

Role of Extra-Ovarian Oestrogens in the Regulation of Gonadotropin Releasing Hormone mRNA Expression in the Rat Brain

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Abstract

To further understand the role of oestrogens in the regulation of gonadotropin releasing hormone (GnRH) mRNA expression in the female rat brain, the effect of EM-652.HCl, a pure anti-oestrogen, was studied in intact and ovariectomized rats as well as in rats chronically treated with a GnRH agonist D-trp⁶, des-Gly-NH₂¹⁰ GnRH ethylamide (GnRH-A), a treatment which blocks ovarian steroidogenesis. Quantitative *in situ* hybridization was used to measure GnRH mRNA at the cellular level in the preoptic-anterior hypothalamic area. It was found that, 49 weeks after ovariectomy (OVX), the number of silver grains per cell corresponding to GnRH mRNA was increased by 34%. Long-term administration (49 weeks) of EM-652.HCl to OVX rats resulted in a further increase (11% over the levels measured in OVX rats) in the hybridization signal. By contrast, in intact female rats, treated during 52 weeks with EM-652.HCl, a 49% increase in the GnRH hybridization signal was detected. In rats treated with GnRH-A during the same period, a 20% decrease in GnRH mRNA was observed. When EM-652.HCl was administered concomitantly with GnRH-A, a further 63% increase over the mRNA levels recorded in GnRH-A treated rats was found. Thus, long-term treatment with the anti-oestrogen EM-652.HCl can upregulate GnRH mRNA expression in intact female rats, OVX rats and female rats chronically treated with a GnRH-A. It is suggested that the pure anti-oestrogen EM-652.HCl can exert an influence on the oestrogen feedback mechanism involved in the regulation of GnRH neuronal activity by neutralizing the action of locally produced or low circulating levels of oestrogens remaining after OVX or GnRH-A treatment.

The hypothalamic hormone gonadotropin-releasing hormone (GnRH) is the primary determinant in the control of secretion of follicle-stimulating hormone and luteinizing hormone (LH), the two pituitary hormones that regulate ovarian and testicular functions. The activity of the hypothalamo-pituitary-gonadal axis is regulated by several neurotransmitters and neuromodulators as well as by circulating sex steroids (1). GnRH biosynthesis, as reflected by changes in GnRH mRNA expression, has been studied under different sex steroid conditions in order to define the role of circulating steroids in GnRH neuronal regulation. We have previously shown that 14-day ovariectomy induced an increase in GnRH mRNA expression as measured by *in situ* hybridization (2). By contrast, chronic administration (14 days) of 17 β -oestradiol (E₂) or dihydrotestosterone to ovariectomized rats prevented the postcastration increase in GnRH mRNA expression.

Another study has also shown that, 7 days after ovariectomy, 2 days of oestrogen treatment decreased mRNA expression (3). Moreover, cellular levels of GnRH mRNA have been reported to be lowest in the morning of proestrus (4) when plasma oestrogen concentrations are highest (5). There are also reports indicating that ovariectomy does not modify or induce a decrease in GnRH mRNA expression (6, 7). A positive influence of oestrogens on GnRH mRNA concentrations in ovariectomized rats has also been reported (6, 8). These differing results might be attributable to differences in hormonal treatment models, anatomical specimens analysed, length of time after castration, and so on.

The contribution of neurosteroids to the regulation of GnRH neuronal activity has not been extensively studied. We have recently shown that, in adrenalectomized and castrated male rats, administration of trilostane, an inhibitor of

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3β -hydroxysteroid dehydrogenase (3β -HSD) activity could induce an increase in GnRH mRNA expression (9). This suggests that some steroids produced in the brain, including oestradiol (10), can directly or indirectly modulate GnRH mRNA expression.

So far, the effect of antiestrogens of GnRH mRNA expression has not been studied in the rat brain. In an attempt to further clarify the influence of oestrogens in the regulation of GnRH mRNA expression in the female rat, we have studied the effects of EM-652.HCl, a pure anti-oestrogen (11, 12), in ovariectomized rats and rats receiving a long-term treatment with a GnRH agonist (GnRH-A) that markedly decreases ovarian function (13–15). To measure GnRH mRNA expression at the cellular level in the preoptic-anterior hypothalamic area, quantitative *in situ* hybridization was used.

