5.6[b]	27.0 ± 4.0[c]
Rate constant (hr^{-1})	0.11 ± 0.06	0.16 ± 0.07	0.17 ± 0.07	0.33 ± 0.06[c]	0.28 ± 0.05[c]
Initial concentration (pg/μg protein)	99.1 ± 6.2	104.3 ± 9.2	104.3 ± 9.2	105.1 ± 8.5	96.9 ± 3.5
SCN					
Turnover rate	1.6 ± 1.9	9.3 ± 4.8	17.3 ± 5.7[c]	16.4 ± 3.5[c]	2.05 ± 0.17[d]
Rate constant	0.08 ± 0.12	0.19 ± 0.13	0.36 ± 0.15	0.32 ± 0.08	0.06 ± 0.06[a]
Initial concentration	19.5 ± 3.2	48.4 ± 7.0[c]	48.4 ± 7.0[c]	50.7 ± 4.9[c]	34.9 ± 2.1[c]
ME					
Turnover rate	9.1 ± 8.7*	20.2 ± 6.2[b]	17.6 ± 4.0[b]	17.7 ± 4.9[b]	0.18 ± 3.3[d]
Rate constant	0.10 ± 0.10*	0.19 ± 0.07[c]	0.16 ± 0.06[c]	0.19 ± 0.06[c]	0.003 ± 0.07[a]
Initial concentration	94.1 ± 13.3	107.1 ± 3.8	107.1 ± 3.8	92.0 ± 6.7	61.5 ± 5.6[d]

Note. n = 22–24 for turnover rate, 14–16 rats for rate constant, 5–8 rats for initial concentration.
* Slope >0.
[a] $P < 0.05$ compared to 1500–1700 hr control.
[b] $P < 0.01$ compared to 0900–1100 hr control.
[c] $P < 0.05$ compared to 0900–1100 hr control.
[d] $P < 0.01$ compared to 1500–1700 hr control.

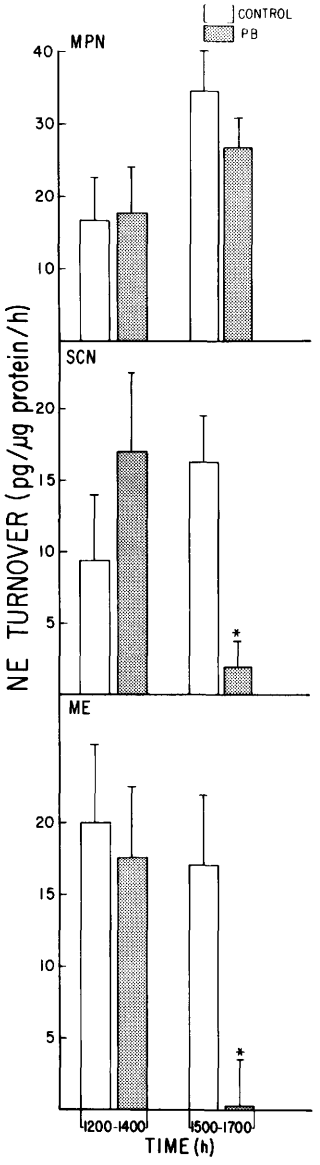

FIG. 2. Norepinephrine turnover rates (pg/μg protein/hr) in saline- and phenobarbital-injected proestrous rats. Phenobarbital, when given at 1200 hr, markedly suppresses NE turnover rates between 1500 and 1700 hr in SCN and ME but not MPN. Vertical lines above bars represent SEM.

treated at either 0900 or 1200 hr. Ovarian ovulatory thresholds to LH also are elevated by PB (unpublished observations). How PB affects ovarian function is not known.

To test pituitary responsiveness to LHRH in PB-treated rats, studies were performed during the morning hours of proestrus to avoid any possibility of endogenous LHRH priming the pituitary gland which could give spurious results when exogenous LHRH was injected. In these OXV animals, plasma E_2 levels were maintained by Silastic E_2 capsule implants. In rats which received PB, pituitary responsiveness to LHRH was significantly greater than in controls and potentiation of anterior pituitary gland responsiveness to LHRH by barbiturates has been previously reported (17). The present studies support previous experiments (7, 17) in eliminating the pituitary gland as the site of action of barbiturates in blocking LH release.

We believe an important physiological event occurs within the preoptico–suprachiasmatic–tuberoinfundibular system (PSTS) of the hypothalamus between 0900 and 1200 hr proestrus. This is the accumulation of new LHRH, particularly within the ME (1). We suggest that it is this LHRH which is released to activate the surge of LH and FSH on proestrous afternoon. PB treatment at 0900 hr proestrus does not affect this proestrous morning rise in ME-LHRH.

