Potentiation of Luteinizing Hormone-Releasing Factor Activities Following Pentobarbital Anesthesia in the Steroid-Blocked Castrated Rat[1] (37727)

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(Introduced by A. R. Midgley, Jr.)

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In many species including the rat and human (1, 2), the median eminence region of the hypothalamus contains a factor which is capable of stimulating the release of luteinizing hormone (LH). Materials extracted from porcine hypothalami have been purified (3), and the synthetic duplicate of the active molecule has been shown to induce the release of both follicle-stimulating hormone (FSH) and LH (4). In the proestrous rat, properly timed pentobarbital anesthesia prevents the preovulatory increase in serum LH (5, 6), presumably either by preventing the release of LH-releasing factor (LRF) from the hypothalamus or by impairing the ability of LRF to stimulate the release of LH from the anterior pituitary gland. These presumed effects of anesthesia on LRF secretion are, of course, based on the additional assumption that LRF is of physiological significance in the process of preovulatory LH release (7).

It is well-established that the pituitaries of barbiturate-anesthetized proestrous rats can respond to exogenous LRF. Ovulation has been induced in such rats by the intracarotid injection of purified porcine LH-releasing hormone (LH-RH) and by intrapituitary infusion of crude extracts of rat and bovine hypothalami (8–10). However, the quantitative effects of pentobarbital anesthesia on the response to LRF have not been established. Therefore, we have investigated the effects of such anesthesia in the ovariectomized estrogen- and progesterone (EP)-treated rat (11), a highly sensitive preparation in which hypothalamic extracts, purified LRF, and synthetic LH-RH preparations cause significant increases in serum LH concentrations (12, 13).

Materials and Methods. Adult female rats (280–320 g) of the Holtzman strain, ovariectomized 1 month earlier, were housed in air-conditioned quarters (70 ± 5° F) with Rockland rat chow and water available *ad libitum*. They were injected sc with estradiol valerate (50 μg) and progesterone (25 mg) on day 1. Three days later, they were anesthetized with ether, cannulated via the left carotid artery (14), and used approximately 4 hr after the operation. Injections of heparin, hypothalamic extract (H. E.), highly purified LH-RH, or brain extract (B. E.) were injected into, and blood samples were obtained from, the carotid cannula. At the beginning of each experiment, the rats were injected with 100 U of heparin in a volume of 100 μl. Prior to obtaining each blood sample, 40 μl of blood was withdrawn and discarded to minimize contamination of blood samples. Following each injection or withdrawal of blood, 40 μl of warm physiologic saline was injected into the cannula.

Crude extracts of rat hypothalami were

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prepared by homogenization of either fresh or frozen tissue in 0.1 *N* HCl (10 hypothalami/ml) at 4°. The next day the supernatant was clarified by low speed centrifugation (1000*g*) for 30 min at 4°. Just prior to use, the supernatant was neutralized to pH 6–7 with 1 *N* NaOH as indicated by pH indicator paper. The precipitate formed during neutralization was removed by centrifugation (1000*g*) at 4° for 10 min. Rat brain extracts (25 mg of frontal lobe tissue/ml in 0.1 *N* HCl) were prepared in a similar manner. Fifty micrograms of highly purified porcine LH-RH (AVS-77–3; tubes 219–230) prepared by, and obtained through the courtesy of, Dr. A. V. Schally (15), were dissolved in 3 ml of phosphate-buffered saline containing 1% egg white (PBS-EW). Aliquots of this stock solution were further diluted with PBS-EW to contain 0.032–0.080 μg LH-RH/100 μl and stored at −20°.

Each rat received two identical injections of H. E., B. E., or LH-RH in volumes of 100 μl (except for 3 animals which received two hypothalamic equivalents in 200 μl). The first injection was given prior to anesthesia while the second injection was given 30 min after pentobarbital anesthesia. Samples of blood (50 μl) were taken 15.5 min and prior to the first injection of H. E., B. E., or LH-RH and at intervals of 8, 20, 30, 40, 60, and 80 min after each injection. Ninety minutes after the first series of blood samples (170 min after the first injection), the rats were anesthetized with pentobarbital (30 mg/kg, ip), and 50-μl samples of blood were taken 15 and 30 min later. Immediately after the second postanesthetic blood sample was obtained, a second (identical) injection of H. E., B. E., or LH-RH was administered. Two blood samples (50 and 10 μl) were taken at 8-, 20-, 30-, 40-, 60-, and 80-min intervals following these injections. In the animals which received two injections of brain extract, H. E., was subsequently injected to determine if the animal was capable of responding to LRF. A 50-μl sample of blood was taken 8 min after this H. E. injection.

