

# Effects of the new generation selective estrogen receptor modulator EM-652 and oral administration of estradiol valerate on circulating, brain, and adrenal $\beta$ -endorphin and allopregnanolone levels in intact fertile and ovariectomized rats

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**Objective:** To investigate the effects of oral estradiol valerate (EV); EM-652, a new-generation selective estrogen receptor modulator; and both agents on central and peripheral  $\beta$ -endorphin ( $\beta$ -EP) and allopregnanolone levels in fertile and ovariectomized rats.

**Design:** Prospective study.

**Setting:** Animal laboratory in an academic research environment.

**Animals:** Thirteen groups of eight Wistar female rats received oral EV (0.01 or 0.05 mg/kg of body weight daily), EM-652 (0.1, 1, or 5 mg/kg daily), or EV (0.05 mg/kg daily) and EM-652 (0.1, 1, or 5 mg/kg/daily) for 14 days.

**Intervention(s):**  $\beta$ -Endorphin levels content in the hypothalamus, hippocampus, anterior and neurointermediate pituitary, and plasma were measured. Allopregnanolone levels in the hypothalamus, hippocampus, anterior pituitary, adrenal glands, and serum were measured.

**Main Outcome Measure(s):**  $\beta$ -Endorphin and allopregnanolone levels.

**Result(s):** In ovariectomized rats, administration of EV or EM-652 reverses changes in  $\beta$ -EP and allopregnanolone levels induced by ovariectomy. Administration of EM-652 plus EV prevents the increase in  $\beta$ -EP and allopregnanolone levels induced by EV in the hippocampus, hypothalamus, and pituitary but not in the adrenal glands and serum.

**Conclusions:** In ovariectomized rats, EM-652 has an estrogen-like action that becomes antiestrogenic in the presence of EV administration. In fertile animals, EM-652 exerts estrogen-like or slight antiestrogenic effects. (Fertil Steril® 2002;77:1018–27. ©2002 by American Society for Reproductive Medicine.)

**Key Words:** EM-652, selective estrogen receptor modulator, estradiol,  $\beta$ -endorphin, allopregnanolone, ovariectomy

Selective estrogen receptor modulators (SERMs) are compounds that activate the estrogen receptor but block or mimic the effect of estradiol in a tissue-specific manner. The best known SERMs are triphenylethylenes (clomiphene, tamoxifen, and tamoxifen derivatives) and benzothiophenes, which include raloxifene and its analogues, benzopyrans (EM-652 and analogues) (1). These compounds, which have variable estrogenic and antiestrogenic activities, act on the estrogen receptors ER $\alpha$  and ER $\beta$  and play different roles in various tissues (2).

Researchers are interested in SERMs for their possible use as a hormone replacement therapy that will not produce some of the unwanted effects of estrogens. Clinical studies have demonstrated that toremifene and chlorotamoxifene, two tamoxifen analogues, slightly reduce serum LH and FSH levels, thus demonstrating weak estrogen-like properties (3, 4). Raloxifene has been shown to exert a potent antagonist effect on short-term suppressive and long-term stimulatory effects of estrogens on LH release in vitro (5, 6), acting as a

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“pure antiestrogen” in pituitary gonadotrophs (7). In contrast, raloxifene seems to have a neurotrophic effect in nerve growth factor-treated PC12 cells, thus showing an estrogen-like effect. Combined administration of estradiol and raloxifene induces neurite growth more potently than does administration of either agent alone (8).

The raloxifene analogue LY-117018 has been shown to act on central and peripheral  $\beta$ -endorphin ( $\beta$ -EP) levels through an estrogen-like mechanism in ovariectomized rats, thus increasing  $\beta$ -EP levels in the pituitary, hypothalamus, and plasma. In contrast, LY-117018 shows a clear antiestrogenic effect on  $\beta$ -EP levels both in fertile and in ovariectomized rats treated with  $17\beta$ -estradiol (9). These data suggest that LY-117018 has a different influence on the opioidergic pathways according to the presence or absence of endogenous or exogenous estradiol.

Similarly, LY-117018 reproduced the estrogen-induced changes in allopregnanolone levels in cerebral areas, pituitary, and adrenals in ovariectomized rats (10). However, when administered together with  $17\beta$ -estradiol to ovariectomized rats, LY-117018 suppressed the estrogen-induced changes in allopregnanolone levels in the brain, thus showing a clear antiestrogenic effect. This antiestrogenic effect was not observed in the adrenals and serum. When administered to fertile rats, LY-117018 showed an antiestrogenic effect on cerebral, adrenal, and serum allopregnanolone levels, with a castration-like effect (10).

A new-generation SERM, EM-652, was recently synthesized for prevention and therapy for breast cancer. This compound avoids the problem of endometrial stimulation (11). EM-652 has the highest affinity for the estrogen receptor compared with estradiol, ICI 182780, hydrotamoxifen, raloxifene, droloxifene, and hydroxytoremifene (12) and is unique among SERMs in exerting a pure antiestrogenic activity in both human breast and endometrial tissue (11, 12). In addition, EM-652 prevents loss of bone mineral density and decreases serum cholesterol levels in the rat (12).

We sought to evaluate the effects of EM-652 on the neuroendocrine function of intact fertile and ovariectomized rats. In particular, we studied the effect of EM-652 on the opioidergic pathway and on allopregnanolone, a neuroactive steroid acting as a  $\gamma$ -aminobutyric acid-A (GABA<sub>A</sub>) receptor modulator. These compounds were chosen because recent evidence indicates that  $\beta$ -EP and allopregnanolone play a major role in the modulation of mood and cognitive performance (13–17). Therefore, we evaluated central and peripheral levels of  $\beta$ -EP and allopregnanolone after 14 days of administration of EM-652, estradiol valerate, or both.

## MATERIALS AND METHODS

### Animals

We studied 104 fertile female Wistar rats. The rats were divided into 13 groups of 8 rats each, which received 14 days

of treatment with different regimens. The animals had 14 hours of illumination daily (lights on at 6 A.M. and off at 8 P.M.) and free access to standard rat chow and tap water.

