PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, 147 434-437 (1974)

Luteinizing Hormone Releasing Hormone-Biosynthesis by Rat Hypothalamus *in Vitro*. Influence of Castration[1] (38357)

JAIME A. MOGUILEVSKY[2], MARIA A. ENERO, AND BERTA SZWARCFARB

(Introduced by Virgilio G. Foglia)

Sección de Neuroendocrinología. Instituto de Fisiología. Facultad de Medicina, Universidad de Buenos Aires and Instituto de Investigaciones Farmacológicas, Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, República Argentina

It has been shown that changes in the secretion of gonadotrophins and/or sexual hormones are accompanied by modifications in the oxidative and protein metabolism of hypothalamus (1, 2). For instance, protein synthesis in the hypothalamus of proestrus and castrated rats is higher than in normal diestrus rats (3). Considering that changes in the protein synthesis of hypothalamus could, in some way, reflect modifications in the hypothalamic synthesis of peptides involved in the control of the anterior pituitary gland, the present experiments were conducted in order to study the effect of gonadectomy on the synthesis of luteinizing hormone releasing hormone (LRH) by rat hypothalamus *in vitro*. The procedure as well as the data obtained are described in the present paper.

Material and Methods. Fragments of hypothalamic tissue from ten adult normal rats (in each experiment) were incubated in one ml Krebs solution containing 10 m*M* glucose (pH 7.4) and 50 μc of (−) tyrosine 3–5 ^{3}H (sp act 25 Ci/ m*M*, New England Nuclear) during 60 min at 37° with continuous shaking under a O_2:CO_2 95%:5% atmosphere. After the incubation acetic acid to give a final concentration of 1 *N* was added to the medium. The mixture was frozen, homogenized and centrifuged at 11,000 rpm (30 min). After centrifugation, an aliquot of the supernatant was taken to determine protein by the method of Lowry (4) and 1.2 ml was passed through a Sephadex G-25 column (0.8 ± 120 cm) equilibrated with 0.2 *N* acetic acid at room temperature. The material was eluted with 0.2 *N* acetic acid. The fractions were monitored at 230 and 280 nm and tested for radioactivity and LRH activity.

The fractions obtained from Sephadex columns with LRH activity were combined, adjusted to pH 8.0 with ammonium hydroxide and evaporated to dryness at 40° under vacuum. The residue was dissolved in 0.1 *N* acetic acid to pH 5.0 and extracted with cloroform:methanol (3:2;v:v) then the peptide material was recovered in the upper layer adjusted to pH 8.0 with ammonium hydroxide and evaporated to dryness as described above. The residue was dissolved in 50 m*M* ammonium acetate (pH 5.0) and chromatographed on CM-Sephadex C-25 column (0.5 × 7.0 cm). A linear gradient from 50 m*M* ammonium acetate (pH 5.0) to 200 m*M* (pH 7.0) was used for the elution at room temperature. The fractions were monitored at 230 and 280 nm and tested for radioactivity and LRH activity.

Radioactivity was determined in a solution containing toluene 666 ml, Triton X-100 333 ml, PPO (2.5-diphenyloxazole) 5.5 g and POPOP (1.4 bis[2-(5 phenyl)-oxazolyl] benzene) 0.1 g as scintillator. Correction for quenching was obtained by the addition of known amounts of tritiated water. Radioactivity incorporated was expressed as specific activity: dpm per mg of initial protein content.

The *in vivo* determination of LRH activity in the different fractions was performed by using

[1] Supported by a Grant from the Consejo Nacional de Investigaciones Científicas y Técnicas.

[2] Established Investigator. Consejo Nactional de Investigactiones Científicas y Técnicas.

Printed in U.S.A.

adult female rats from the strain of the Institute of Physiology. After ovariectomy and 72 hr before the administration of the samples, the rats were injected with 50 μg of estradiol benzoate (im) and 25 mg of progesterone (im). Under pentobarbital anesthesia, control blood samples were drawn from the jugular vein, and then the samples for assay were injected into this vein. Blood samples were taken again after 15 min.

LH concentration in serum, from individual rats, was assayed at two doses levels using the rat LH radioimmunoassay, described by Niswender *et al.* (5). The results were expressed as NIAMD-Rat-LH-RP-1 standard which biological potency is equivalent to 0.03 × NIH-LH-S1.

The castration was carried out under ether anesthesia 4–6 weeks prior to sacrifice.

