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Absence of Immunoreactive Luteinizing Hormone-Releasing Hormone in Ovine Cerebrospinal Fluid Collected from the Third Ventricle (39641)[1]

R. J. COPPINGS, P. V. MALVEN, AND V. D. RAMIREZ

Department of Animal Sciences, Purdue University, West Lafayette, Indiana 47907 and Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801

Substantial quantities of luteinizing hormone-releasing hormone (LHRH) can be recovered from the median eminence (1). Additional brain areas have been implicated either in the synthesis of LHRH or its storage because their cell bodies contained material histochemically identified as LHRH (2) or because tissue extracts contained LHRH activity (1). Among these, the circumventricular organs (3), including the pineal organ (4), are especially pertinent to the present paper because it has been hypothesized (5) that the ventricular system might be a route for transport of LHRH and other hypophysiotropic factors. According to this hypothesis, LHRH might be secreted by one of the circumventricular organs into the cerebrospinal fluid (CSF), recovered from the CSF by ependymal cells lining the floor of the third ventricle, and transported across the median eminence (ME) to be released into the portal veins supplying the adenohypophysis. The second and third aspects of this hypothesis have already been proven feasible since LHRH can be recovered from portal blood following its intraventricular infusion (6, 7). Furthermore, the intraventricular route of LHRH administration (6, 8, 9) readily causes pituitary release of luteinizing hormone (LH).

If endogenous LHRH is transported by the ventricular system as hypothesized (5), endogenous LHRH should be recoverable from third ventricle CSF. Unfortunately, published results on this important point are contradictory. Joseph *et al.* (10) reported that CSF, collected after death from ovariectomized rats, contained large quantities (2300 ng/ml) of immunoreactive LHRH. Conversely, Cramer and Barraclough (11) could not detect immunoreactive LHRH in artificial CSF perfused through the third ventricle of anesthetized female rats electrically stimulated to release LH. Technical differences in methods of CSF collection and of LHRH radioimmunoassay make difficult any resolution of the contradictory results from these two reports. Therefore, the present study was conducted with ovariectomized sheep, a larger experimental species and one in which substantial volumes of CSF could be collected repetitively from the third ventricle of conscious animals. Furthermore, LH release could be both inhibited and stimulated by estradiol administration in this preparation.

Materials and methods. Intraventricular cannulae made from 20-gauge stainless steel hypodermic needles were permanently implanted into the third ventricle of three adult ewes ovariectomized 3 months earlier. Stereotaxic procedures (Malven, unpublished) for locating the third ventricle were aided by dorsal-ventral radiographs obtained by the method of Tarttelin (12) with minor modifications. When the cannula tip was in the third ventricle, CSF readily flowed to the top of the cannula. Following recovery from surgery, a teflon cannula was introduced into the jugular vein for collection of blood plasma. During experimental trials, CSF (0.4 to 0.5 ml) and blood (2 to 4 ml) were collected simultaneously at 1or 2-hr intervals for periods of about 24 hr. In three such trials, 50 μg of estradiol-17β ($E_2\beta$) was injected intramuscularly 2 hr after the initiation of sample collection. There was one control trial in which no $E_2\beta$ was administered. Extra blood samples were sometimes collected at 10- or 20-min intervals for periods of 4 to 6 hr in order to analyze the pattern of episodic LH release. One additional trial was performed in which 50 μg of synthetic LHRH was rapidly injected through the jugular vein cannula.

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Blood was collected at 2.5- to 10-min intervals from 90 min before to 150 min after LHRH injection. Third ventricle CSF was sampled at −90, −30, +5, +15, +30, +90, and +150 min relative to LHRH injection.

All samples of CSF were frozen on dry ice within 1 min after collection. Concurrently collected samples of blood were immediately centrifuged, and the separated plasma was frozen within 20 min. Additional blood samples, without a paired CSF sample, were kept at 4° for up to 6 hr prior to centrifugation. All samples of blood plasma and CSF were stored at −20° until immunoassayed for LH or LHRH.

In order to estimate potential losses of endogenous LHRH during storage and assay procedures, known quantities of synthetic LHRH were added to seven samples of freshly collected CSF. Synthetic LHRH (300 to 2700 pg) was also added to 2-ml aliquots of heparinized whole blood which were then incubated at 4° for 30 to 120 min prior to separation of plasma and storage at −20°. The pituitary stalk-median eminence region was dissected immediately after death in two of these ewes, weighed, and frozen for subsequent extraction and LHRH assay. The location of the cannula tip within the third ventricle of each ewe was verified by gross dissection of the ventral surface of the brain.

