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Glutamic Acid Decarboxylase Messenger Ribonucleic Acid Is Regulated by Estradiol and Progesterone in the Hippocampus*

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ABSTRACT

Ovarian steroids modulate learning, memory, and epileptic seizure activity, functions that are mediated in part by the hippocampus. Normal function depends on precise interactions between the inhibitory γ-aminobutyric acid (GABA)ergic and excitatory glutamatergic neurons of the hippocampus. To determine whether estradiol and progesterone interact with GABAergic neurons, the levels of mRNA for glutamic acid decarboxylase (GAD), the rate-limiting enzyme for GABA synthesis, were measured by *in situ* hybridization histochemistry with ^{35}S-labeled riboprobes complimentary to the feline GAD cDNA. The levels of mRNA for GAD were analyzed in selected region of the dorsal hippocampus and medial basal hypothalamus in ovariectomized, ovariectomized estradiol-treated, and ovariectomized estradiol- and progesterone-treated rats. In estradiol-treated rats, GAD mRNA levels increased in GABAergic neurons associated with the CA1 pyramidal cell layer, but not in the stratum oriens of CA1 or any other region of the hippocampus. Estradiol plus progesterone treatment reversed the estradiol-induced increase in GAD mRNA in CA1 and induced a small decrease in the hilus. No effect of estradiol or progesterone was observed in the dorsomedial, ventromedial, or arcuate nuclei of the hypothalamus. Estradiol or progesterone may alter cognitive performance and seizure activity by increasing or decreasing, respectively, the activity of GABAergic neurons in the hippocampus. (*Endocrinology* **131:** 2697–2702, 1992)

IN ADDITION to their primary role in the maintenance and regulation of reproductive capacity, ovarian steroid hormones influence more general physiological functions, such as learning and memory. In both women (1, 2) and laboratory animals (3–6), estradiol and testosterone alter certain aspects of cognitive performance. Ovarian steroids also alter the susceptibility to epileptiform activity. Estradiol decreases the threshold to seizure activity in animal models of convulsive disorders (7–11) as well as in epileptic women (12), and this effect is reversed by progesterone. The frequency of seizure activity in women also varies with the stage of the menstrual cycle and appears to depend on the relative concentrations of estradiol and progesterone in the serum (12).

The mechanisms and sites of action for the effects of estradiol on cognitive performance and epileptic seizure activity have not been established, but one probable site is the hippocampus, a sexually dimorphic steroid-responsive region of the brain (6, 13). The hippocampus exhibits long-term potentiation, a use-dependent increase in synaptic efficacy that has been implicated as being the substrate for memory and, in the extreme, epileptic activity. Normal hippocampal function is dependent on precise interactions between the excitatory glutamatergic and inhibitory γ-aminobutyric acid (GABA)ergic neuronal systems. Excess stimulation (14) or inhibition (15) of GABA activation can result in epileptiform activity. Repeated subconvulsive electrical stimulation of the limbic system results in increased excitability, referred to as "kindling," an animal model for epilepsy. Kamphuis *et al.* (16, 17) suggested that changes in GABAergic function are important factors contributing to long term increased excitability in the course of kindling.

GABA activation in the hippocampus influences learning and memory. The retention of newly acquired information can be influenced by posttraining administration of GABAergic drugs. Agonists such as muscimol and baclofen impair, and antagonists such as bicuculline enhance subsequent retention (18). The drugs' effects are blocked by lesions of the dorsal hippocampus or amygdala, indicating that these regions may be involved in mediating neuromodulatory influences on memory storage (18).

The effects of estradiol on GABA cell function have been examined in some brain regions. In the hypothalamus, estradiol increases the turnover (19) and release (20) of GABA as well as the activity of the rate-limiting enzyme for GABA synthesis, glutamic acid decarboxylase (GAD) (21, 22). Levels of GAD mRNA are dependent on the degree of GABA neuronal activation [for review, see Erlander and Tobin (23)]. To determine whether estradiol influences the activity of GABA neurons in the hippocampus, we measured the levels of GAD mRNA using *in situ* hybridization histochemistry to allow for specific localization of discrete populations of GABA neurons not only by region, CA1, CA3, or dentate gyrus, but by cellular layer within each region.