Each blood sample was promptly added to cold PBS-EW in an assay tube such that the final volume of serum plus PBS-EW equalled 500 μl. The diluted samples of whole blood were then stored at 4° for 1–3 days before being assayed for LH by radioimmunoassay (RIA). In calculating serum LH concentrations, all hematocrits were assumed to approximate 50%. All blood samples and representative aliquots of H. E., brain extract, and LH-RH were assayed in a double-antibody radioimmunoassay for LH (16) to determine the LH content or LH-like activity. The techniques used anterior pituitary gland extract, 1 mg of which is equivalent to 0.17 mg of NIH-LH-S_1 as determined by ovarian ascorbic acid depletion bioassay.

Results. Injections of hypothalamic extract. In the EP-treated rat, serum LH levels decrease from approximately 200 ng/ml prior to steroid treatment to approximately 30 ng/ml on the third day following steroid injections (12). In such unanesthetized rats, the injection of H. E. caused a rapid increase in serum LH levels which could not be explained by the LH-like activity of H. E. (Table I). The injection of an identical dose of H. E. into the same rat following pentobarbital anesthesia resulted in a potentiation of LH release (Table I). Increases in serum LH concentrations in anesthetized animals were 2.5–14-fold greater than the increases previously attained in the same (unanesthetized) animals. The time required for the peak concentrations of LH to appear in the serum (approximately 8 min after injection of hypothalamic extract) is in accord with values reported previously (12, 14). In both unanesthetized and anesthetized rats, serum LH levels declined rapidly following the initial increase and disappeared with a half-life of approximately 30 min, a value in agreement with previously reported estimates of LH half-life in rats (17–19). Serum luteinizing hormone levels did not increase following the injections of brain extract into either anesthetized or unanesthetized EP-treated rats (Table II), although such rats were capable of responding to LRF as evidenced by the increase in serum LH levels measured 8 min after a test injection of H. E.

Injections of luteinizing hormone-releasing hormone. Doses of purified porcine LH-RH

TABLE I. Effect of Pentobarbital Anesthesia on the Response[a] to Crude Rat Hypothalamic Extracts (H.E.).

Rat no.	Dose of H.E. (hypothalamic equivalents)	Increase in serum LH (ng B160/ml) 8 min after H.E. injections: Unanesthetized	Increase in serum LH (ng B160/ml) 8 min after H.E. injections: Anesthetized[b]
D108–16–1	2.0[c]	105	489
D108–16–2	2.0	110	437
D108–19–3	2.0	32	277
D108–19–1	0.5	46	400
D108–19–2	0.5	21	308
D108–58–1	1.0[d]	270	450
D108–96–1	1.0	131	245
D108–84–2	0.6	92	259
D108–84–1	0.2	47	116
D108–96–2	0.1	20	104

[a] In ovariectomized rats injected (sc) with estradiol valerate (50 μg) and progesterone (25 mg).

[b] Pentobarbital (30 mg/kg, ip) was injected 30 min before the ia injection of H.E.

[c] Indicated dose of previously frozen H.E. was injected prior to and following anesthesia (LH contamination was less than 20 ng B160/hypothalamic equivalent).

[d] Recently homogenized H.E. was injected prior to and following anesthesia (LH contamination was less than 30 ng B160/hypothalamic equivalent).

ranging from 0.032 to 0.080 μg/rat resulted in a rapid 3–7-fold increase in serum LH levels in unanesthetized rats. As indicated in Table III, the LH-like activity of LH-RH was not adequate to explain the observed responses. Injection of an identical dose of LH-RH into the same rat following anesthesia with pentobarbital resulted in a potentiation of LH release (Table III) similar to that observed for H. E. Maximum serum LH levels in the pentobarbital-anesthetized rats were increased up to 2.3-fold as compared with maximum serum levels attained prior to anesthesia. One of the rats which responded most dramatically to the first injection (an increase in serum LH or 337 ng/ml) failed to demonstrate a potentiation of LH release following anesthesia, suggesting that this animal might have achieved maximal release rates.