Doses of EV and EM-652 were diluted in 1 mL of saline and administered through an oral cannula. The rats were housed together and were in the same estrous cycle stage, as indicated by vaginal smears obtained every day. At the end of treatment, each animal was euthanized by decapitation on the same day, as described elsewhere (18). The anterior pituitary, neurointermediate pituitary, hypothalamus, hippocampus, and adrenal glands were removed and weighed. Two blood samples were taken from each rat before euthanization (18); one was collected in a heparinized plastic tube and one in a nonheparinized plastic tube. Within 40 days after euthanization,  $\beta$ -EP levels were measured in the anterior pituitary, neurointermediate pituitary, hypothalamus, hippocampus, and plasma, and allopregnanolone levels were measured in the anterior pituitary, hypothalamus, hippocampus, adrenal glands, and serum.

Approval from the institutional review board was obtained before the start of the study.

### Protocol

Three groups of intact fertile rats received EM-652, 0.1 mg/kg of body weight, 1 mg/kg, or 5 mg/kg daily. One group of intact fertile rats received placebo (control group).

Nine groups of rats were castrated and, after 2 weeks, were treated orally for 14 days with (1) placebo; (2) estradiol valerate (Sigma Aldrich, St. Louis, MO) 0.01 mg/kg/day; (3) EV 0.05 mg/kg/day; (4) EM-652 0.1 mg/kg/day; (5) EM-652 1 mg/kg/day; (6) EM-652 5 mg/kg/day; (7) EV 0.05 mg/kg/day + EM-652 0.1 mg/kg/day; (8) EV 0.05 mg/kg/day + EM-652 1 mg/kg/day; (9) EV 0.05 mg/kg/day + EM-652 5 mg/kg/day.

### $\beta$ -Endorphin Assay

The blood sample collected in the heparinized tube was centrifuged at 3,500 rpm for 10 minutes, and plasma was stored at  $-20^{\circ}\text{C}$  until assay. The anterior pituitary, neurointermediate lobe, hypothalamus, and hippocampus were collected in 4% acetic acid, then boiled and homogenized. The homogenate was centrifuged at 1,200 rpm for 15 minutes at  $4^{\circ}\text{C}$ . The supernatant of tissue homogenates and the plasma were passed through a C-18 Sep-Pak (Waters Corp., Milford, MA) cartridge that had been equilibrated with methanol. After washing with water and acetic acid (0.5 N),  $\beta$ -EP was eluted with a solution containing acetic acid (0.5 N) and acetonitrile 25/75 v/v, and dried under a stream of nitrogen.

Levels of  $\beta$ -EP were measured with a specific radioimmunoassay (18), using camel  $\beta$ -EP as standard (Sigma Chemicals). The antiserum (supplied by Dr. P. Sacerdote, Milan, Italy) was used at a final dilution of 1:130,000, allowing a detection limit of 10 pg/tube. The antiserum, labeled hormone (Amersham International, London, England), and standard curve material were dissolved in 0.5%

bovine serum albumin-phosphate buffer (pH, 7.4). Recovery of [<sup>125</sup>I]-β-EP corresponded to 85% ± 11% of the total amount after acetic acid extraction. The sensitivity of the assay was 2.5 pg/mL, and the intraassay and interassay coefficients of variation were 6.2% and 9.3%, respectively. The protein content was determined in the whole homogenate by using the method of Bradford (19). Protein content and weight of organs in each group of rats did not significantly differ.

### Allopregnanolone Assay

The hypothalamus, hippocampus, pituitary, and adrenals were homogenized in ice-cold 50% aqueous methanol containing 1% acetic acid with 3,000 dpm of internal recovery standard of tritium-labeled steroid. The homogenate was centrifuged at 1,200 rpm for 15 min at 4°C. The blood sample collected in the nonheparinized plastic tube was centrifuged at 3,500 rpm for 10 minutes; and the serum was stored at -20°C until assay. The supernatant of tissue homogenates and serum were passed through a C-18 Sep-Pak cartridge that had been equilibrated with homogenizing buffer. The cartridge was sequentially washed with homogenizing buffer and 50% aqueous methanol. The unconjugated steroid fraction was eluted with absolute methanol and dried under nitrogen. Analytical grade solvents were purchased from Merck (Darmstadt, Germany).

Allopregnanolone levels were measured by using a radioimmunoassay method described elsewhere (2). The sensitivity of the assay was 15 pg/mL, and the mean (±SE) recovery after extraction and chromatography was 85.5% ± 12.7%. The intra-assay and inter-assay coefficients of variation were 7% and 9%, respectively (2).

### Statistical Analysis

Data are reported as means (±SD). Allopregnanolone and β-EP levels in fertile controls and ovariectomized controls were compared by using a two-sample *t*-test or Mann-Whitney test, as appropriate. One-way analysis of variance or Kruskal-Wallis one-way ANOVA on ranks, as appropriate, were used to compare allopregnanolone and β-EP levels in fertile controls and fertile rats treated with EM-652, ovariectomized controls and ovariectomized rats treated with estradiol valerate, ovariectomized controls and ovariectomized rats treated with EM-652, and ovariectomized rats treated with estradiol valerate alone and ovariectomized rats treated with estradiol valerate and EM-652. The Bonferroni multiple comparison test with  $\alpha$  set at 0.05, 0.01, and 0.001 was used to compare treatment groups.

## RESULTS

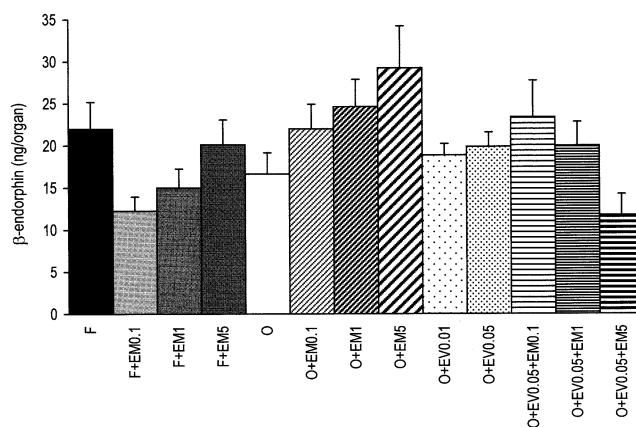
### Hippocampus

#### β-Endorphin

Administration of EM-652 to intact fertile rats decreased β-EP content in the hippocampus (at ANOVA:  $F = 24.1$ ,

**FIGURE 1**

β-Endorphin levels in the hippocampus. F = fertile; EM0.1, EM1, EM5 = EM-652 at daily doses of 0.1, 1, or 5 mg/kg; EV0.01, EV0.05 = estradiol valerate at daily doses of 0.01 or 0.05 mg/kg; O = ovariectomized.  $P < .05$  (O vs. O + EM0.1);  $P < .01$  (F + EM1 vs. F + EM5; F vs. O; O vs. O + EV0.05; O + EM0.1 vs. O + EM5);  $P < .001$  (F vs. F + EM0.1 and EM1; F + EM0.1 vs. F + EM5; O vs. O + EM1 and EM5; O + EV0.05 + EM5 vs. O + EV0.05, O + EV0.05 + EM0.1, O + EV0.05 + EM1).



