In Vivo Inhibition of Protein Synthesis in Specific Hypothalamic Nuclei by 17β-Estradiol[1] (34771)

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Estrogen has several actions on the brain as evidenced by effects on sex behavior, central nervous system excitability (1), and on control of hypothalamic gonadotropin-releasing factors (2). Estradiol (3, 4) or ovarian fragments (5) implanted into various hypothalamic sites decrease gonadotropin function which in turn may reflect decreased availabiltiy of gonadotropin-releasing factors.

Neurons of the arcuate nucleus have been implicated in the synthesis of gonadotropin-releasing factor(s), a function which is inhibited by estradiol (3). Since gonadotropin-releasing factors may be small polypeptides (6), it seemed possible that estrogen might influence the synthesis of specific brain proteins and that this would be reflected by altered uptake and incorporation of amino acids into these compounds. It was of interest therefore to determine if estradiol would influence the uptake, incorporation, and localization of amino acids in brain protein in general.

Although the hypothalamus is the brain area of primary interest to this study, the cerebellum and motor cortex were also chosen because they have been shown to have high uptake and incorporation of ^{14}C-lysine into brain protein (7). ^{14}C-Lysine was selected for this purpose because it is rapidly taken up by the brain and in short-term experiments the label is attributable to lysine as either the free amino acid or bound to protein (8).

Administration of a pharmacologic dose (approx 6 mg/month) of estradiol over a 4-month period was chosen in an attempt to simulate the prolonged use of estrogens as antiovulatory agents in humans. The monthly dose of estrogen used in the sequential oral contraceptives is approximately 2 mg (9).

Materials and Methods. Long-Evans female rats (180 to 200 g), placed on a schedule of 14-hr light and 10-hr dark, received water and Purina rat chow *ad libitum*. One week ofter ovariectomy, the experimental group was injected sc for 4 months with a daily dose of 100 μg of 17β-estradiol dipropionate dissolved in sesame oil/100 g of body weight (b wt). Controls received injections of sesame oil.

Tissue preparation. After 4 months of treatment the rats were injected ip with 1 μCi/40 g of b wt of uniformly labeled L-lysine ^{14}C-monohydrochloride (sp act = 150 mCi/mmole, Nuclear Chicago Corp., Des Plaines, Ill.). Rats were decapitated 10, 20, and 30 min after injection and the blood was collected in heparinized tubes placed in an ice bath. The brain was rapidly removed and rinsed free of adhering blood. The left cerebellar lobe, hypothalamus, and motor cortex were removed, weighed, and frozen on Dry Ice.

Because uptake and synthesis of ^{14}C-lysine into protein by the three areas of the brain was very low, two rats from the same group were sacrificed consecutively and the corresponding tissues were combined. Each tissue pool was then treated as one sample.

Immediately after removal, the frozen tissues were homogenized in 10% trichloroacetic acid (1 ml of TCA/40 mg of tissue) and centrifuged at 0° for 15 min at 1500 rpm. Blood samples were centrifuged and 0.5 ml of each of the two plasma samples was added to 4.0 ml of TCA and centrifuged. Tissue and

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plasma supernatants contained ^{14}C-lysine in the free amino acid pool, *i.e.*, ^{14}C-lysine which had not been incorporated into protein. The TCA precipitates (ppt) from plasma and tissue were washed twice with 2 ml of 5% TCA containing 0.2% unlabeled lysine. Specific activity of the washes was negligible and they were discarded. Tissue and plasma supernatants were frozen overnight and the tissue TCA insoluble ppt were stored at 2°.

Specific activity of ^{14}C-lysine in the free amino acid pool. Duplicate samples of TCA extracts (0.25 ml) were plated on aluminum planchettes and dried under an infrared lamp. Counts per minute (cpm) corrected for background, were determined with a Nuclear Chicago gas flow counter. Specific activity was expressed as cpm per mg of wet sample weight.

Specific activity of ^{14}C-lysine incorporated into protein. TCA-insoluble residues were resuspended in 5% TCA and were heated in a 90° water bath for 15 min to remove nucleic acids (8). Lipids were extracted by the method of Roberts (10).