Discussion. In pentobarbital-blocked proestrous rats, injections of median eminence extracts directly into the pituitary gland or carotid artery induce LH release as indicated by the restoration of ovulation (8, 10). In such experiments, an altered responsiveness of the adenohypophysis during anesthesia could not have been determined, since serum LH levels could not be measured, and, using ovulation as the endpoint, the relative re-

TABLE II. Failure of Rat Cerebral Extracts to Alter Serum LH Levels in the Unanesthetized or Anesthetized Rat.[a]

Rat no.	Serum LH (ng B160/ml) at −1 to +80 min following injections of rat brain extracts (B.E.)[b]: Unanesthetized[d] + B.E. −1	+8	+20	+60	+80	Anesthetized + B.E. −1	+8	+20	+60	+80	Increase in serum LH (ng B160/ml) following injection of H.E.[c]: +8 min
D108–93–1	28	32	29	32	33	34	32	33	27	32	237
D108–93–2	33	28	20	32	33	33	32	32	30	27	226
D108–93–3	30	29	33	31	30	32	31	29	31	29	215
D108–93–4	28	29	32	32	34	33	31	33	33	30	214

[a] In ovariectomized rats injected sc with estradiol valerate (50 μg) and progesterone (25 mg).

[b] Extract of 2.5 mg of brain (frontal lobe) tissue/injection.

[c] One hypothalamic equivalent/rat. (LH contamination less than 20 ng B160/hypothalamic equivalent.)

[d] Pentobarbital (30 mg/kg, ip) given 30 min before injection of brain extract and 110 min before injection of H.E.

TABLE III. Effect of Pentobarbital Anesthesia on the Response to Purified LH-RH.[a]

Rat no.	LH-RH (μg/rat)[b]	Increase in serum LH (ng B160/ml) 8 min after LH-RH injection: Unanesthetized	Increase in serum LH (ng B160/ml) 8 min after LH-RH injection: Anesthetized[c]
D108–59–1	0.032	90	205
D108–59–2	0.048	98	241
D108–95–1	0.080	211	259
D108–95–2	0.080	158	251
D108–95–3	0.080	337	337
D108–95–4	0.080	176	247

[a] In ovariectomized rats injected sc with estradiol valerate (50 μg) and progesterone (25 mg).

[b] Highly purified porcine LH-RH (AVS-77-3, Nos. 219–230) was injected at the indicated dose both before and after anesthesia.

[c] Treated with pentobarbital (30 mg/kg, ip) 30 min before injection of LH-RH.

sponsiveness of the unanesthetized animal to exogenous LRF could not be determined. More recently, it has been demonstrated that pentobarbital anesthesia of the proestrous rat prevents the preovulatory increase in serum LH concentration (6) and induces a rapid increase in serum prolactin (20). The effects of pentobarbital treatment are generally accepted as evidence for an alteration in the activity of neurons regulating LH and prolactin secretion. For LH, in particular, it is assumed that anesthesia prevents the secretion of an LH-releasing factor, but there is little direct evidence to support the generally accepted theory that the proestrous "surge" of LH in the rat is preceded and caused by a comparable "surge" of LRF release. Malacara, Seyler, and Reichlin (21) have demonstrated increased LRF activity in the peripheral plasma of women during the period of preovulatory LH release, but a comparable increase in circulating LRF activity during the critical period of the proestrous rat has not been reported.

Although we had reasoned that the blockade of LH release in the anesthetized proestrous rat might be at least partially due to an altered pituitary blood flow or a decrease in pituitary sensitivity to LH-releasing factor, the data presented here support an entirely different conclusion; *i.e.*, that pentobarbital anesthesia actually enhances the response to releasing factors. Such anesthesia should, in the presence of continuing LRF secretion, cause an increase rather than a decrease in the rate of LH release. Given the assumption that intact proestrous and steroid-blocked castrated rats respond similarly to LRF following pentobarbital anesthesia, these results suggest that pentobarbital does not block ovulation by acting at the pituitary level. Given the additional assumption that the preovulatory LH surge is caused by LRF, the absence of an LH surge in anesthetized proestrous rats indicates that LRF release is absent or much reduced and suggests a depression of LRF-secreting neurons.

Our initial observations regarding the pentobarbital potentiation of induced LH release were obtained using crude hypothalamic extracts. Since there was the possibility that pharmacologically active substances other than LRF were responsible for this phenomenon, we injected a highly purified LH-RH preparation into both unanesthetized and pentobarbital-anesthetized EP-treated rats. These experiments also indicated a potentiation of LH-RH activity in the anesthetized rat, although the potentiation was less dramatic for LH-RH than for crude hypothalamic extracts. Thus, the possibility that hypothalamic substances other than LRF were responsible for the potentiation is remote. The specificity of the response is also supported by the observation that no change in serum LH concentrations were seen in either the unanesthetized or anesthetized rats following injections of brain extracts.