Bernardi. Effect of EM-652 on β-endorphin and allopregnanolone. *Fertil Steril* 2002.

$P < .001$  an ANOVA); at Bonferroni test: fertile rats vs. 0.1 and 1 mg/kg/day,  $P < .001$ ; 0.1 vs. 5 mg/kg,  $P < .001$ ; 1 vs. 5 mg/kg,  $P < .01$  (Fig. 1). Hippocampal β-EP levels were lower in ovx rats than in intact fertile rats ( $P < .01$ ) (Fig. 1). Administration of estradiol valerate to ovariectomized rats increased β-EP content in a dose-related manner (at ANOVA,  $F = 5.9$ ,  $P < .05$ ; on Bonferroni test, ovariectomized rats vs. 0.05 mg/kg/day,  $P < .01$ ). Similarly, administration of EM-652 to ovx rats increased β-EP content in a dose-related manner (at ANOVA:  $F = 17.6$ ,  $P < .001$ ; at Bonferroni test, ovariectomized vs. 1 and 5 mg/kg,  $P < .001$ ; ovariectomized vs. 0.1 mg/kg/day,  $P < .05$ ; 0.1 vs. 5 mg/kg/day,  $P < .01$ ) (Fig. 1). Administration of both estradiol valerate and the highest dose of EM-652 reduced hippocampal β-EP content to levels significantly lower than those observed in ovariectomized animals treated with estradiol valerate alone (at ANOVA,  $F = 21.5$ ,  $P < .001$ ; at Bonferroni test, EV + EM-652, 5 mg/kg/day vs. all other groups,  $P < .001$ ) (Fig. 1).

#### Allopregnanolone

Administration of EM-652 to intact fertile rats decreased allopregnanolone levels in the hippocampus in a dose-related manner (Kruskal-Wallis ANOVA,  $H = 20.9$ ,  $P < .001$ ; at Bonferroni test: fertile rats vs. 0.1, 1, and 5 mg/kg/day,  $P < .001$ ; 0.1 vs. 5 mg/kg/day,  $P < .05$ ) (Fig. 2). Hippocampal allopregnanolone levels were lower in ovariectomized rats

than in intact fertile rats ( $P < .01$ ) but higher than in fertile rats treated with EM-652 at the highest doses ( $P < .05$ ) (Fig. 2). Administration of estradiol valerate to ovx rats increased allopregnanolone levels in a dose-related manner (at ANOVA:  $F = 4.1$ ,  $P < .05$ ; at Bonferroni test: ovariectomized rats vs. 0.05 mg/kg/day,  $P < .05$ ). Similarly, administration of EM-652 to ovariectomized rats increased allopregnanolone levels in a dose-related manner (at ANOVA:  $F = 5.7$ ,  $P < .01$ ; at Bonferroni test: ovariectomized rats vs. 5 mg/kg/day,  $P < .01$ ; 0.1 vs. 5 mg/kg/day,  $P < .05$ ) (Fig. 2). Administration of both estradiol valerate and EM-652 reduced hippocampal allopregnanolone levels with respect to ovariectomized animals treated with EV, reaching levels similar to those of castrated animals (at ANOVA,  $F = 10.8$ ,  $P < .001$ ; at Bonferroni test: estradiol valerate vs. estradiol valerate + EM-652 0.1 mg/kg/day,  $P < .05$ ; estradiol valerate vs. estradiol valerate + EM-652 1 and 5 mg/kg/day,  $P < .001$ ) (Fig. 2).

## Hypothalamus

### $\beta$ -Endorphin

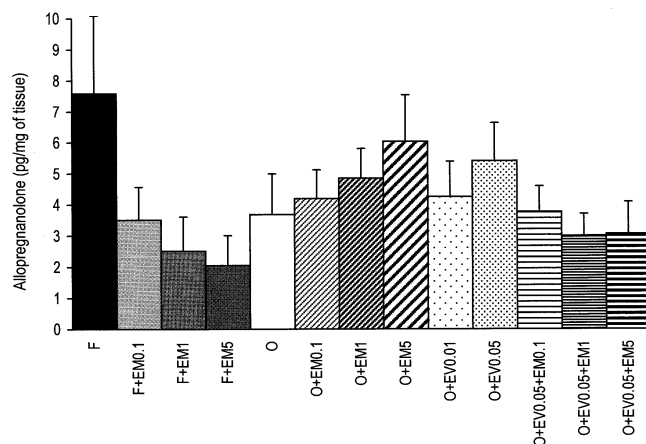
Administration of EM-652 in intact fertile rats increased hypothalamic  $\beta$ -endorphin levels at the highest dose used (at ANOVA,  $F = 5.4$ ,  $P < .01$ ; at Bonferroni test: fertile vs. 5 mg/kg/day,  $P < .05$ ; 0.1 vs. 5 mg/kg/day,  $P < .01$ ) (Fig. 3). Hypothalamic  $\beta$ -EP levels in ovariectomized rats were lower than those in intact fertile rats ( $P < .05$ ) (Fig. 3). Administration of EV significantly increased  $\beta$ -endorphin levels in ovariectomized rats (at ANOVA,  $F = 71.8$ ,  $P < .001$ ; at Bonferroni test, ovariectomized rats vs. 0.01 and 0.05 mg/kg/day,  $P < .001$ ). Administration of EM-652 to ovariectomized rats increased  $\beta$ -endorphin levels in a dose-related manner (at ANOVA:  $F = 23.0$ ,  $P < .001$ ; at Bonferroni test: ovariectomized rats vs. 1 and 5 mg/kg,  $P < .001$ ; ovariectomized rats vs. 0.1 mg/kg/day,  $P < .01$ ; 0.1 vs. 5 mg/kg/day,  $P < .01$ ) (Fig. 3). In contrast, administration of both estradiol valerate and EM-652 decreased hypothalamic  $\beta$ -EP levels in ovariectomized rats compared with administration of EV alone, in a dose-related manner (at ANOVA,  $F = 79.4$ ,  $P < .001$ ; at Bonferroni test: estradiol valerate vs. estradiol valerate + EM-652 0.1 mg/kg/day,  $P < .01$ ; estradiol valerate vs. estradiol valerate + EM-652 1 and 5 mg/kg/day,  $P < .001$ ; estradiol valerate + EM-652 0.1 mg/kg/day, vs. estradiol valerate + EM-652 1 and 5 mg/kg/day,  $P < .001$ ; estradiol valerate + EM-652 0.1 mg/kg/day, vs. estradiol valerate + EM-652 1 mg/kg/day vs. estradiol valerate + EM-652 5 mg/kg/day,  $P < .01$ ); at the highest dose,  $\beta$ -EP levels were lower than those in untreated ovariectomized rats (Fig. 3).