Weighed portions of the residues were removed for the determination of protein (11). The remaining residues were weighed and solubilized in 1.5 ml of 88% formic acid in a 60° water bath for several hours. One ml of the suspensions was plated and treated as described above. Specific activity was expressed as cpm per mg of protein.

Radioautographic localization of ^{14}C-lysine incorporated into protein. After 4 months of treatment, 4 controls and 4 estradiol-treated rats were each injected with ^{14}C-lysine (1 μg/40 g of b wt). Twenty-five min later, sodium pentobarbital (5 mg/100 g of b wt) was administered ip. Thirty min after the injection of lysine the degree of anesthesia was sufficient to permit perfusion of the brain via the left ventricle of the heart. Solutions used for the perfusion are described by Koenig *et al.* (12). Brains were embedded with Paraplast and 6-μ sections were coated with Kodak nuclear liquid emulsion NTB 2 utilizing the radioautographic technique of Kopriwa and Leblond (13). After an exposure interval of 11 weeks, brain sections were developed, stained with thionin, and silver grains were determined with a micrometer disc and cell counter from randomly chosen areas totaling either 25 or 56 μ^2 for each area of the brain investigated. Counts (corrected for background radiation) were made by the same person at a magnification of 1250.

Statistics. The Student's t test was used to detect significance of differences between control and experimental groups. $p \leqq 0.05$ was considered significant.

Results. Organ weights. The effectiveness of the estradiol treatment is confirmed by the results, *i.e.*, the lower body weight and hematocrits and the larger anterior pituitaries and uteri.

Specific activity of ^{14}C-lysine in the free amino aicd pool of various brain areas (Table I). Specific activity of ^{14}C-lysine in the free amino acid pool of the hypothalamus, cerebellum, and motor cortex reached a maximum in all rats at 20 min after the ip administration of ^{14}C-lysine; however, in rats treated with estradiol, specific activity was significantly lower than that of controls in the hypothalamus and cerebellum. A less marked decrease occurred in the motor cortex of estradiol-treated rats.

Specific activity of ^{14}C-lysine in the free amino acid pool of plasma reached a maximum value either at /or prior to sacrifice 10 min following the administration of the amino acid.

Specific activity of ^{14}C-lysine incorporation into protein (Table II). Incorporation into protein increased with time for all three areas of the brain for all rats. By 30 min in rats treated with estradiol, incorporation was significantly lower than that of the controls in the hypothalamus and cerebellum. A less marked inhibition in incorporation occurred in the motor cortex of the estradiol-treated rats ($p<0.1$).

Concentration of protein in the TCA-insoluble residues. No differences in protein content of the three areas of the brain were found between the two groups of rats for any time interval. Average protein content of the three areas of the brain for both groups of rats was 872 ± 44 μg/mg of TCA-insoluble residue.

Radioautographic localization of ^{14}C-lysine

TABLE I. Specific Activity of ^{14}C-Lysine in the Free Amino Acid Pool (cpm/mg of wet tissue) 10, 20, and 30 min Following Administration.

Tissue	Time (min): 10	20	p^a	30	p^a
Hypothalamus					
Control	2.13 ± 0.14[b]	3.17 ± 0.34	<.02	1.94 ± 0.50	—
Estradiol	1.54 ± 0.26	1.64 ± 0.22	—	1.14 ± 0.18	—
p^c	—	<.001		—	
Cerebellum					
Control	1.65 ± 0.11	3.32 ± 0.17	<.001	1.93 ± 0.27	<.01
Estradiol	1.36 ± 0.09	1.63 ± 0.27	—	1.39 ± 0.19	—
p^c	—	<.001		—	
Motor cortex					
Control	1.63 ± 0.24	2.33 ± 0.27	—	1.33 ± 0.29	<.05
Estradiol	1.58 ± 0.17	1.57 ± 0.27	—	0.92 ± 0.23	—
p^c	—	—		—	
Plasma					
Control	6.97 ± 0.25	4.69 ± 0.67	<.02	1.55 ± 0.36	—
Estradiol	6.23 ± 0.37	3.96 ± 0.13	—	1.59 ± 0.25	<.01
p^c	—	—		—	

[a] p values for significance of difference between means for a given brain area in control or estradiol-treated rats between specified time intervals.