Materials and methods

Animals and treatment

Ten to 12-week-old female Sprague-Dawley rats [Cri:CD(SD)Br] (Charles River Laboratory, St Constant, Canada) weighing approximately 210–240 g at the start of treatment, were used. The rats were acclimatized to the environmental conditions (temperature $22 \pm 3^\circ\text{C}$; humidity $50 \pm 20\%$; 12-h light/dark cycle, lights on at 07.15 h) for 1 week before starting the experiment. The rats were housed individually and were allowed free access to tap water and a pelleted certified rodent feed (Lab Diet 5002, Ralston Purina, St Louis, MO, USA). The experiments were conducted in a Canadian Council on Animal Care approved facility in accordance with the CCAC Guide for Care and Use of Experimental Animals.

In the first experiment, 45 rats were randomly distributed between three groups of 15 rats each as follows: (i) intact controls; (ii) ovariectomized (OVX) controls; and (iii) OVX + EM-652.HCl (2.5 mg/kg, body weight). On day 1 of the study, rats from the appropriate groups were bilaterally ovariectomized under isoflurane anaesthesia. Treatment with EM-652.HCl suspended in 0.4% methylcellulose or the vehicle alone (control groups) was initiated on day 2 of the study and was given once daily by oral gavage in 0.5 ml/rat for 49 weeks. The daily dose of EM-652.HCl was selected on the basis of previous experiments indicating that there was a maximal effect on oestrogen-sensitive parameters at 2.5 mg/kg (11, 12).

In the second experiment, rats were randomly assigned to four groups of 15 rats per group as follows: (i) intact control; (ii) intact + EM-652.HCl (2.5 mg/kg); (iii) intact + GnRH-A (0.008 mg/kg); and (iv) intact + GnRH-A + EM-652.HCl. EM-652.HCl was administered once daily as described above while GnRH-A (D-trp⁶, des-Gly-NH₂¹⁰ GnRH ethylamide) was injected subcutaneously once daily as solution in 0.3% NaCl, 0.25% NaH₂PO₄·H₂O, pH 5.6–6.2. Rats were treated for 52 weeks. The daily dose of GnRH-A was chosen on the basis of previous reports showing that there was maximal inhibition of ovarian steroidogenesis at 0.008 mg/kg (13, 14).

In situ hybridization

Approximately 24 h after last dosing, overnight fasted rats (five rats per group) were perfused under ketamine-xylazine anaesthesia with 200 ml of 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were quickly frozen and 10 μm coronal sections were serially cut through an area extending from the medial preoptic area to the anterior hypothalamus. *In situ* hybridization was performed as described previously (16). The probe chosen was a [³⁵S]-labelled 48 bp oligonucleotide complementary to the GnRH coding region of the rat cDNA (bases 102–149). After hybridization, the sections were dehydrated and coated with liquid photographic emulsion (Kodak NTB-2). They were then processed after 7 days of exposure. To assess the specificity of the hybridization signal, consecutive sections were alternatively hybridized with the labelled oligonucleotide probe encoding GnRH and a labelled sense oligomer directed to the complementary DNA strand. For each experimental group, the mean number of silver grains per cell was calculated from at least 250 ± 10 cells from five rats per group.

Hormone measurements

Blood samples were collected from overnight fasted rats (10 rats per group) under isoflurane anaesthesia by exsanguination at the abdominal aorta. Oestradiol was measured in the second experiment by radioimmunoassay following methanol and diethyl ether extraction and chromatography LH-20 columns, as described in detail elsewhere (17).

Statistical analysis

Statistical significance was measured according to the multiple-range test of Duncan–Kramer (18). Data are expressed as means \pm SEM.