### Allopregnanolone

In intact fertile rats, administration of EM-652 reduced hypothalamic allopregnanolone content in a dose-related manner to levels similar to those in ovariectomized animals (at Kruskal-Wallis ANOVA:  $H = 24.6$ ,  $P < .001$ ; at Bonferroni

**FIGURE 2**

Allopregnanolone levels in the hippocampus. F = fertile; EM0.1, EM1, EM5 = EM-652 at 0.1, 1, or 5 mg/kg; EV0.01, EV0.05 = estradiol valerate at daily doses of 0.01 or 0.05 mg/kg; O = ovariectomized.  $P < .05$  (F + EM0.1 vs. F + EM5; O vs. F + EM5; O vs. O + EV0.05; O + EM0.1 vs. O + EM5; O + EV0.05 vs. O + EV0.05 + EM0.1);  $P < .01$  (F vs. O; O vs. O + EM5);  $P < .001$  (F vs. F + EM0.1, F + EM1, F + EM5; O + EV0.05 vs. O + EV0.05 + EM1, O + EV0.05 + EM5).



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test: fertile rats vs. all other groups,  $P < .001$ ; 0.1 vs. 5 mg/kg/day,  $P < .05$ ) (Fig. 4). In ovariectomized rats, hypothalamic allopregnanolone content was significantly lower than in intact fertile rats ( $P < .001$ ) (Fig. 2); this value increased after daily administration of estradiol valerate 0.05 mg/kg (at ANOVA:  $F = 21.1$ ,  $P < .001$ ; at Bonferroni test: ovariectomized rats and 0.01 mg/kg/day vs. 0.05 mg/kg/day,  $P < .001$ ). Administration of EM-652 to ovariectomized rats markedly increased allopregnanolone levels in a dose-related manner (at ANOVA:  $F = 64.9$ ,  $P < .001$ ; at Bonferroni test: 1 vs. 5 mg/kg,  $P < .05$ ; all other comparisons,  $P < .001$ ) (Fig. 4).

The effects of EM-652 on allopregnanolone levels in the hypothalamus appear to be greater than those of estradiol. Administration of estradiol valerate and EM-652 in ovariectomized rats induced a significant dose-related decrease in allopregnanolone levels compared with administration of estradiol valerate at 0.05 mg/kg (at ANOVA:  $F = 36.9$ ,  $P < .001$ ; at Bonferroni test: estradiol valerate vs. all other groups,  $P < .001$ ; estradiol valerate + EM-652 0.1 mg/kg/day vs. estradiol valerate + EM-652 5 mg/kg/day,  $P < .01$ ); at the highest dose, allopregnanolone levels decreased to levels lower than those in untreated ovariectomized rats (Fig. 4).

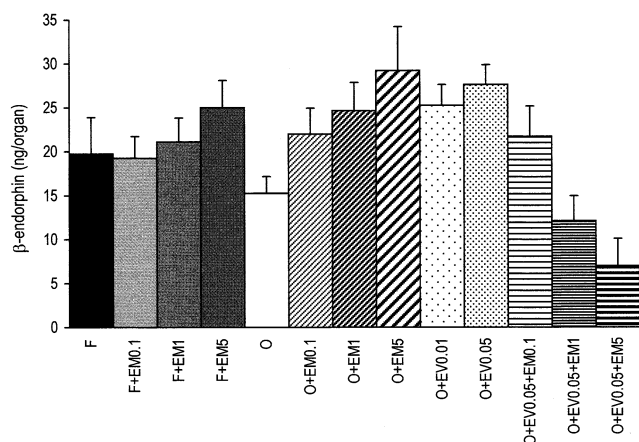
## Neurointermediate Pituitary

### $\beta$ -Endorphin

Administration of EM-652 to intact fertile rats increased  $\beta$ -endorphin content in the neurointermediate lobe at all

**FIGURE 3**

$\beta$ -Endorphin levels in the hypothalamus. F = fertile; EM0.1, EM1, EM5 = EM-652 at daily doses of 0.1, 1, or 5 mg/kg; EV0.01, EV0.05 = estradiol valerate at daily doses of 0.01 or 0.05 mg/kg; O = ovariectomized.  $P < .05$  (F vs. F + EM5; F vs. O);  $P < .01$  (F + EM0.1 vs. F + EM5; O vs. O + EM0.1; O + EM0.1 vs. O + EM5; O + EV0.05 vs. O + EV0.05 + EM0.1; O + EV0.05 + EM1 vs. O + EV0.05 + EM5);  $P < .001$  (O vs. O + EV0.01, O + EV0.05; O vs. O + EM1, O + EM5; O + EV0.05, O + EV0.05 + EM0.1 vs. O + EV0.05 + EM1, O + EV0.05 + EM5).

