

Bidirectional Transcription Regulation of Glial Fibrillary Acidic Protein by Estradiol *in Vivo* and *in Vitro**

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ABSTRACT

Glial fibrillary acidic protein (GFAP) expression shows cyclic variation in the rat hypothalamus and hippocampus during the normal estrous cycle. To elucidate the role of transcription in the regulation of GFAP, we examined levels of GFAP intron 1 by *in situ* hybridization in the hypothalamus and hippocampus of normal, cycling rats. On the afternoon of proestrus, when plasma estradiol levels are highest, GFAP transcription and messenger RNA were both increased in the arcuate nucleus of the hypothalamus and decreased in the outer molecular layer of the dentate gyrus. In the hilus of the hippocampus, neither GFAP transcription nor messenger RNA changed during the

estrous cycle. *In vitro*, astrocytes showed bidirectional responses, such that estradiol treatment increased GFAP transcription in monotypic astrocytic cultures but decreased GFAP transcription in astrocytes cocultured with neurons. The functionality of an estrogen response element in the 5'-upstream region of the GFAP promoter was established by site-directed mutagenesis and binding of human recombinant estrogen receptor in gel shift assays. We conclude that estrogen may act directly upon astrocytes by estrogen receptor binding, and that the direction of the transcriptional response is influenced by astrocyte-neuron interactions. (*Endocrinology* **139**: 3202-3209, 1998)

IN THE hypothalamus, modulation of neurosecretion of GnRH by estrogen is subject to changes in glial fibrillary acidic protein (GFAP) that modify astrocyte-neuron interactions (1, 2). However, the mechanisms underlying this process are not completely understood. In the arcuate nucleus of the hypothalamus, GFAP expression varies cyclically during the normal estrous cycle, with messenger RNA (mRNA) elevated on proestrus (3) and protein increased 12 h later on estrus (4). These cyclic variations in GFAP levels may mediate the preovulatory surge of GnRH. Before the LH surge, there are decreases in perikaryal membrane in contact with presynaptic terminals and synapse number in the arcuate nucleus (1). It has been suggested that these synapses are primarily inhibitory (2, 5). The decreased number of contacts is hypothesized to be an integral step in a cascade that results in disinhibition and increased neurosecretion by GnRH neurons. Astrocytes are implicated in this process, because the decrease in synapse number is associated with an increase in the amount of perikaryal membrane covered by GFAP-positive astrocytic processes (1). Treatment with antiestrogens blocks both the drop in synapse number and the LH surge (2).

The hippocampus shows similar transient synaptic remodeling across the estrous cycle, in which there is a gradual increase in both dendritic spine density and synapses on dendritic spines in CA1 pyramidal neurons, followed by a

rapid decrease during the 24 h between proestrus and estrus (6, 7). Although GFAP levels vary cyclicly in the hippocampus as well, there are obvious differences. Most strikingly, GFAP immunoreactivity in the hilus of the hippocampus is significantly increased on the afternoon of proestrus (8), 12 h before peak GFAP immunoreactivity in the hypothalamus. This suggests differential regulation of GFAP transcription by 17 β -estradiol (E₂) in different brain regions.

To further elucidate the role of E₂ in regulation of GFAP, we examined several aspects of GFAP transcription. Regional differences in transcription were examined *in vivo* by *in situ* hybridization with an intron probe with which we have observed transcriptional regulation of GFAP by glucocorticoids (9) and increases in transcription with age (10). We also examined the effects of astrocyte-neuron interactions on E₂-mediated GFAP transcription, because the direction of the effect of glucocorticoids on GFAP transcription is influenced by astrocyte-neuron interactions (9), and because the effect of E₂ on another astrocytic mRNA (apolipoprotein E) is dependent upon cell-cell interactions (11). Finally, because estrogen receptor (ER α) mRNA has been detected in astrocytes (12), the role of a putative estrogen response element (ERE) in the GFAP promoter was studied by site-directed mutagenesis and ER α binding.

Materials and Methods

Animals

Three-month-old female F344 rats (n = 50) were kept on a 12-h light, 12-h dark schedule (lights on at 0700 h, lights off at 1900 h). Vaginal cytology in vaginal lavages (1000 h) were followed for 1 month to determine regularly cycling animals, of which a total of 36 were selected for study. Animals were killed at one of four cycle stages (diestrous morning, proestrous morning, proestrous evening, or estrous morning) between 1000-1200 h (morning) or between 1730-1830 h (evening).