[b] Mean ± standard error; the mean represents an average of six values, and each value represents combined tissues from two rats.

[c] p values represent significance of difference between means of controls and estradiol-treated rats.

incorporated into brain protein (Table III). Of the eight hypothalamic nuclei examined, grain counts of the paraventricular, periventricular, and arcuate nuclei of the rats treated with estradiol were significantly lower than the control rats.

No difference in incorporation of ^{14}C-lysine into protein of the motor cortex occurred between the two groups of rats by 30 min (Table II). The radioautographic data confirms this finding (Table III). Incorporation of ^{14}C-lysine into protein of the entire left cerebellar lobe was significantly depressed in the rats treated with estradiol (Table II), however, this difference is not localized in the three layers of the cerebellar cortex which was the only area examined (Table III). In this case decreased incorporation may be due to decreased uptake in other regions of the cerebellum or may simply reflect decreased availability of ^{14}C-lysine in the free amino acid pool.

In contrast to gray matter (motor cortex, cerebellum, hypothalamus), the corpus callosum (predominantly white matter) of both groups of rats incorporated significantly less ^{14}C-lysine into protein. This is in agreement with work cited by Altman (14).

Within the same group, incorporation of ^{14}C-lysine into protein of the chorioid plexus (an area outside the blood–brain barrier) was significantly higher than that of all areas of the brain examined with the exception of the paraventricular nucleus. Incorporation into the paraventricular nucleus was the same as that for the chorioid plexus ($p>0.2$). The ability of the paraventricular nucleus to synthesize large amounts of amino acids into protein has been noted (15).

Discussion. Uptake of ^{14}C-lysine into the free amino acid pool of hypothalamus and cerebellum was significantly lower in estradiol-treated rats 20 min following amino acid administration, whereas the lower uptake in

TABLE II. Specific Activity of ^{14}C-Lysine Incorporated into Protein (cpm/mg of protein) 10, 20, and 30 min Following Administration.

Tissue	Time (min): 10	20	p^a	30	p^a
Hypothalamus					
Control	2.24[d]	5.18 ± 0.88[b]	—	11.25 ± 0.82	<.01
Estradiol	1.80 ± 0.20	6.64 ± 1.42	—	6.72 ± 1.00	—
p^c	—	—		<.02	
Cerebellum					
Control	3.33[d]	6.08 ± 0.93	—	10.86 ± 0.92	<.01
Estradiol	1.52 ± 0.22	7.16 ± 1.03	—	6.29 ± 0.95	—
p^c	—	—		<.001	
Motor cortex					
Control	1.48[d]	11.52 ± 1.68	—	17.58 ± 2.51	—
Estradiol	2.17 ± 0.42	10.76 ± 1.68	—	12.14 ± 0.64	—
p^c	—	—		—	

[a] p values for significance of difference between means for a given brain area in control or estradiol-treated rats between specified time intervals.

[b] Mean ± standard error; the mean represents an average of four to six values, and each value represents combined tissues from two rats.

[c] p values represent significance of difference between means of control and estradiol-treated rats.

[d] Means of only two or three values and therefore not included in the statistical evaluation.

the motor cortex was less marked (Table I). An increase in incorporation of ^{14}C-lysine into protein at the 20-min interval for the estradiol-treated rats is not responsible for the lower concentration of lysine in the free amino acid pool, since at 20 min, synthesis into brain protein was the same for both groups of animals (Table II).

At 30 min, incorporation of ^{14}C-lysine into protein was significantly lower in the cerebellum and hypothalamus, and to a lesser extent in the motor cortex, of the estradiol-treated rats. However, since the concentration of ^{14}C-lysine in the free amino acid pool is also depressed at 20 min, the decreased synthesis into brain protein of the estradiol-treated rats may merely reflect decreased availability of free lysine rather than a true inhibition of protein synthesis. The radioautographic data differentiates between these two possibilities with respect to the hypothalamus and is discussed below.

The data presented indicate that chronic treatment with estradiol significantly impaired the entry of ^{14}C-lysine into the free amino acid pool of both hypothalamus and cerebellum. In view of the evidence that lysine enters the brain by an active transport

TABLE III. Silver Grain Counts for Various Brain Areas Following 4-Months Daily Treatment with Estradiol Dipropionate in Adult Ovariectomized Rats.