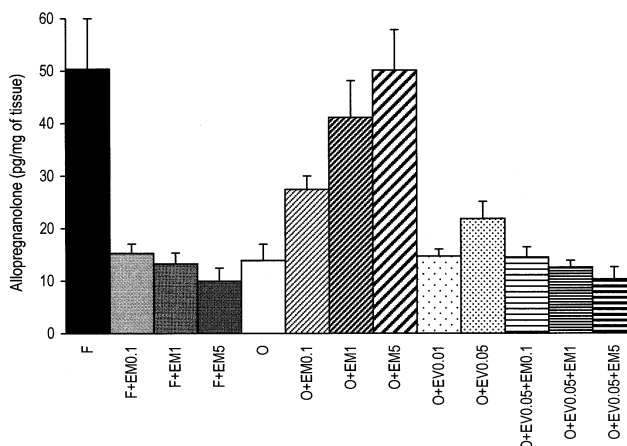


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doses (at ANOVA:  $F = 28.8$ ,  $P < .001$ ; at Bonferroni test: fertile rats vs. all other groups,  $P < .001$ ; 0.1 vs. 5 mg/kg/day,  $P < .05$ ; 1 vs. 5 mg/kg/day,  $P < .01$ ) (Table 1).  $\beta$ -endorphin levels were significantly lower in ovariectomized rats than in intact controls ( $P < .001$ ) (Table 1). Administration of estradiol valerate to ovariectomized rats significantly increased  $\beta$ -endorphin content at the higher dose (at ANOVA:  $F = 93.6$ ,  $P < .001$ ; at Bonferroni test: ovariectomized rats and 0.01 mg/kg/day vs. 0.05 mg/kg/day,  $P < .001$ ). Administration of EM-652 significantly increased  $\beta$ -endorphin levels in the neurointermediate lobe of ovariectomized rats in a dose-related manner (at ANOVA:  $F = 44.6$ ,  $P < .001$ ; at Bonferroni test: ovariectomized rats vs. 1 and 5 mg/kg/day,  $P < .001$ ; ovariectomized rats vs. 0.1 mg/kg/day,  $P < .05$ ; 1 vs. 5 mg/kg/day,  $P < .05$ ) (Table 1). Administration of estradiol valerate plus EM-652 decreased  $\beta$ -endorphin levels in ovariectomized rats in a dose-related manner compared with EV alone (at ANOVA:  $F = 17.0$ ,  $P < .001$ ; at Bonferroni test: estradiol valerate vs. estradiol valerate + EM-652 1 and 5 mg/kg/day,  $P < .001$ ; estradiol valerate + EM-652 0.1 mg/kg/day vs. estradiol valerate + EM-652 5 mg/kg/day,  $P < .01$ ; estradiol valerate + EM-652 1 mg/kg/day vs. estradiol valerate + EM-652 5 mg/kg/day,  $P < .05$ ) (Table 1).

**FIGURE 4**

Allopregnanolone levels in the hypothalamus. F = fertile; EM0.1, EM1, EM5 = EM-652 at daily doses of 0.1, 1, or 5 mg/kg; EV0.01, EV0.05 = estradiol valerate at daily doses of 0.01 or 0.05 mg/kg; O = ovariectomized.  $P < .05$  (F + EM0.1 vs. F + EM5; O + EM0.1 vs. O + EM5);  $P < .01$  (O + EV0.05 + EM0.1 vs. O + EV0.05 + EM5);  $P < .001$  (F vs. O; O + EV0.01 vs. O + EV0.05; O vs. O + EM0.1, O + EM1, O + EM5; O + EM0.1 vs. O + EM1, O + EM5; O + EV0.05 vs. O + EV0.05 + EM0.1, O + EV0.05 + EM1, O + EV0.05 + EM5).



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## Anterior Pituitary

### $\beta$ -Endorphin

Administration of EM-652 to intact fertile rats decreased  $\beta$ -endorphin levels in a dose-dependent manner (at ANOVA:  $F = 21.8$ ,  $P < .001$ ; at Bonferroni test: fertile rats vs. all other groups,  $P < .001$ ; 0.1 vs. 5 mg/kg/day,  $P < .05$ ) (Table 1). Levels of  $\beta$ -endorphin in the anterior pituitary of ovx rats were lower than intact controls ( $P < .001$ ) (Table 1). Administration of estradiol valerate (at ANOVA:  $F = 11.8$ ,  $P < .001$ ; at Bonferroni test: ovariectomized rats vs. 0.01 mg/kg/day,  $P < .05$ ; ovariectomized rats vs. 0.05 mg/kg/day,  $P < .001$ ) and EM-652 (at ANOVA:  $F = 37.8$ ,  $P < .001$ ; at Bonferroni test: ovariectomized rats vs. 1 and 5 mg/kg/day,  $P < .001$ ; ovariectomized rats vs. 0.1 mg/kg,  $P < .05$ ; 0.1 vs. 1 mg/kg/day,  $P < .01$ ; 0.1 vs. 5 mg/kg/day,  $P < .001$ ) increased  $\beta$ -endorphin content in the anterior pituitary of ovariectomized rats in a dose-dependent manner (Table 1).

The effects of EM-652 on  $\beta$ -endorphin levels in the anterior pituitary are greater than those of estradiol valerate. Administration of EV plus EM-652 decreased anterior pituitary  $\beta$ -EP levels in ovariectomized animals compared with EV alone (at ANOVA:  $F = 12.8$ ,  $P < .001$ ; at Bonferroni test: estradiol valerate vs. estradiol valerate + EM-652 1 mg/kg/day,  $P < .01$ ; estradiol valerate vs. estradiol valerate +

EM-652 5 mg/kg/day,  $P < .001$ ; estradiol valerate + EM-652 0.1 mg/kg/day vs. estradiol valerate + EM-652 1 mg/kg/day,  $P < .01$ ; estradiol valerate + EM-652 1 mg/kg/day vs. estradiol valerate + EM-652 5 mg/kg,  $P < .05$ ) (Fig. 4).

### Allopregnanolone

Administration of EM-652 to intact fertile rats significantly decreased pituitary allopregnanolone levels in a dose-dependent manner (at ANOVA:  $F = 203.2$ ,  $P < .001$ ; at Bonferroni test: fertile rats vs. all other groups,  $P < .001$ ; 0.1 vs. 5 mg/kg/day,  $P < .01$ ) (Table 1). Pituitary levels of allopregnanolone were significantly lower in ovariectomized rats than in intact fertile rats ( $P < .001$ ) (Table 1) and increased after administration of estradiol valerate at 0.05 mg/kg/day (at ANOVA:  $F = 9.4$ ,  $P < .01$ ; at Bonferroni test: ovariectomized controls and 0.01 mg/kg/day vs. 0.05 mg/kg/day,  $P < .01$ ) or EM-652 at all doses (at ANOVA:  $F = 68.1$ ,  $P < .001$ ; at Bonferroni test: ovariectomized controls vs. all other groups,  $P < .001$ ; 0.1 vs. 5 mg/kg/day,  $P < .001$ ; 1 vs. 5 mg/kg/day,  $P < .01$ ) in a dose-related manner (Fig. 4).