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Materials and Methods

Animals

Animal studies were conducted in accord with the principles and procedures of the NIH Guide for the Care and Use of Laboratory

Animals. Female Sprague-Dawley rats (Charles River, Wilmington, MA) were maintained in a controlled environment on a 14-h light, 10-h dark cycle, with lights on at 0500 h. Thirty animals were bilaterally ovariectomized under ketamine and PromAce anesthesia. Two weeks later, all animals were adrenalectomized to remove the adrenal source of progesterone and were maintained on 0.9% saline. At the same time, 20 of the animals received Silastic capsules containing 180 μg 17β-estradiol/ml sesame oil. Two days later at 900 h, 10 of the estradiol-treated rats received two Silastic capsules containing 50 mg progesterone/ml sesame oil while under Metofane (methoxyflurane) anesthesia. The ovariectomized and estradiol-treated animals were anesthetized for an equivalent time (3–8 min), but received no implants. This hormone regimen was selected to provide serum levels within physiological ranges that approximate levels observed on the afternoon of the day of proestrus and induce gonadotropin release and sexual behavior. All animals were killed by decapitation at 1500 h, and the brains were removed, frozen on dry ice, and stored at −70 C. Trunk blood was collected for RIA of estradiol and progesterone. Sections of brain were cut in a cryostat (8 μm), thaw mounted on gelatin-coated slides, and stored at −70 C.

In situ hybridization

In situ hybridization histochemistry was performed according to the methods of Wise *et al.* (24). Feline GAD cDNA sense in SP65–13 and antisense in SP65–16 plasmids, to generate antisense and sense riboprobes, respectively, were generously provided by Dr. A. Tobin (25). The feline GAD cDNA has 95% identity to the rat GAD_{67} gene (23). Riboprobes (2.6 kilobases) were transcribed complementary to the sense and antisense cDNAs for GAD using [^{35}S]UTP (New England Nuclear, Boston, MA) and hydrolyzed to fragments with a mass average of approximately 200 basepairs. The hydrolyzed probe will not distinguish GAD_{67} from GAD_{65}. On the day of hybridization, sections were thawed, rapidly dried, and fixed in phosphate-buffered 4% paraformaldehyde. After fixation, the sections were washed sequentially in phosphate buffer, diethylpyrocarbonate-treated water, triethanolamine buffer with acetic anhydride, and 2 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate). Hybridization buffer (55 μl) containing either antisense or sense GAD cRNA (0.6 μg/ml), formamide, dextran sulfate, NaCl, Tris buffer, dithiothreitol, EDTA, Denhardt's solution, and excess transfer RNA was applied to the tissue sections. In a preliminary experiment, a saturation curve was generated using 0.3–1.0 μg GAD cRNA/ml hybridization buffer, and saturation of the endogenous GAD mRNA occurred between 0.5–0.7 μg/ml. Glass coverslips were applied, and sections were incubated overnight (16 h) at 50 C in humidified covered glass dishes. After hybridization, coverslips were removed, and slides were rinsed in 4 × SSC containing dithiothreitol, treated with RNase-A at 37 C, and washed in RNase buffer (37 C), 2 × SSC, and 0.1 × SSC (60 C). Slides were dehydrated and exposed to Kodak SB-5 film (Eastman Kodak, Rochester, NY) for 6 days. Films were developed using conventional photographic techniques. The slides were then dipped in NTB2 emulsion for 5 weeks, developed, and stained with cresyl violet.

Autoradiography

The films were quantified using the BrainV2.00 IBM/AT Dumas Image Analysis System (Drexel University, Philadelphia, PA). The specific gray level was determined by subtracting the gray level of a region of the thalamus that contains no GAD-labeled cells from the gray level in the region of interest. For the CA1 and CA3 regions of the hippocampus, the area analyzed included strata oriens, pyramidale, and radiatum. Analysis of the emulsion-coated slides was performed using the Microcomp Integrated Image Analysis System (Southern Micro Instruments, Inc., Atlanta, GA). Individual cells were circumscribed, and the number of grains per cell was determined for all GAD cells for each region evaluated. Background was determined by measuring the number of grains over unlabeled cells.