Results

GnRH mRNA expression

Analysis of the autoradiograms indicated that a strong signal could be obtained after 7 days of exposure. The specificity of the signal could be established by the following criteria: (i) labelled cells have the same localization as reported by immunocytochemical staining of GnRH (19, 20) and (ii) hybridization with a sense oligomer produced no labelling in adjacent sections. Moreover, using a combination of immunocytochemistry and *in situ* hybridization, we have previously shown that the GnRH hybridization signal could be detected only in neurones containing immunoreactive GnRH (2). Cells expressing GnRH mRNA were observed by decreasing order of intensity in the preoptic area, anterior hypothalamus, diagonal band of Broca and ventral septal area. The greatest number of labelled cells (24 in a single 10- μm section) was observed in the area of the organum vasculosum of the lamina terminalis (OVLt).

As illustrated in Fig. 1, 49 weeks after OVX, the mean value of silver grains per cell corresponding to GnRH mRNA expression was increased by 34% ($P < 0.001$) over that measured in intact rats. Administration of EM-652.HCl to OVX rats resulted in a further 11% increase ($P < 0.001$) of the mRNA levels compared to the value measured in the OVX rats.

In the second series of experiments, we evaluated the effect of treatment with EM-652.HCl in intact rats and in rats with a

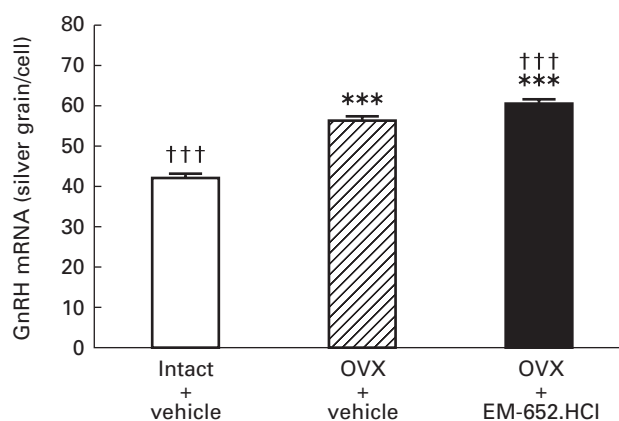


FIG. 1. Effect of ovariectomy and EM-652.HCl administration for 49 weeks to ovariectomized rats on gonadotropin releasing hormone (GnRH) mRNA levels *** $P < 0.001$, intact versus the other experimental groups. + + + $P < 0.001$ ovariectomized rats versus the other groups.