In normal proestrous rats, ME-LHRH declines between 1200 and 1500 hr and a concomitant rise in serum gonadotropins occurs. Presumably, this decline in ME-LHRH represents the discharge of the decapeptide into the portal circulation since elevated portal plasma LHRH has been observed about this time of day on proestrus (18). In PB-treated rats ME-LHRH also declines by about the same order of magnitude as controls although portal plasma LHRH concentrations do not increase (19) and serum LH and FSH levels remain basal. Thus the decline in ME-LHRH does not represent a release of ME-LHRH in PB-treated rats. Perhaps, if newly accumulated ME-LHRH is not released it is rapidly degraded by endopeptidases which exist in high concentrations in the hypothalamus (20).

The most striking effects of PB treatment are on the hypothalamic noradrenergic system. In a preceding study NE turnover rates were low in MPN, SCN, and ME between 0900 and 1100 hr and increase signifi-

TABLE III. DOPAMINE ACTIVITY DURING PROESTRUS IN SALINE (CONTROL) AND PHENOBARBITAL-INJECTED (PB) RATS

	TIME (hr)				
ME	0900–1100 Control	1200–1400 Control	1200–1400 PB	1500–1700 Control	1500–1700 PB
Turnover rates (pg/μg protein/hr)	142.0 ± 11.8	213.6 ± 19.6[a,d]	205.6 ± 22.6	110.0 ± 25.1	71.4 ± 21.0
Rate constant (hr^{-1})	0.48 ± 0.08	0.59 ± 0.06	0.57 ± 0.07	0.35 ± 0.10	0.34 ± 0.12
Initial concentration (pg/μg protein)	293.6 ± 27.0	362.0 ± 11.9	362.0 ± 11.9	315.0 ± 34.1	210.5 ± 12.4[b,d]

Note. n = 22–24 for turnover rate, 14–16 rats for rate constant, 5–8 rats for initial concentration.
[a] $P < 0.05$ compared to 0900–1100 hr control.
[b] $P < 0.01$ compared to 0900–1100 hr control.
[c] $P < 0.05$ compared to 1500–1700 hr control.
[d] $P < 0.01$ compared to 1500–1700 hr control.

cantly in ME between 1200 and 1400 hr proestrus (2). PB treatment does not affect the increased ME-NE or DA turnover rates observed during this time interval. However, while NE turnovers are increased in MPN, SCN, and ME between 1500 and 1700 hr, PB treatment dramatically inhibits such turnover rates in SCN and ME but not MPN. We previously proposed that the increased NE and DA turnover rates in ME between 1200 and 1400 hr concomitant with a decline in ME-LHRH between 1200 and 1500 hr could represent processes involved in initiation of the preovulatory gonadotropin surge (2). While the present data do not support this conclusion, it is possible that PB affected CA turnover rates as early as 1300–1500 hr. On the other hand, the important changes in CA turnover rates to induce LHRH release could be occurring between 1500 and 1700 hr (or 1400–1700 hr). Only additional time-course studies could provide this information. Regardless of the time course of effect of PB, NE turnover rates are dramatically suppressed in SCN and ME but not MPN. These data further support the concept that the effective site of NE in evoking LHRH releases is not solely the MPN but also involves other component parts of the preoptico–suprachiasmatic–tuberoinfundibular system. In PB-treated proestrous rats preovulatory gonadotropin surges do not occur and in such animals a decline in both SCN and ME-NE turnover rates is observed. Since PB does not affect the normal rise in ME-DA turnover rates which occur between 1200 and 1400 hr nor the declines which are observed between 1500 and 1700 hr, it seems apparent that this drug does not affect release of DA in the tuberoinfundibular system. The selective suppression of ME- and SCN-NE turnover rates but not ME-DA turnovers by PB provides further support for the concept that NE is the important CA involved in preovulatory gonadotropin surges (21).

Barbiturates reduce NE turnover rates in rat cerebral cortex (22, 23) but these effects are not due to a direct drug action on NE nerve terminals since phenobarbital does not affect [^{3}H]NE uptake or stimulation-induced release of [^{3}H]NE from rat cerebral cortical slices *in vitro* (24). The reticular activating system is sensitive to the depressant effects of barbiturates (4, 5) and cell bodies of the noradrenergic axon terminals which innervate MPN, SCN, and ME (25) are part of this system (8). Consequently, the decrease in NE turnover rates in ME and SCN could represent a depressant effect of PB on NE cells in the brainstem lateral tegmental areas (25) or in the RAS synaptic input to these neurons.

A direct effect of PB on NE terminals in SCN and ME cannot be excluded as reductions in CNS neurotransmitter release by barbiturates have been described (26). In addition, Blaustein (27) has demonstrated a barbiturate-induced decrease in Ca^{2+} uptake in synaptosomal preparations and NE release is a Ca^{2+}-dependent event (28). However, if this direct effect of PB occurs, why do increased NE turnover rates persist in MPN?

In conclusion, these data suggest that PB

blockade of preovulatory gonadotropin surges is not due to a direct effect on the anterior pituitary gland or on ME-LHRH accumulation but may be via its action in depressing hypothalamic NE release during proestrus afternoon.

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