Hormone assays

RIA of estradiol and progesterone was performed using ^{125}I-labeled hormones and antibodies form ICN Biomedicals, Inc. (Costa Mesa, CA). For measurement of estradiol, 0.5 ml serum was extracted in anhydrous ether, dried, and reconstituted in assay buffer before assay. The lower limits of detection were 5 pg/ml for estradiol and 1 ng/ml for progesterone.

Data analysis

Statistical evaluation of the data was performed using an analysis of variance, followed by Tukey HSD *post-hoc* comparison (26) to determine which groups were different.

Results

Estradiol concentrations in serum were below detectable levels in the ovariectomized rats and were 22.2 ± 3.0 and 23.5 ± 3.4 pg/ml in the estradiol- and estradiol- plus progesterone-treated rats, respectively. Progesterone concentrations were 1.4 ± 0.2, 1.6 ± 0.4, and 140 ± 19.8 ng/ml serum in ovariectomized, estradiol-, and estradiol- plus progesterone-treated animals, respectively.

The distribution of cells labeled with the GAD antisense cRNA was similar to that previously reported (14, 27, 28). No specific labeling was observed with hybridization using the radiolabeled sense probe for GAD (Fig. 1). Quantitative analysis of the films demonstrated a small increase in GAD mRNA levels in the CA1 region of the hippocampus after estradiol treatment. Treatment with estradiol and progesterone significantly decreased the levels of GAD mRNA in the CA1 and dentate hilus compared to treatment with estradiol alone (Fig. 2). In the CA1 region, the GAD-labeled cells were sparsely distributed, such that, on film, the optical density of the background between the labeled cells contributed significantly to the optical density of the larger region. In addition, it was not possible on film to determine with accuracy whether individual cells were in the oriens or pyramidal cell layer, so the analysis included both cell layers. Therefore, the slides were also dipped in photographic emulsion to allow for cell by cell analysis of GAD mRNA levels. Three populations of GABA neurons were quantified: 1) cells adjacent to or within the CA1 pyramidal cell layer, 2) cells dispersed in the stratum oriens of CA1, and 3) cells in the dentate hilus. Treatment with estradiol caused a 30% increase in GAD mRNA levels per cell in the cells associated with the pyramidal layer in CA1; this was reversed by estradiol plus progesterone administration (Fig. 3). Estradiol and/or progesterone did not alter the levels of GAD mRNA in any of the other hippocampal regions measured. The number of GAD-positive cells in a given region was not altered by treatment with the ovarian steroid hormones (Table 1).

No statistically significant steroid-induced changes in the levels of GAD mRNA were detected in regions of the medial basal hypothalamus in any group (Fig. 4). In agreement with previous reports (27), the arcuate and dorsal medial nuclei contained large numbers of GAD mRNA-positive cells, whereas the ventromedial nucleus contained very few (<1/8-μm section). GAD-labeled cells were periventricular or lateral to and not within the VMN.