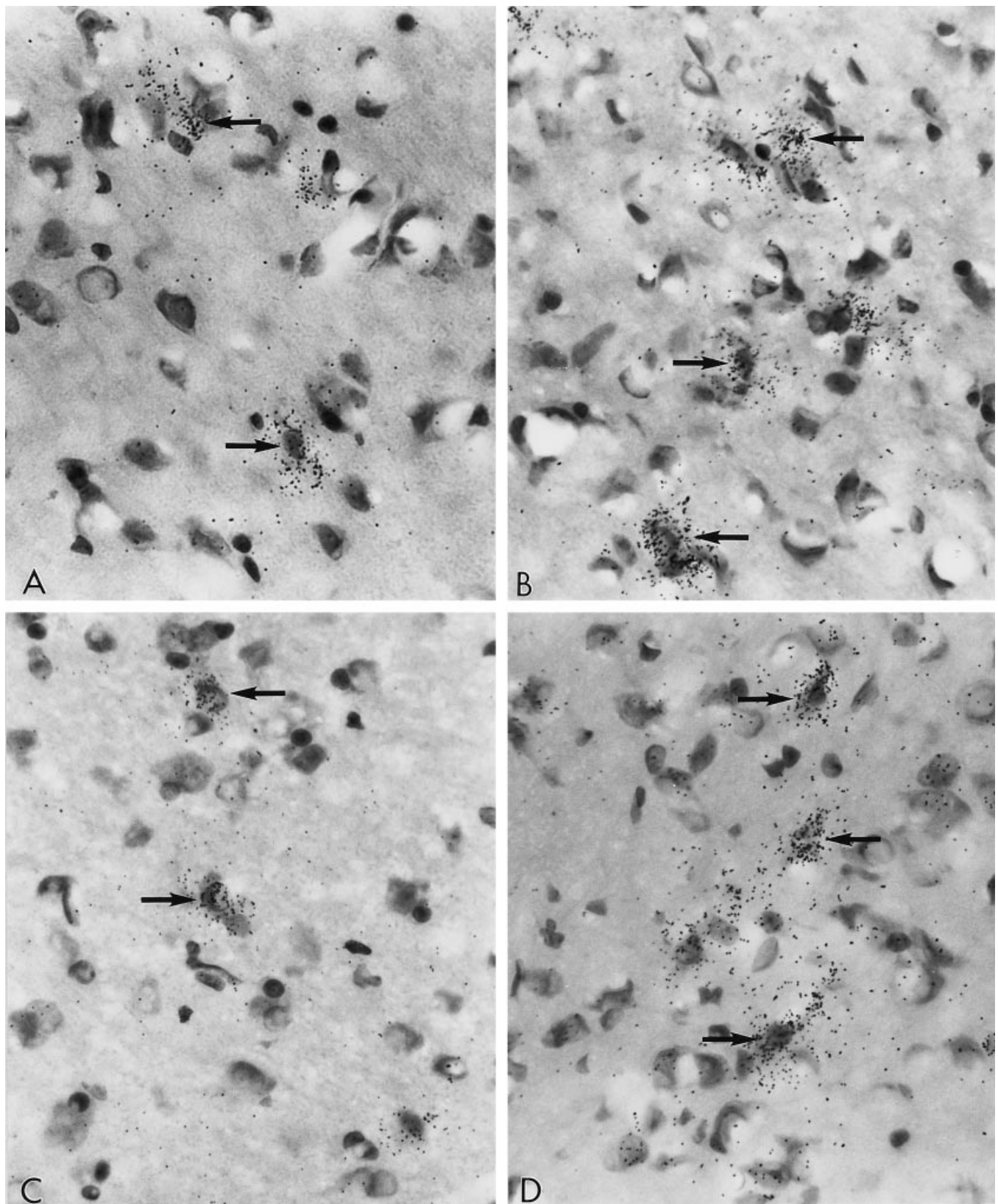


FIG. 2. Representative light microscope autoradiographs illustrating the signal obtained in the medial preoptic area of female rats following hybridization with the labelled GnRH probe. Silver grains are concentrated over a few neurones (\rightarrow). (A) Vehicle-treated, (B) EM-652.HCl-treated, (C) gonadotropin releasing hormone agonist (GnRH-A)-treated and (D) GnRH-A and EM-652.HCl-treated rats ($\times 800$).

decreased ovarian function induced by the chronic administration of a GnRH-A. As observed in the OVX rats, which are deprived of circulating oestrogens, chronic treatment with the anti-oestrogen induced a significant increase (49% over intact control levels; $P < 0.001$) (Figs 2B and 3) in the hybridization signal. In rats treated with GnRH-A, we observed a 20% decrease ($P < 0.001$) in mRNA levels compared to the levels observed in rats which received the vehicle alone (Figs 2C and 3). When EM-652.HCl was administered concomitantly with GnRH-A, a 63% increase ($P < 0.001$) over the mRNA levels recorded in GnRH-A-treated rats was observed (Figs 2D and 3). When compared with the GnRH mRNA levels detected in the vehicle-treated intact rats, the combination of both treatments resulted in a 21% increase in GnRH mRNA levels ($P < 0.001$). Treatment of intact rats with EM-652.HCl induced a marked increase in serum levels of estradiol (50.06 ± 8.49 versus 8.07 ± 2.52 pg/ml for control intact rats; $P < 0.001$). In the two groups of GnRH-A-treated rats, serum oestradiol could not be detected.

Discussion

The present results obtained in long-term ovariectomized rats are in agreement with previous data showing an increase in GnRH mRNA expression, 7 days (3) and 14 days (2) following ovariectomy. Interestingly, the present data demonstrate that, even in the absence of circulating oestrogens, the anti-oestrogen EM-652.HCl could further increase the hypothalamic expression of GnRH mRNA. This effect may be explained by an anti-oestrogenic action of EM-652.HCl at the level of the central nervous system which could counteract the negative influence of locally produced oestrogens. It has already been shown that the enzymes leading to the synthesis of oestradiol from cholesterol are present in the brain, and that oestradiol can be *de novo* synthesized in the rat brain (10, 21). Moreover, we have previously reported that trilostane, a 3β -HSD inhibitor, could induce an increase in GnRH mRNA expression in castrated and adrenalectomized male rats (9). Such data suggest that steroids including