The effects of EM-652 on allopregnanolone levels in the anterior pituitary are greater than those of estradiol ( $P < .001$ ). Administration of EV and the highest doses of EM-652 in ovariectomized rats induced a significant dose-related decrease in allopregnanolone levels compared with administration of estradiol valerate alone (at ANOVA:  $F = 7.2$ ,  $P < .001$ ; at Bonferroni test: estradiol valerate vs. estradiol valerate + EM-652 5 mg/kg/day,  $P < .05$ ; estradiol valerate + EM-652 0.1 mg/kg/day vs. estradiol valerate + EM-652 5 mg/kg/day,  $P < .01$ ; estradiol valerate + EM-652 0.1 mg/kg/day vs. EV + EM-652 1 mg/kg/day,  $P < .05$ ) (Fig. 4).

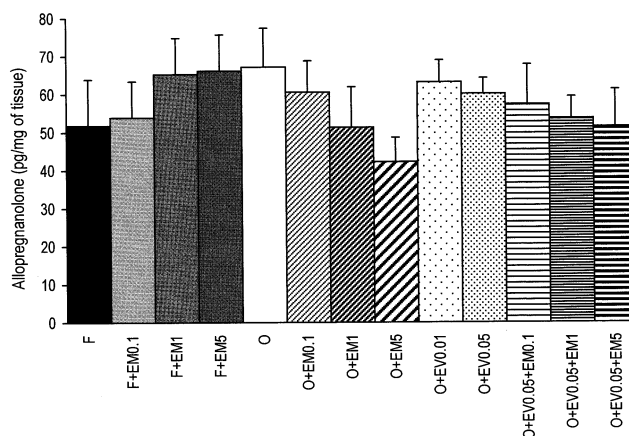
## Adrenal Glands

### Allopregnanolone

Administration of EM-652 (1 and 5 mg/kg/day) to intact fertile rats significantly increased adrenal allopregnanolone levels (at Kruskal–Wallis ANOVA:  $H = 9.6$ ,  $P < .05$ ; at Bonferroni test: untreated rats and 0.1 mg/kg/day vs. 1 and 5 mg/kg/day,  $P < .05$ ) (Fig. 5). Adrenal allopregnanolone levels were significantly higher in ovariectomized rats than in intact fertile rats ( $P < .01$ ) (Fig. 5). Administration of EV at 0.05 mg/kg/day to ovariectomized rats significantly decreased adrenal allopregnanolone content (at ANOVA:  $F = 3.7$ ,  $P < .05$ ; at Bonferroni test: ovariectomized controls vs. 0.05 mg/kg/day,  $P < .05$ ) (Fig. 5). In contrast, administration of EM-652 produced a dose-related decrease in adrenal allopregnanolone levels compared with untreated ovariectomized rats (at ANOVA:  $F = 15.0$ ,  $P < .001$ ; at Bonferroni test: ovariectomized controls vs. 5 mg/kg/day,  $P < .001$ ; ovariectomized controls vs. 1 mg/kg/day,  $P < .01$ ; 0.1 vs. 5 mg/kg/day,  $P < .001$ ), reaching levels similar to those in fertile animals (Fig. 5). Administration of EV plus EM-652 significantly decreased allopregnanolone levels compared

**FIGURE 5**

Allopregnanolone levels in the adrenal glands. F = fertile; EM0.1, EM1, EM5 = EM-652 at daily doses of 0.1, 1, or 5 mg/kg; EV0.01, EV0.05 = estradiol valerate daily doses of 0.01 or 0.05 mg/kg; O = ovariectomized.  $P < .05$  (F, F + EM0.1 vs. F + EM1, F + EM5; O vs. O + EV0.05; O + EV0.05 vs. O + EV0.05 + EM5);  $P < .01$  (F vs. O; O vs. O + EM1);  $P < .001$  (O, O + EM0.1 vs. O + EM5).



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with estradiol valerate alone (at ANOVA:  $F = 3.2$ ,  $P < .05$ ; at Bonferroni test: estradiol valerate vs. estradiol valerate + EM-652 5 mg/kg/day,  $P < .05$ ) (Fig. 5).

## Circulating $\beta$ -Endorphin and Allopregnanolone

### $\beta$ -Endorphin

Administration of EM-652 to intact fertile rats decreased plasma levels of  $\beta$ -endorphin in a dose-related manner (at Kruskal–Wallis ANOVA:  $H = 21.1$ ,  $P < .001$ ; at Bonferroni test: fertile rats vs. all other groups,  $P < .001$ ; 0.1 vs. 5 mg/kg/day,  $P < .01$ ) (Table 1). Plasma levels of  $\beta$ -endorphin were lower in ovariectomized rats than in intact controls ( $P < .001$ ) (Table 1). Administration of estradiol valerate (at ANOVA:  $F = 5.9$ ,  $P < .01$ ; at Bonferroni test: ovariectomized rats vs. 0.01 and 0.05 mg/kg/day,  $P < .05$ ) and EM-652 (at ANOVA:  $F = 25.2$ ,  $P < .001$ ; at Bonferroni test: ovariectomized rats vs. 1 and 5 mg/kg/day,  $P < .001$ ; ovariectomized rats vs. 0.1 mg/kg/day,  $P < .01$ ; 0.1 mg/kg/day vs. 5 mg/kg/day,  $P < .001$ ; 1 vs. 5 mg/kg/day,  $P < .01$ ) to ovariectomized rats increased plasma  $\beta$ -endorphin levels in a dose-dependent manner (Table 1).

As observed in the anterior pituitary, the effects of EM-652 on circulating levels of  $\beta$ -endorphin are greater than those of estradiol valerate. In contrast, administration of estradiol valerate plus EM-652 at 5 mg/kg/day decreased circulating levels of  $\beta$ -endorphin in ovariectomized animals

compared with EV alone (at ANOVA:  $F = 6.5$ ,  $P < .01$ ; at Bonferroni test: estradiol valerate + EM-652 5 mg/kg vs. all other groups,  $P < .01$ ) to levels lower than those in untreated ovariectomized rats ( $P < .01$ ) (Table 1).