Radioimmunoassays (RIA). Concentrations of LH in blood plasma were quantified by procedures described previously (13). The antiserum developed and characterized by Kerdelhue *et al.* (14) was used to measure LHRH in CSF, blood plasma, and stalk-median eminence extracts. Synthetic LHRH (Beckman lot No. 1 337702) was used as standard, and the same material was radioiodinated (^{125}I) by the chloramine-T method and purified by gel filtration on Sephadex G-25. Radioactively labeled LHRH was added to the assay in a volume of 100 μl. The assay diluent was 0.1% gelatin in 0.01 *M* phosphate-buffered saline, pH 7.5, (gelatin–PBS), and it was added to each assay tube in variable volumes so that the total volume of diluent plus standard or diluent plus sample equaled 500 μl. Antiserum against LHRH (1:50,000) was added in a volume of 200 μl, yielding a final concentration of 1:200,000. These components were incubated at 4° for 24 hr, and, then, 2 ml of cold (4°) absolute ethanol was added (15). Following centrifugation, the supernatant was decanted, and the radioactivity in the precipitate was determined.

In addition to the standard curve of synthetic LHRH, each assay included serial dilutions of acetic acid extracts of ovine stalk-median eminence (SME). Inhibition curves resulting from increasing amounts of standard LHRH and SME extract were always parallel. Aliquots of freshly thawed samples of CSF were assayed at volumes of 100 and 200 μl each. Since the minimum LHRH standard that could be reliably distinguished from zero was 20 pg/tube, the minimum detectable concentration of LHRH in the 200-μl aliquots of CSF was 100 pg/ml.

Prior to LHRH assay, all samples of blood plasma were subjected to a rigorous extraction procedure. The proteins were precipitated from 1-ml aliquots of plasma by addition of 1 ml of a 0.2 *M* acetic acid–absolute ethanol mixture (1:1) followed by centrifugation. The supernatant was then boiled at 75° for 5 min, resulting in the formation of a second precipitate. The supernatant from this precipitation step was then mixed with 0.5 ml of the acetic acid–absolute ethanol mixture and centrifuged again. The supernatant thus obtained was boiled to dryness, and 500 μl of gelatin–PBS was added to start the assay. The minimum plasma LHRH concentration that could be reliably distinguished from zero was 20 pg/ml.

Results and discussion. Table I summarizes the data and shows that immunoreactive LHRH could not be detected in natural CSF collected from the third ventricle of conscious ewes. The data for the three trials in which $E_2\beta$ was injected were divided according to time periods after $E_2\beta$. Coppings and Malven (16) observed initial inhibition of LH release for the period 2–10 hr after injection and, then, facilitation at later time periods (12–20 hr). The inhibition of plasma LH was also observed herein, but in only two of the present trials was the facilitation sufficient to induce typical LH surges peaking at plasma LH concentrations of approximately 30 ng/ml. In the third $E_2\beta$ trial,

TABLE I. CONCENTRATIONS OF LH IN PLASMA AND LHRH IN PLASMA AND CSF IN UNTREATED AND $E_2\beta$-TREATED EWES AND AFTER INTRAVENOUS LHRH INJECTION.

Hormone measured	Endocrine treatment of ewes				
	Untreated (n = 1[a])	$E_2\beta$ Treated (n = 3)		LHRH treated (n = 1)	
		Inhibitory periods (2–10 hr)	Stimulatory periods (12–20 hr)	Before LHRH	After LHRH
Plasma LH (ng/ml)	9.9 ± 0.8[b] (14)	1.5 ± 0.1[c] (31)	11.4 ± 1.1 (36)	1.4 ± 0.3[c] (10)	42.0 ± 4.6[c] (19)
CSF LHRH (pg/ml)	<100 (14)	<100 (20)	<100 (24)	<100 (3)	<100 (5)
Plasma LHRH (pg/ml)	66 ± 7 (14)	67 ± 9 (31)	43 ± 6[d] (36)	73 ± 4 (3)	251 ± 47[c] (9)

[a] n = Number of experimental trials.
[b] All means are presented ± SE with the number of samples in parentheses.
[c] Significantly different ($P < 0.01$) from average in untreated trials (Student's t-test).
[d] Significantly different ($P < 0.05$) from average in untreated trials (Student's t-test).

no LH surge took place although pulsatile LH release recurred between 12 and 20 hr after $E_2\beta$ injection. The failure of a sustained LH surge to occur in this one trial resulted in a mean plasma LH lower then expected, 12–20 hr after $E_2\beta$ (Table I).