Brain area	Silver grains/1.56 μ^2 Controls[a]	Estradiol[a]	p
Motor cortex	9 ± 0.6	9 ± 0.6	—
Cerebellar cortex	7 ± 0.6	6 ± 0.3	—
Corpus callosum	3 ± 0.3	4 ± 0.3	—
Chorioid plexus	14 ± 0.3	14 ± 0.5	—
Preoptic nuclei	8 ± 0.3	8 ± 0.8	—
Anterior hypothalamic nuclei	7 ± 0.3	8 ± 1.2	—
Suprachiasmatic nucleus	8 ± 0.3	6 ± 0.8	—
Paraventricular nucleus	16 ± 0.5	10 ± 1.3	<.01
Periventricular nucleus	7 ± 0.0	6 ± 0.3	<.02
Arcuate nucleus	12 ± 0.6	8 ± 0.5	<.01
Ventromedial nucleus	7 ± 0.3	6 ± 0.4	—
Mamillary nuclei	8 ± 0.9	6 ± 0.3	—

[a] Mean ± standard error; the mean represents the value obtained from four rat brains per group.

carrier system (16), it is possible that estradiol interferes with this system. Two characteristics of the data suggest that estradiol might be competing with lysine for the same carrier: (i) Significant differences in specific activity of ^{14}C-lysine in the free amino acid pool occurred between the 10–20- and 20–30 min intervals in four out of six cases for the three areas of the brain from the control rats (Table I). None of these intervals were significant for the three areas of the brain from the rats treated with estradiol. This is suggestive of a possible saturation of the carrier system in the rats treated with estradiol. (ii) Uptake is inhibited in all three regions of the brain and this is consistent with the report that lysine is actively taken up and synthesized into protein by all regions of the brain (7); therefore, any agent which interferes with this carrier transport system would be followed by decreased uptake of lysine by all areas of the brain.

The presence of radioactivity in brain sections following administration of a labeled amino acid has been entirely ascribed to newly-formed protein (17). Consequently, radioautography was employed to localize hypothalamic sites which might be involved in protein systhesis as well as to eliminate the possibility of the decrease in incorporation of ^{14}C-lysine into protein merely reflecting decreased availability of the free amino acid.

Of the eight hypothalamic nuclei examined, only the arcuate, paraventricular, and periventricular nuclei in rats treated with estradiol incorporated less ^{14}C-lysine into protein than the controls (Table III). If the depressed synthesis observed at 30 min in the rats treated with estradiol reflected the decreased availability of the free amino acid observed at 20 min, then lower grain counts should have been observed for all eight nuclei. The fact that significantly lower grain counts were found for only three nuclei of the estradiol-treated rats is indicative that estradiol selectively inhibited incorporation of ^{14}C-lysine into protein of specific hypothalamic nuclei. It may be of especial physiological interest that estradiol inhibits protein synthesis in those hypothalamic areas which have been found to accumulate selectively tritiated estradiol, *i.e.*, anterior hypothalamus and median eminence (18), paraventricular and supraoptic nuclei (19), and the arcuate and periventricular nuclei (20). The effects of physiological levels of estradiol on protein synthesis in the brain is currently under investigation.

Since the completion of this work we have become aware of several studies in which specific hypothalamic peptidase activity was reported to vary directly as a function of the estrogen level (21, 22).

Summary. The influence of chronic administration of a pharmacologic dose of 17β-estradiol in adult ovariectomized rats on uptake and incorporation of ^{14}C-lysine into protein was determined for the hypothalamus, cerebellum, and motor cortex, 10, 20, and 30 min after injection of the amino acid. Localization of ^{14}C-lysine incorporated into protein was determined by radioautography for eight hypothalamic nuclei. The data indicate that estradiol inhibits entry of ^{14}C-lysine into the free amino acid pool of the hypothalamus and cerebellum, and inhibits incorporation of ^{14}C-lysine into protein of specfic hypothalamic nuclei, *i.e.*, arcuate, paraventricular, and periventricular.

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