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After death (by decapitation under nembutal anesthesia), brains were frozen in isopentane (-18°C) and stored at -70°C until sectioning ($16\ \mu\text{m}$); coronal sections $2.8\ \text{mm}$ posterior to bregma allowed simultaneous examination of the arcuate nucleus, dentate gyrus, and hilus. Sections were mounted on polylysine-coated slides.

LH assay

Trunk blood was collected immediately after decapitation for LH assay. LH levels were measured using the DELFIA rat LH assay (Wallac, Oy, Finland). Briefly, serum was incubated in streptavidin-coated plates with europium-(Eu)-labeled and biotinylated antirat LH antibodies at room temperature. After washing and enhancement, fluorescence was counted (Wallac 1230 Arcus Fluorometer; courtesy of Stephanie Griffith, Harbor-University of California-Los Angeles Medical Center). Rat LH reference preparation (AFP-7187B) was obtained from the National Hormone and Pituitary Program (NIDDK, Bethesda, MD). Blood LH levels were transiently increased 30-fold on proestrous evening ($P < 0.0001$), suggesting that proestrous evening rats were killed after the elaboration of glial processes and the decrease in synapse number, as these precede the LH surge (2).

In situ hybridization

Sections were fixed in 4% buffered paraformaldehyde, washed in PBS, and dehydrated in an ethyl alcohol series (30–100%). Sections were prehybridized for 1 h at 55°C (prehybridization buffer: 0.75 M NaCl, 50% formamide, 10% dextran sulfate, and 0.05 M phosphate, pH 7.4) and hybridized with a ^{35}S -labeled complementary RNA (cRNA) probe (9, 10). Antisense ^{35}S -labeled cRNA was transcribed from the pBluescript transcription vector containing the 0.9-kb rat GFAP intron I or 2.7 kb of rat GFAP complementary DNA including coding and 3'-untranslated region. Sections were hybridized for 3 h at 55°C (mRNA probe) or for 18 h at 50°C (intron probe). Slides were then covered with NTB2 emulsion (Eastman Kodak, Rochester, NY) and exposed for 1 week (mRNA) or 3 weeks (intron) for cellular analysis. After development, slides were counterstained with cresyl violet. Grain density per cell was measured by computer videodensitometry for the 10 cells that most clearly had signal higher than background in the arcuate nucleus, hilus, and outer molecular layer of the dentate gyrus for each brain (see Fig. 1). Background measurements came from quantification of unlabeled cells. Unlabeled cells had a signal intensity less than 1% of labeled cells. A detailed analysis of frequency distributions of signal intensity for all regions is in preparation.

Cell culture

Mixed glia were isolated from the cerebral cortex after mechanical dissociation (13) from 1- to 3-day-old F344 rat pups. Cells were plated onto plastic culture dishes at 2×10^5 cell/cm 2 and maintained in DMEM-Ham's F-12 culture medium (Mediatech, Herndon, VA) supplemented with 10% FBS (Life Technologies, Grand Island, NY), 100 U/ml penicillin, and 50 U/ml streptomycin (Sigma Chemical Co., St. Louis, MO) at 37°C in a 5% CO $_2$ -95% air incubator. Medium was renewed every 2–3 days until confluence. Confluent cultures were purified from containing microglia by shaking; the purity of monotypic cultures was confirmed (>99%) by immunohistochemical staining (9). Primary neurons were derived from the cerebral cortex of 18-day-old embryos. After mechanical dissociation, 5 million neurons were plated on the bed layer of confluent astrocytes (15 million cells/flask) and maintained in mixed sandwich cocultures for 5–8 days in serum-free, chemically defined medium (9). Pure astrocyte cultures or astrocyte-neuron cocultures were treated with E $_2$ (Sigma) for 24 h in serum-free, phenol red-free medium.

Northern blot hybridization

Total RNA was extracted from cell culture using Tri-Reagent (Molecular Research Center, Cincinnati, OH). Total RNA (5 mg) was electrophoresed on a 1% denaturing agarose gel (0.66 M formaldehyde) and transferred to nylon membranes. Membranes were hybridized with a [^{32}P]GFAP cRNA probe (9) in buffer (50% formamide, $1.5 \times \text{SSPE}$ (0.15 M NaCl, 1 mM EDTA, 11.5 mM NaH $_2$ PO $_4$, pH 7.4) 1% SDS, 0.5% powdered milk, 0.1 mg/ml yeast RNA, and 0.3 mg/ml single stranded

DNA) for 12 h at 54°C , followed by high stringency washing [$0.2 \times \text{SSC}$ (standard saline citrate)-0.2% SDS; 73°C]. Membranes were placed against PhosphorImager exposure cassettes, from which relative radioactivity of bands was analyzed with a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA).