Both Table I and Fig. 1 contain data from one trial in which blood plasma and CSF were collected before and after intravenous injection of 50 μg of synthetic LHRH. As expected, LHRH injection immediately increased plasma concentrations of immunoreactive LHRH with a maximum value of 420 pg/ml occurring at 2.5 and 5.0 min after injection. Plasma LHRH concentrations decreased rapidly thereafter, reflecting a fast clearance of the exogenous hormone from the blood plasma. Plasma LH also increased 2.5 min after injection, but, in contrast to LHRH levels, plasma LH continued to increase, reaching a maximum of 80 ng/ml at 70 min after LHRH injection. In both the present study and that of Jonas *et al.* (17), plasma LHRH returned to pretreatment levels prior to the maximal plasma LH concentration. Despite the large increase in plasma LHRH concentration following its injection, no immunoreactive LHRH could be detected in CSF samples (minimum detectable concentration = 100 pg/ml). These results and those of Cramer and Barraclough (11) in rats suggest that significant amounts of LHRH did not pass from blood into CSF.

The failure to find immunoreactive LHRH in natural CSF collected from the

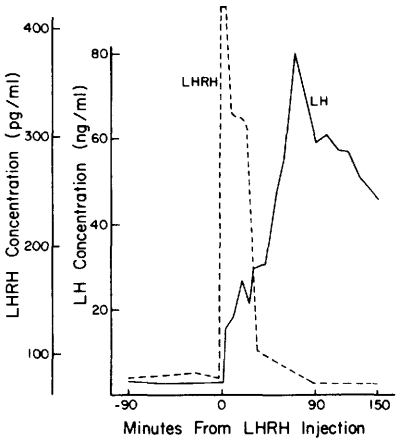

FIG. 1. Plasma concentrations of LH (solid line) and LHRH (dotted line) before and after intravenous injection of 50 μg of synthetic LHRH in one trial.

third ventricle cannot be attributed to the lack of a suitable RIA. The two SME extracts contained an average of 26 ng/ovine SME which was comparable to the 30 ng/ovine hypothalamus reported previously (14). Also, the collection and storage procedures for CSF did not appear to destroy immunoreactive LHRH. The recovery of exogenous LHRH, added to freshly collected CSF, averaged 142 ± 7%.

The present results with natural CSF confirm the observations of Cramer and Barraclough (11), using artificial CSF perfused through the third ventricle of anesthetized female rats. The present results contradict those in which relatively high levels of LHRH were recovered from CSF collected

from ovariectomized female rats (10) and from male rats (18) after death. Using a bioassay for LHRH rather than an immunoassay, Gradwell and Symington (19) also failed to find LHRH-like activity in bovine CSF collected from the spinal region.

In contrast to CSF, immunoreactive LHRH was detected in blood plasma but in highly variable concentrations. Recovery of exogenous LHRH, added to whole blood, averaged only 49%, but the rigorous extraction procedure probably contributed to this low average. LHRH concentrations in plasma samples from an untreated ovariectomized ewe averaged 66 pg/ml. (Table I). Plasma concentrations of LHRH did not change during inhibitory periods in $E_2\beta$ trials; however, during stimulatory time periods after $E_2\beta$ (12–20 hr), plasma LHRH tended to be reduced ($P < 0.05$). This inverse relationship between plasma LHRH and plasma LH concentrations might reflect a more efficient pituitary uptake of endogenous LHRH from portal vein blood during periods of $E_2\beta$-stimulated LH release. This interpretation of the present data contradicts the assumption of previous workers that concentrations of LHRH in the peripheral blood plasma of sheep might reflect the rate of release of endogenous LHRH from the hypothalamus. In previous reports, a change in plasma LHRH was not correlated with spontaneous or $E_2\beta$-induced LH surges in sheep (17, 20). Differences in methodology may partially explain these dissimilar results. The procedure employed in the present experiments to extract LHRH from blood plasma prior to assay differed from that used by Jonas *et al.* (17), whereas Nett *et al.* (20) did not use any extraction procedure. Moreover, the endogenous immunoreactivity measured by Jonas *et al.* (17) was shown to be different from exogenous LHRH. In this regard, Jeffcoate and Holland (21) reported chromatographic evidence that LHRH immunoreactivity in the peripheral blood of the sheep could be separated into multiple peaks. Interestingly, none of the LHRH peaks resolved chromatographically by these authors corresponded to exogenous LHRH. This suggests that the interaction of LHRH with the pituitary may modify the conformation or composition of LHRH. Therefore, the significance of plasma LHRH measurements made by using antisera against synthetic LHRH is unclear.

Summary. Cerebrospinal fluid (CSF) was collected repetitively from the third ventricle of three ovariectomized sheep. Immunoreactive LHRH could not be detected (<100 pg/ml) in any of 70 samples of CSF collected from untreated, estradiol-treated, and LHRH-treated ewes. The LHRH immunoassay accurately measured the hormone in extracts of ovine stalk-median eminence tissue. Furthermore, no immunoreactive LHRH was lost when synthetic LHRH was added to freshly collected CSF and stored in the manner used for the attempted measurement of endogenous LHRH. Intravenous injection of LHRH increased plasma concentrations of the hormone to 420 pg/ml but LHRH could not be detected (<100 pg/ml) in CSF collected concurrently.

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