Results and Discussion. In Fig. 1 can be seen the elution pattern of normal hypothalamic tissue as well as tubes combined for LRH activity assay and radioactivity after the chromatography on Sephadex G-25. The radioactivity incorporated in different peptides is shown in Table I. The high size radioactive peptides emerged in fraction S-1, while in fraction S-2 the small radioactive peptides with LRH activity were eluted. In the experiments without incubation, no radioactivity was found in fractions S-1 and S-2. This seems to indicate that the radioactivity found in these fractions represented ^{3}H-Tyr incorporated into the peptides. ^{3}H-Tyr emerged from Sephadex column in tubes 31–38 thus, the fraction S-3 represented the elution of part of the labeled aminoacid.

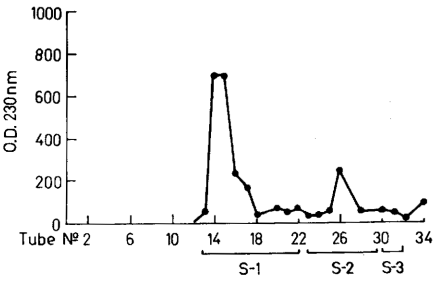

FIG. 1. Gel filtration of rat hypothalamic extract on a column of Sephadex G-25 (0.8 × 120 cm). Solvent 0.2 *N* acetic acid. Fraction size 1.5 ml. Flow rate 0.21 ml/min. Each point represents the mean of four experiments. Tubes combined for LRH activity assay and radioactivity are indicated.

Absorbancy in Fig. 2 represents the behavior of the hypothalamic peptide material from control tissue, that emerged in fraction S-2, in CM-Sephadex.

Table II shows the ^{3}H-Tryr incorporated in hypothalamic extracts from normal and castrated rats and LRH activity of the fraction S-2 from Sephadex G-25 after chromatography in CM-Sephadex.

As can be seen in fraction CM-3 the material with LRH activity emerged and only in this fraction the radioactivity incorporated is significantly higher in castrated than in control rats ($P < 0.01$). On this basis it can be postulated

TABLE I. Radioactivity and Biological Activity of Acetic Acid Extracts of Rats Hypothalamus after Separation in a Sephadex G-25 Column.

		Biological activity[a]	^{3}H-Tyr (dpm/mg protein)	
			Time incubation	
Fraction	Tubes	ngLH released ml serum	0 min[b]	60 min[c]
Control	Acetic acid 1N (pH 7.0)	230 ± 34 (3)	—	—
S-1	13–23	172 ± 47 (4)	0	23,754 ± 1,647 (5)
S-2	24–29	2450 ± 686*(3)	0	59,352 ± 8,553 (5)
S-3[d]	30–32	342 ± 80 (4)	220,532	299,801 ± 53,680 (5)

[a] Pool of the hypothalamic extracts from ten normal rats was used in each experiment. The biological activity is expressed as ng LH/ml serum over the basal values.

[b] Hypothalamic tissue plus 50 μc ^{3}H-Tyr without incubation. Means of two experiments.

[c] Similar to b but incubated during 60 min at 37°C.

[d] The radioactivity of this fraction represents part of the ^{3}H-Tyr eluted in the column.

*Significantly different ($P < 0.025$) from control values using *t* test.

Number of experiments is shown between parenthesis.

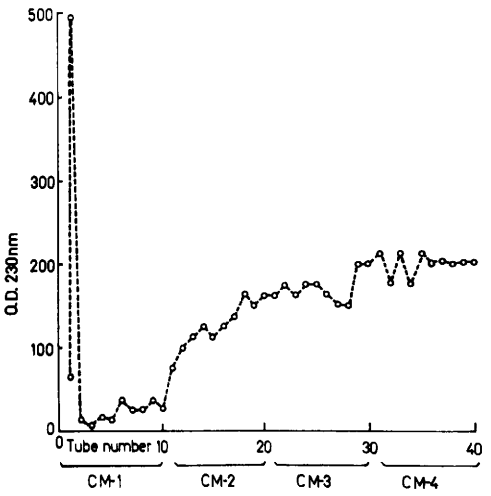

FIG. 2. Ion-exchange chromatography of fraction S-2 from Sephadex G-25 (see Fig. 1) on a CM-25 column (0.5 × 7.0 cm). Column equilibrated with ammonium acetate 50 m*M*, pH 6.0. Gradient to 200 m*M*, pH 7.0 started at tube 1. Fraction size 2.0 ml. Flow rate 0.5 ml/ min. Tubes combined for LRH assay and radioactivity are indicated.

that the differences found in the radioactivity of Fraction CM-3 are related to an increase in the hypothalamic synthesis of LRH in castrated rats. The fact that gonadectomy increased LRH activity in the rat hypophysial portal blood further supported this point of view (6).