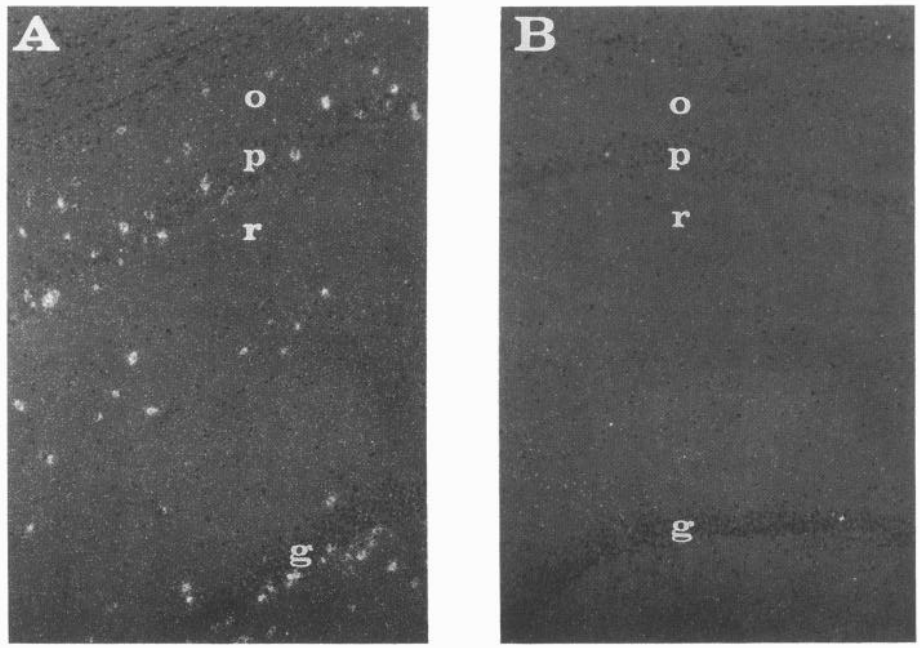

FIG. 1. Darkfield photomicrograph of [^{35}S]cRNA for GAD antisense (A) and sense (B) in the CA1 region of the hippocampus. o, Stratum oriens; p, stratum pyramidale; r, stratum radiatum; g, dentate granule cells.

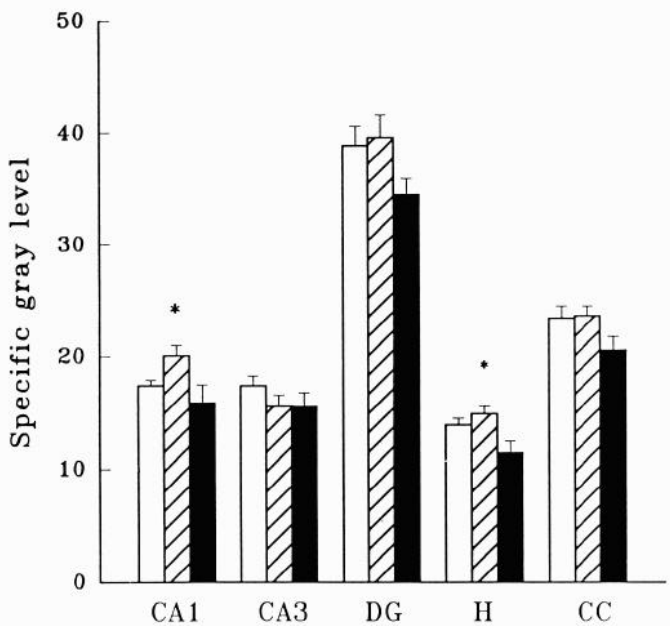

FIG. 2. Levels of GAD mRNA in ovariectomized (□), ovariectomized estradiol-treated (▨), and estradiol- plus progesterone-treated (■) rats in CA1, CA3, dentate granule cells (DG), and hilus (H) of the hippocampus and the cingulate cortex (CC). Brain sections hybridized with ^{35}S-labeled GAD cRNA were exposed to Kodak SB5 film. For background determination, the relative optical density over a region of thalamus containing no GAD-positive cells was measured and subtracted from the relative optical density of the region of interest, resulting in the specific gray level. *Bars* represent the mean ± SEM. *, $P < 0.05$ compared to estradiol- plus progesterone-treated rats.