### *Allopregnanolone*

Administration of EM-652 to intact fertile rats significantly decreased serum allopregnanolone levels in a dose-dependent manner (at ANOVA:  $F = 171.9$ ,  $P < .001$ ; at Bonferroni test: all comparisons,  $P < .001$ ) (Table 1). Serum levels of allopregnanolone were significantly lower in ovariectomized rats than in intact fertile rats ( $P < .001$ ) (Table 1). A dose-dependent increase in serum allopregnanolone levels was observed after administration of EV (at ANOVA:  $F = 33.7$ ,  $P < .001$ ; at Bonferroni test: ovariectomized controls vs. 0.01 mg/kg/day,  $P < .05$ ; ovariectomized controls and 0.01 mg/kg/day vs. 0.05 mg/kg/day,  $P < .001$ ) or EM-652 (at ANOVA:  $F = 68.1$ ,  $P < .001$ ; at Bonferroni test: ovariectomized controls vs. 1 and 5 mg/kg/day,  $P < .001$ ; 0.1 mg/kg/day vs. 1 and 5 mg/kg/day,  $P < .001$ ) (Table 1). Administration of EV plus EM-652 did not change serum allopregnanolone levels compared with EV alone (at ANOVA:  $F = 3.0$ ,  $P < .10$ ; at Bonferroni test: 0.1 mg/kg/day vs. 5 mg/kg/day,  $P < .10$ ) (Table 1).

## DISCUSSION

EM-652 is an orally active nonsteroidal antiestrogen that has the most potent antiestrogenic activity known thus far (11, 12). In particular, this compound has the highest affinity for the estrogen receptor compared with estradiol, ICI 182780, hydrotamoxifen, raloxifene, droloxifene, and hydroxytoremifene (12). Accordingly, it has the most potent inhibitory activity on estrogen receptor- $\alpha$  and estrogen receptor- $\beta$  compared with other antiestrogens (ICI 182780, hydrotamoxifen, raloxifene, droloxifene, and hydroxytoremifene) (12). In addition, EM-652 inhibits both the AF1 and AF2 functions of estrogen receptors, whereas hydrotamoxifen acts only on AF2 (12).

EM-800, a prodrug of EM-652, prevents the development of 7,12 dimethylbenz[a]anthracene-induced mammary carcinoma in the rat, a widely used model for human breast cancer. Moreover, EM-800 exerts the strongest inhibition of all antiestrogens on the growth of human breast cancer cell lines in vitro and in vivo (11, 12). Furthermore, this compound reduces uterine size to those seen after castration and, unlike tamoxifen and raloxifene, has no stimulatory effect on alkaline phosphatase activity in human uterine Ishikawa carcinoma cells, an estrogen-sensitive variable (11, 12). These observations, in addition to the positive effect of EM-652 on bone and cholesterol levels (12), warrant the interest in neoadjuvant and adjuvant used EM-652, and its use as a possible hormone replacement therapy that prevents breast and uterine cancer.

The brain is a target organ for estrogens (20–22), and the expression of estrogen receptor- $\alpha$  (ER $\alpha$ ) and estrogen receptor- $\beta$  (ER $\beta$ ) differs in various regions of rat brain; with anatomical evidences of distinct roles of each ER subtype (23, 24). Hybridization histochemical studies have shown that both receptors are present in the rat cortex (mostly ER $\beta$  but also ER $\alpha$ ), pituitary, and hypothalamus (ER $\alpha$  mostly in arcuate and ventromedial nuclei and ER $\beta$  mostly in paraventricular and supraoptic nuclei) (25–27). The cerebellum expresses only ER $\beta$ , whereas ER $\alpha$  predominates in the hippocampus, the component of the limbic system involved in the modulation of cognitive functions (25, 26, 28, 29).

Gonadal steroids regulate reproductive function by modulating the synthesis, release, and metabolism of several neurotransmitters and neuropeptides and the expression of their receptors (30). Steroidal hormones control  $\beta$ -endorphin modulation of LH secretion by inhibition of hypothalamic GnRH-producing neurons. In addition, administration of estradiol increases hypothalamic proopiomelanocortin (POMC) mRNA in the rat; the presence of cellular colocalization of  $\beta$ -endorphin immunoreactivity and cFos immunoreactivity may indicate a intracellular coregulation of these peptides by an estrogen-dependent mechanism (31).

Previous reports described decreased levels of  $\beta$ -endorphin in the hypothalamus, pituitary, and plasma after ovariectomy (32–38). In ovariectomized rats, estradiol replacement increases central and peripheral levels of  $\beta$ -EP and restores a normal circadian  $\beta$ -endorphin pattern in the medial basal hypothalamus (34, 35). In addition, antiestrogens such as clomiphene and cyclophenil have been reported to reduce hypothalamic  $\beta$ -endorphin content in fertile rats by acting as antiestrogens, and to increase hypothalamic levels of  $\beta$ -endorphin in ovariectomized rats, in which they exert an estrogen-like action (33).

It has been reported that administration of tamoxifen plus estradiol prevents the estradiol-mediated induction of rat hypothalamic  $\beta$ -endorphin immunoreactive cells, whereas tamoxifen alone does not affect the number of  $\beta$ -endorphin immunoreactive cells (31). A similar effect has been described at the transcriptional level; whereas estradiol administration increases hypothalamic POMC mRNA, administration of tamoxifen or of estradiol plus tamoxifen does not change levels of POMC mRNA (31). In contrast, recent findings indicate that a raloxifene analogue exerts an estrogen-like effect on central and peripheral levels of  $\beta$ -endorphin, indicating that some SERMs may exert estrogen-like effect on the neuroendocrine system (9).

Our study shows that in ovx rats, the effects of EM-652 on the neuroendocrine system are similar to those of estradiol valerate, as assessed by measurement of  $\beta$ -endorphin and allopregnanolone. In fact, EM-652 increases  $\beta$ -EP levels in the hypothalamus, pituitary, and plasma, thus showing estrogen-like effects. In contrast, in fertile animals, EM-652 had a clear antiestrogenic effect on  $\beta$ -EP levels in the hip-

TABLE 1

Levels of  $\beta$ -endorphin and allopregnanolone levels in anterior pituitary, neurointermediate lobe, serum, and plasma.