Transfection of primary astrocyte cultures

Rat GFAP promoter constructs with a luciferase reporter (9) were transiently transfected into pure astrocyte cultures and astrocyte-neuron cocultures by lipofection using the DOTAP transfection reagent (Boehringer Mannheim, Mannheim, Germany). Eighteen hours after transfection, the culture medium was changed. Twenty-four hours later, transfected cells were treated with E $_2$ (final concentration, 1 pM) in serum-free, phenol red-free medium and lysed after an additional 24 h. Transfection efficiency was assayed by cotransfection with a simian virus 40 promoter-driven β -galactosidase gene (pSV- β -galactosidase control vector, Promega, Madison, WI). Luciferase activity was measured in cell lysates by the luciferase assay system (Promega). The activity was normalized to total protein with the Coomassie protein assay (Pierce, Rockford, IL) and β -galactosidase (Promega). Data were expressed as a percentage of the vehicle-treated control value (mean \pm SEM).

GFAP promoter luciferase constructs

Some 1.9 kb of the GFAP 5'-upstream region were excised from BSSK (Stratagene, La Jolla, CA) using *KpnI* and *XbaI* sites of the cloning cassette. After directional subcloning into the *KpnI* and *NheI* sites of the pGL3 Basic plasmid (Promega), the insert was confirmed by sequencing.

GFAP promoter constructs were cloned into pGL3 Basic (Fig. 4). Construct A contains the full 1.9-kb 5'-upstream sequence. The other constructs result from consecutive deletions of the GFAP 5'-upstream sequence (Fig. 4A, numbering according to the transcription start site). Fragments were cut out using internal restriction sites (Fig. 4A) in combination with *KpnI*, *SmaI*, and *ApaI* sites in the pGL3 basic vector. Cut sites were blunt ended using T4 DNA polymerase and religated. Fragment A8 was constructed using one of the internal *XhoII* sites in combination with *BglII* in the pGL3 basic vector and religated into the pGL3 basic vector cut with *BglII*.

Site-directed mutations in ERE $_1$ were introduced into fragment A3 by standard PCR (30 cycles). Briefly, two PCR primers were used: mutated, 5'-CCTTGACTCTGTGTTTCAGTAGCCTTGGTGGGG-3'; and reversed, 5'-GTTTCCTGTGAACACCAGCCTG-3'. The wild-type A3 sequence was used as a template. PCR products were gel purified, religated, and verified by sequencing. The sequence of ERE consensus in frog and chick vitellogenin (14) is GGTCA nnn TGACC. The ERE $_1$ in GFAP promoter (at -149 bp) is GGGTA cag TGACC. The mutated sequence is GTGTT cagTAGACC.

Gel shift

Two complementary 32-bp oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer (Foster City, CA): wild-type containing the putative ER-binding site (ERE $_1$) found in the rat GFAP promoter (CCTTGACTCTGGGTACAGTAGCCTTGGTGGGG, coordinates -172 to -141), and ERE mutant (CCTTGACTCTGTGTTTCAGTAGCCTTGGTGGGG). *Underlining* = mutation site of ERE. Double-stranded oligomers were constructed by annealing complementary DNA in saline-Tris-EDTA after heating to 95°C for 5 min and cooling slowly to 4°C . The wild-type DNA fragment was then labeled using [^{32}P]deoxy-ATP in a polynucleotide transferase reaction. Human recombinant ER (functionally active ER α ; 6.25 pM; Panvera, Madison, WI) were incubated in binding buffer [10 mM HEPES (pH 7.9), 2 mM MgCl $_2$, 50 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 10% glycerol, 0.1% Nonidet P-40, 200 mg BSA/ml, and 0.2 mM phenylmethylsulfonylfluoride] with 0.12 pM labeled wild-type DNA and 0.06–12 pM wild-type or mutant competitor DNA without radioactive label. Twenty minutes later, samples were loaded on a 20-cm, low cross-link, 8% polyacrylamide gel while running at 150 V at 4°C . The gel was run at 350 V for 4 h, dried, exposed to a phosphorimaging screen, and analyzed using ImageQuant software (Molecular Dynamics).