The fact that fraction CM-3 showed LRH activity (See Table II) appear to indicate that our experimental condition does not alter the biological activity of natural LRH from rat hypothalamus. On the other hand, in experiments which will be published in detail, we also found no modifications in the biological activity of synthetic LRH with the experimental procedure of purification described in the present paper.

It is important to note the low rate of aminoacid incorporation into the peptide material emerged in fraction CM-3. In this respect Johansson *et al.* (7), incubating porcine hypothalamus during 4 hr and with a different medium, also have shown a low rate of aminoacid incorporation into LRH. It is probable that the small synthesis of LRH obtained *in vitro* is representative of a physiological situation in the living animal, where this active peptide is synthetized in a very small quantity by the hypothalamus under normal conditions. Nevertheless more experimental evidence is needed before conclusions can be reached.

Summary. The biosynthesis of the luteinizing hormone releasing hormone (LRH) from ^{3}H-tyrosine by rat hypothalamus *in vitro* has been studied in normal and castrated male rats. After 1 hour of incubation with ^{3}H-Tyr the hypothalamic extracts were chromatographed on Sephadex G-25. Fractions with LRH activity determined by *in vivo* assays, eluted from this column were passed through a CM-Sephadex C-25 column with a gradient of ammonium acetate 50 m*M*, pH 5.0–200 m*M* pH 7.0. Radioactivity which emerged in the fraction with LRH activity was considered as ^{3}H-Tyr incorporated into peptides with LRH activity and this incorporation is higher in castrated than in normal rats.

TABLE II. Radioactivity and Biological Activity of Fraction S-2 from Sephadex G-25 after Separation in a CM-Sephadex C-25 Column.

Fraction	Tubes	Biological activity[a]	^{3}H-Tyr(dpm/mg protein)[b]	
		ngLH released ml serum	Normal	Castrated
Control	Acetic acid 1N	230 ± 34(3)	—	—
CM-1	1–10	206 ± 63(3)	28,026 ± 1,902 (5)	34,278 ± 8,465 (5)
CM-2	11–20	235 ± 70(3)	152 ± 26 (5)	240 ± 96 (5)
CM-3	21–30	996 ± 207*(3)	82 ± 10 (5)	155 ± 15** (5)
CM-4	31–40	235 ± 64 (3)	65 ± 18 (5)	66 ± 19 (5)

[a] Pool of the hypothalamic extracts from 10 normal rats was used in each experiment. The biological activity is expressed as ng LH/ml serum over the basal values.

[b] Radioactivity incorporated in hypothalamic tissue.

*Significantly different ($P < 0.05$) from control values using t test.

**Significantly different ($P < 0.01$) from normal values using t test.

Number of experiments is shown between parenthesis.

The authors are grateful to Dr. Laura Caligaris and Dr. S. Taleisnik for the radioimmunoassays and to Dr. N. Aparicio and Dr. S. Z. Langer for the generous advice and help.

1 Moguilevsky, J. A., and Malinow, M. R., Amer. J. Physiol. **206,** 855 (1964).

2. Moguilevsky, J. A., Scacchi, P., and Christot, J., Proc. Soc. Exp. Biol. Med. **137,** 653 (1971).

3. Moguilevsky, J. A., and Christot, J., J. Endocrinol. **55,** 147 (1972).

4. Lowry, O. H., Rosebrough, N. S., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).

5. Niswender, G. D., Midgley, A. R., Jr., Monroe, S. E., and Reichert, L. E., Jr., Proc. Soc. Exp. Biol. Med. **128,** 807 (1968).

6. Ben-Jonathan, N., Mical, S. R., and Porter, J. C., Endocrinology **93,** 497 (1973).

7. Johansson, N. G., Hooper, F., Sievertsson, H., Currie, B. L., and Folkers, K., Biochem. Biophys. Res. Commun. **49,** 656 (1972).

Received November 26, 1973. P.S.E.B.M., 1974, Vol. 147.