Group	Anterior pituitary		Neurointermediate lobe $\beta$ -endorphin levels (ng/organ)	Serum allopregnanolone levels (pg/mL)	Plasma $\beta$ -endorphin level (ng/mL)
	Allopregnanolone levels (pg/mg of tissue)	$\beta$ -endorphin levels (ng/organ)			
Fertile rats					
No treatment	26.1 $\pm$ 3.4	657 $\pm$ 74	1,810 $\pm$ 256	1,734 $\pm$ 140	2.55 $\pm$ 0.25
EM 0.1 mg/kg	6.7 $\pm$ 1.6	461 $\pm$ 112	2,657 $\pm$ 149	1,278 $\pm$ 181	1.56 $\pm$ 0.54
EM 1 mg/kg	5.5 $\pm$ 1.7	409 $\pm$ 61	2,496 $\pm$ 381	760 $\pm$ 98	1.11 $\pm$ 0.51
EM 5 mg/kg	3.0 $\pm$ 0.7	355 $\pm$ 75	3,091 $\pm$ 285	365 $\pm$ 69	0.80 $\pm$ 0.38
Ovariectomized rats					
No treatment	5.1 $\pm$ 1.8	349 $\pm$ 36	871 $\pm$ 142	475 $\pm$ 88	1.59 $\pm$ 0.19
EV 0.01 mg/kg	5.4 $\pm$ 1.6	485 $\pm$ 100	906 $\pm$ 196	689 $\pm$ 139	1.71 $\pm$ 0.29
EV 0.05 mg/kg	9.1 $\pm$ 2.5	539 $\pm$ 91	2,601 $\pm$ 438	1,123 $\pm$ 224	2.14 $\pm$ 0.47
EM 0.1 mg/kg	17.3 $\pm$ 2.9	559 $\pm$ 113	1,641 $\pm$ 684	518 $\pm$ 80	2.60 $\pm$ 0.52
EM 1 mg/kg	21.5 $\pm$ 3.7	822 $\pm$ 135	3,358 $\pm$ 743	1,030 $\pm$ 161	2.83 $\pm$ 0.53
EM 5 mg/kg	28.1 $\pm$ 4.2	977 $\pm$ 183	4,250 $\pm$ 1010	1,135 $\pm$ 198	3.78 $\pm$ 0.66
EV 0.05 mg/kg	9.8 $\pm$ 1.7	439 $\pm$ 80	2,126 $\pm$ 438	1,101 $\pm$ 108	2.15 $\pm$ 0.32
+ EM 0.1 mg/kg					
EV 0.05 mg/kg	7.0 $\pm$ 1.8	383 $\pm$ 66	1,819 $\pm$ 327	1,129 $\pm$ 149	2.13 $\pm$ 0.53
+ EM 1 mg/kg					
EV 0.05 mg/kg	5.8 $\pm$ 1.6	297 $\pm$ 82	1,362 $\pm$ 232	1,328 $\pm$ 160	1.10 $\pm$ 0.40
+ EM 5 mg/kg					

Note: Doses of EM-652 and estradiol valerate (EV) are daily doses. Data are means ( $\pm$ SD). See text for details on the results of Bonferroni's multiple comparison test.

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poampus, anterior pituitary, and plasma, although an estrogen-like action was observed in the neurointermediate lobe and hypothalamus at the highest EM-652 dose. In ovx rats treated with EV, the highest dose of EM-652 had antiestrogenic effects on  $\beta$ -EP levels in all tissues examined reducing hypothalamic and circulating  $\beta$ EP to levels lower than those observed in untreated ovx rats. These data indicate that the effects of EM-652 on the opioidergic pathways depend on the tissue examined and on the presence of endogenous or exogenous estradiol.

Scanty information is available on the modulation of neurosteroid levels by gonadal steroids. The changes in serum allopregnanolone levels during the estrous cycle and after ovariectomy in the rat and the inhibitory action of allopregnanolone on the ovulatory process suggest that allopregnanolone levels may be modulated by sex-steroid hormones (39). Our study confirms these findings, indicating that ovariectomy significantly decreases allopregnanolone levels in serum and in selected areas of the central nervous system (40, 41) while increasing allopregnanolone adrenal content, probably by modulation of adrenal steroidogenesis (42). A previous study of a raloxifene analogue, LY-117018, demonstrated that this antiestrogen exerts estrogen-like effects on central and peripheral levels of allopregnanolone (10).

In agreement with previous studies, we found that estrogens modulate allopregnanolone levels in various tissues. In

addition, we show for the first time that in ovx rats, EM-652 reproduces estrogen-induced changes in allopregnanolone levels in cerebral areas, the pituitary, and the adrenal glands. Moreover, when administered together with EV to ovx rats, EM-652 suppresses the estrogen-induced changes in allopregnanolone levels in the hippocampus, hypothalamus, and anterior pituitary, thus showing an antiestrogenic effect. This effect is not observed in serum and the adrenal glands, where an estrogen-like effect is present. When administered to intact fertile rats, EM-652 had a castration-like effect on hippocampal, hypothalamic, pituitary, adrenal, and serum allopregnanolone levels. Thus, administration of both estradiol and EM-652 seems to produce different effects in central and peripheral tissues.

The mechanism by which estradiol and SERMs modulate circulating and tissue levels of allopregnanolone is unclear. Estrogens may act directly on the enzymes involved in the biosynthetic pathway of allopregnanolone. Ovariectomy decreases or does not affect hypothalamic  $5\alpha$ -reductase activity (43, 44), but increases adrenal  $5\alpha$ -reductase activity, which is in turn inhibited by estradiol replacement (45). These data indicate that the effects of estradiol on the expression of  $5\alpha$ -reductase differ in the central nervous system and the adrenal glands. In addition, estradiol administration is reported to increase the activity of  $3\beta$ -hydroxysteroid oxidoreductase in rat brain (46).

In conclusion, administration of estradiol valerate to ovx rats increases levels of  $\beta$ -endorphin and allopregnanolone in serum, hypothalamus, and hippocampus while it reduces adrenal levels of allopregnanolone. In ovx rats, EM-652 has an estrogen-like effect on the central nervous system, whereas in ovx animals treated with EV, it acts as an antiestrogen in the hypothalamus and hippocampus. In intact fertile rats, EM-652 increases  $\beta$ -endorphin levels in the hypothalamus and has a slight inhibitory effect on these levels in the hippocampus. On the contrary, EM-652 administration in intact fertile rats increased allopregnanolone levels in adrenal glands. The effects of EM-652 appear to be similar to those of LY-117018 in the same experimental model; however, EM-652 has a stronger estrogen-like effect on  $\beta$ -endorphin and allopregnanolone levels.

These findings provide new perspectives on the use of new generation SERMs as hormone replacement therapy in selected patients. The estrogen-like effects of EM-652 on  $\beta$ -endorphin and allopregnanolone levels in cerebral areas involved in mood and cognition may prove beneficial in the clinical setting. Further studies on the effects of this molecule on the central nervous system are warranted.

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