It has been demonstrated previously that unanesthetized rats of this type used in these studies respond to a second injection of hypothalamic extract with no indication of altered pituitary responsiveness (12). However, the data presented here indicate that pituitary responsiveness to the second injection of hypothalamic extract or purified porcine LH-releasing factor is significantly increased if the animal has been anesthetized with pentobarbital. The mechanism of this enhanced responsiveness is not apparent, but

we have considered the following possibilities:

(a) Altered metabolism of circulating LH or LRF. We have presented calculations (12) which suggest that the increase in serum LH concentrations in reponse to hypothalamic extracts cannot be explained on the basis of an altered rate of removal or inactivation of circulating LH. The same arguments apply to the potentiation of the response observed in these studies, and we conclude that the increased serum LH levels following anesthesia reveal an increase in the quantity of LH *released*. We have no data on the metabolism of LRF activities in the rat, and, therefore, cannot exclude the possibility that pentobarbital anesthesia prolongs the time required for degradation of the injected LRF activity.

(b) Removal of inhibitory influences. The results obtained in this study are compatable with the theory that pentobarbital anesthesia removes or decreases an inhibitory influence which had been preventing maximum LH release. In this case, it seems unlikely that secretion of an inhibiting factor has been prevented by pentobarbital anesthesia, since basal endogenous LH secretion did not increase following treatment with pentobarbital.

(c) Direct effect on the pituitary gland. The possibility that potentiation of LH release is due to a direct effect on cells of the hypophysis or improved circulation to such cells must be considered. In this regard, the data are pertinent to the observation that pentobarbital anesthesia prevents the preovulatory release of LH in the intact proestrous rat (6, 8, 10). If the data presented here can be extrapolated to such a normal animal, it appears that pentobarbital anesthesia must block LH release by preventing the exposure of the sensitized pituitary to endogenous LRF, presumably by preventing totally the release of LRF. An extension of this reasoning indicates that basal LH secretion in the steroid-blocked castrate (and proestrous rat) may not be regulated by LRF secretion, since basal LH levels do not increase in response to the sensitizing effects of pentobarbital anesthesia. Other data are available to suggest that basal LH secretion and the preovulatory "surge" of LH may be controlled by separate mechanisms (22).

Regardless of the mechanism involved, the observed potentiation of LH release in pentobarbital-treated rats complicates the interpretation of responses to releasing factors in anesthetized animals. In many studies, the LH-releasing potencies of hypothalamic extracts or other compounds have been tested by their ability to induce ovulation in the anesthetized proestrous rat. More recently, pentobarbital-anesthetized rats have been used for studies involving the infusion of releasing factors into the portal vessels of the hypophyseal stalk (23, 24) and for the evaluation of the efficacy of biogenic amines or electrical stimulation of the preoptic area in inducing the release of LH (6, 25).

Blake and Sawyer (26) recently reported that intact proestrous rats anesthetized with ether at the time of LH-RH injection were no less sensitive to LH-RH than similar animals anesthetized with pentobarbital or urethane. In EP-treated ovariectomized rats, equal responses were observed in animals anesthetized with urethane or ether, but the effect of pentobarbital on this type of animal was not studied. Although Blake and Sawyer concluded that pentobarbital anesthesia did not increase pituitary sensitivity in proestrous rats, they did not test the responsiveness of totally unanesthetized animals. We, on the other hand, have not studied the effects of anesthesia on the intact proestrous rat. However, the data presented here suggest that quantitative conclusions based on the responses of anesthetized rat may not be directly applicable to the unanesthetized animal and indicate that the inhibitory effects of pentobarbital anesthesia cannot be explained by a direct action on the pituitary gland.

Summary. In steroid-blocked ovariectomized rats, increases in serum LH concentrations were induced, both before and after pentobarbital anesthesia, by systemic injections of crude extracts of rat hypothalamic tissue (0.1–2.0 hypothalamic equivalents/rat) or by similar injections of purified porcine luteinizing hormone-releasing hormone

(LH-RH) at doses of 0.032–0.080 μg/rat. Increases in serum LH concentrations were greater during pentobarbital anesthesia than they had been in the same animals prior to anesthesia. Under comparable conditions, acid extracts of rat cerebral tissue (2.5 mg equivalents/rat) did not cause an increase in serum LH concentrations. These data reveal an increased responsiveness to LH-releasing activities during pentobarbital anesthesia and suggest that barbiturates probably do not block LH release in the proestrous rat by limiting the pituitary response to such stimuli.

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