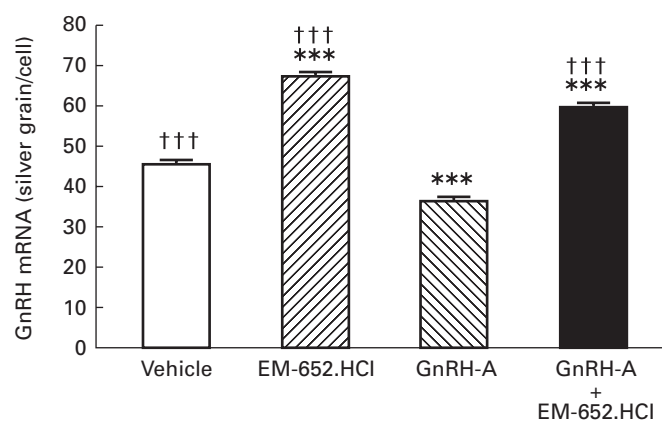


FIG. 3. Effects of EM-652.HCl and gonadotropin releasing hormone agonist (GnRH-A) administered alone or in combination in GnRH mRNA levels. *** $P < 0.001$. Vehicle-treated rats versus all the other experimental groups. + + + $P < 0.001$ GnRH-A-treated rats versus all the other experimental groups.

oestradiol produced in extra-gonadal and extra-adrenocortical sites (possibly the brain) can negatively modulate GnRH neuronal activity.

In the intact rat, chronic administration of the anti-oestrogen resulted in a stimulation of expression of hypothalamic GnRH mRNA, despite the increased serum concentrations of oestradiol. It thus seems that EM-652.HCl is acting as a pure antagonist of oestrogens at the central level, preventing the negative-feedback regulation exerted by oestrogens. On the other hand, chronic treatment with GnRH-A induced a decrease in GnRH mRNA expression, even though the circulating concentrations of oestradiol were markedly depressed (nondetectable), suggesting an inhibitory action of GnRH-A on GnRH neurones. Such a decrease in ovarian function has been well documented and attributed to an inhibition of bioactive LH secretion (13–15). We have previously shown that acute intracerebroventricular injection of GnRH-A depressed GnRH mRNA expressions in hypophysectomized rats (22). Using superfused rat hypothalami, Feleder *et al.* (23) reported that addition of the GnRH agonist busserelin to the superfusion medium inhibited GnRH release. Thus, the decrease in GnRH expression following chronic administration of GnRH-A might be related to an auto-regulation of GnRH neurones involving an ultrashort feedback. It might also be hypothesized that a decrease in GnRH secretion could play a complementary role in the induction of medical hypophysectomy (limited to the gonadotrophs) following long-term administration of a GnRH agonist. The anti-oestrogen induced an increase of GnRH mRNA expression in OVX rats treated with GnRH-A. It might be suggested that, in GnRH-A-treated rats, circulating oestrogens (even at very low concentrations) and/or oestrogens locally produced in the brain can exert a negative regulation on GnRH neuronal activity.

The exact site(s) of action of EM-652.HCl in the central nervous system is undetermined. Until recently, it was believed that GnRH neurones did not possess oestrogen receptors (ER) and that changes in GnRH gene expression were due to the effect of steroids on afferent inputs to GnRH neurones (24–27). During the last few years, there have been reports indicating that GnRH neurones may express both ER α and ER β (28–32). The majority of GnRH neurones are located close to the OVLT (19,20), an area which lacks the blood–brain barrier and thus accessible to any circulating endogenous factor or administered drug. It might be suggested that EM-652.HCl, which exerts a potent inhibitory activity on both ER α and ER β (33), can directly act at GnRH neurones to prevent the action of oestrogens. However, an effect of the anti-oestrogen on afferent inputs to GnRH neurones such as dopamine, norepinephrine, neuropeptide Y, β -endorphin and serotonin (25–27, 34–38), cannot be excluded.

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