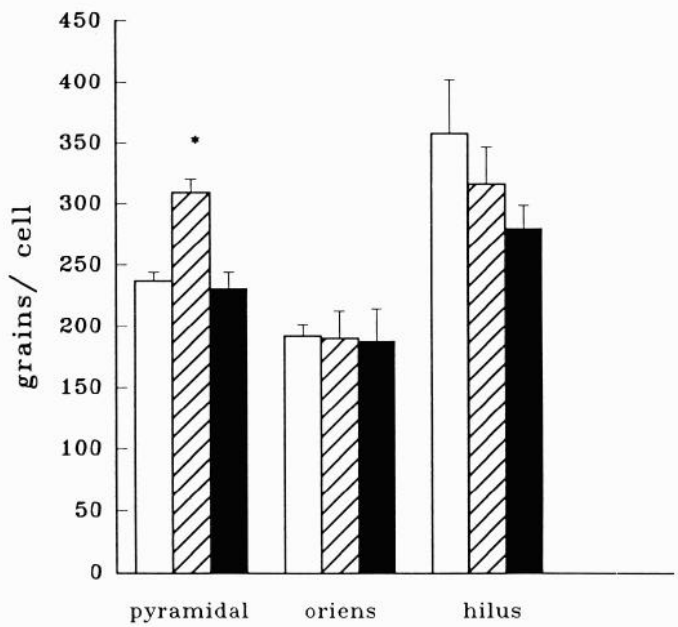

FIG. 3. Levels of GAD mRNA, measured in grains per cell, in the strata pyramidale and oriens of CA1 and the dentate hilus in ovariectomized (□), estradiol-treated (▨), and estradiol- plus progesterone-treated (■) rats. After hybridization with GAD cRNA, slides were exposed to Kodak NTB2 emulsion for a cell by cell analysis. Background over unlabeled cells was subtracted from the mean. *Bars* represent the mean ± SEM. *, $P < 0.001$, estradiol-treated compared to ovariectomized and ovariectomized estradiol- plus progesterone-treated groups.

Discussion

Estradiol and progesterone regulate GAD mRNA levels in a distinct population of GABA neurons associated with the pyramidal layer in the CA1 region of the hippocampus. Estradiol increases and estradiol plus progesterone reverse that increase in the levels of GAD mRNA per cell without altering the number of cells expressing the gene. The distribution of estrogen-responsive GAD mRNA-labeled cells corresponds to the same population of basket cells that is immunopositive for GAD (29), is closely associated with the

TABLE 1. Total number of GAD mRNA-positive cells per 8-μm section

	Stratum oriens CA1	Stratum pyramidale CA1	Dentate hilus
OVX	34 ± 4	58 ± 3	43 ± 2
E	31 ± 3	61 ± 4	41 ± 3
EP	38 ± 3	58 ± 4	38 ± 2

OVX, Ovariectomized; E, ovariectomized plus estradiol treated; EP, ovariectomized plus estradiol and progesterone treated.

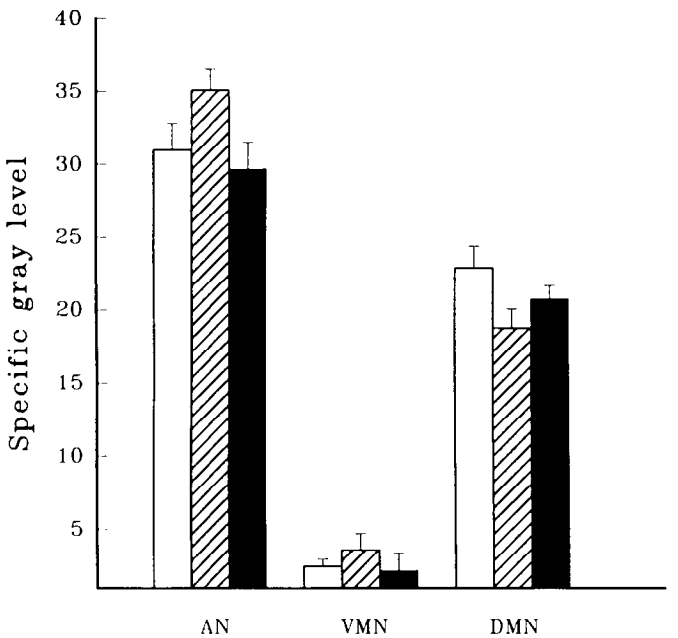

FIG. 4. Levels of GAD mRNA in arcuate (AN), ventromedial (VMN), and dorsomedial (DMN) nuclei of the hypothalamus in ovariectomized (□), ovariectomized estradiol-treated (▨), and estradiol- plus progesterone-treated (■) rats. Specific gray levels were determined from film exposed to brain sections hybridized with ^{35}S-labeled GAD riboprobes. The increase in GAD mRNA levels in the arcuate nucleus of estradiol-treated rats was not significantly different from levels in the other two groups ($P < 0.09$). *Bars* represent the mean ± SEM.

pyramidal cell layer, and parallels the distribution of estradiol-concentrating cells (30) in the CA1 region of the hippocampus. Prior work has established that the CA1 region of the hippocampus is particularly responsive to the effects of estradiol, which include the induction of progesterone receptors (31), an increase in $GABA_A$ receptors (32), an increase in agonist-binding sites for the *N*-methyl-D-aspartate-selective glutamate receptor (33), and an increase in spine density (34) and synapse (35) formation.

Regulation of the release and synthesis of neurotransmitter occurs at multiple sites in GABA neurons, including regulation of the levels of GAD mRNA, concentration of the enzyme, association of enzyme with cofactor pyroxidal phosphate, and possibly even the relative concentrations of the two forms of GAD enzyme [for review, see Erlander and Tobin (23)]. In the rat, two different forms of the GAD enzyme, GAD_{65} and GAD_{67}, are derived from two distinct GAD genes, and the GAD_{65} enzyme is more responsive to pyroxidal phosphate (23). Estradiol may regulate GABA neurons at any one or more of these steps. We have demonstrated estradiol-induced increases in total GAD mRNA levels in cells of the CA1 pyramidal layer of the hippocampus. An increase in the concentration of GAD mRNA has been strongly correlated with an increase in GABA neuronal activity (23). GABA can either hyperpolarize or depolarize hippocampal pyramidal cells depending on the synaptic connections; inhibition may result from the direct connection to pyramidal cells, and excitation from GABAergic connections to other GABA neurons (36), resulting in "disinhibition" (37). It would be of great interest to determine whether estradiol preferentially regulates one type of GABA connection.

Wallis and Luttge (22) found no effect of estradiol on GAD activity in the dorsal hippocampus; however, the inclusion of nonestrogen-responsive regions of the hippocampus may have obscured any existing effects of estradiol in CA1. In the limbic system, some of the effects of estradiol are opposite those observed in the hypothalamus. Estradiol treatment increases $GABA_A$ receptors in the hippocampus (32) and suppresses $GABA_A$ receptors in the arcuate and ventromedial nuclei of the hypothalamus (38, 39). In the septum, GABA activity is suppressed in the morning and increased in the afternoon (40), whereas the reverse occurs in the hypothalamus (19, 20, 40) in estradiol-treated animals. Our data showing increased GAD mRNA in the afternoon in estrogen-treated rats demonstrates that the hippocampus responds to estradiol in a manner similar to the septum.

More extensive evidence exists for the estrogen responsiveness of GABA neurons in the hypothalamus. Estradiol induces an increase in GABA turnover (19) and release (20) in the medial preoptic area in the morning, but not in the late afternoon. These changes parallel the rhythm in GAD activity that occurs in the medial preoptic areas and the medial basal hypothalamus in the proestrous rat (41). In addition, estradiol treatment of ovariectomized rats alters the activity of GAD in some hypothalamic regions (21, 22).

In the present study no changes in GAD mRNA levels were observed in the arcuate nucleus, but the possibility that estradiol stimulates GABA neurons in this region cannot be ruled out for several reasons. First, in the rat, estradiol- and progesterone-responsive neurons may represent a small portion of the GAD-labeled cells in the arcuate nucleus. Since the methods used in the present study do not allow for identification of the steroid-responsive cells, all GAD-positive cells are grouped together for quantification. Secondly, the activity of GABA neurons in the hypothalamus exhibits a hormone-dependent diurnal rhythm (19, 20, 41). This study measured the levels of GAD mRNA at 1500 h, a time when GABA activity in the hypothalamus has been reported to be low compared to morning values in estradiol-treated rats and not different from that in ovariectomized rats (19, 20). The time relationships between GABA release and the transcription of new message for GAD has not been established, making it difficult to identify pertinent times for examining steroid-induced responses. Future studies are warranted to identify steroid-responsive GABA neurons and examine a time course of effect.

The action of progesterone on GAD mRNA may be mediated by the estrogen-induced progesterone receptors (31)

or indirectly by the action of a progesterone metabolite at the steroid-binding site on the $GABA_A$ receptor (42). Earlier work has shown that progesterone can decrease the activity of GAD independently of prior estradiol treatment in the dorsal hippocampus (22), indicating that the effects may not be mediated by the intracellular progesterone receptor.

In summary, estradiol-induced activation and progesterone inhibition of a subset of GABA neurons in the CA1 region of the hippocampus may mediate some of the actions of ovarian steroid hormones on cognitive performance and epileptic seizure activity.

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