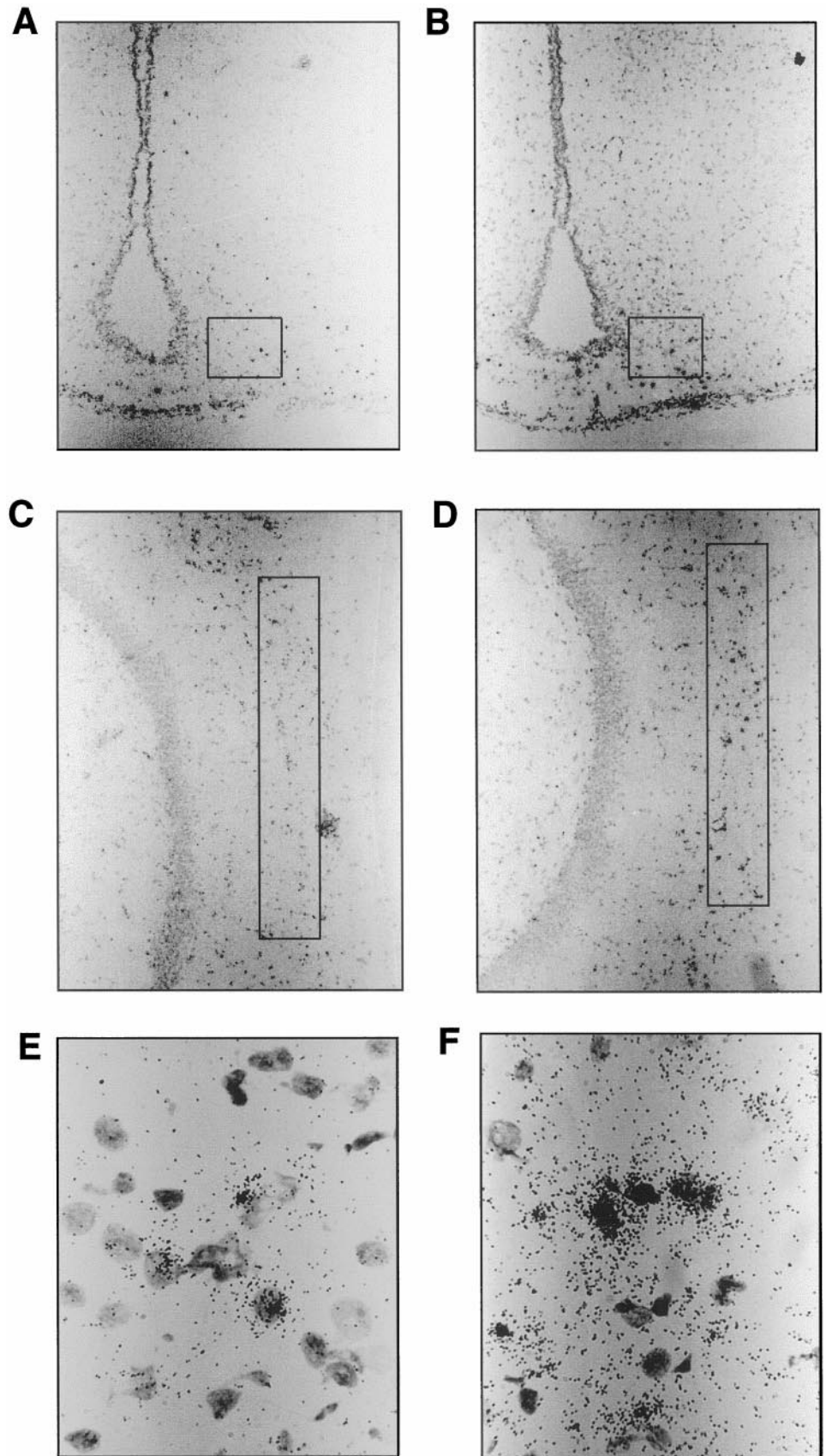


FIG. 1. GFAP intron 1 *in situ* hybridization signal in the arcuate nucleus of the hypothalamus (A and B) and in the outer molecular layer of the dentate gyrus (C and D) during the rat estrous cycle. In the hypothalamus, GFAP intron 1 hybridization signal is higher on proestrus evening (B; when plasma E_2 levels are high) than on diestrus (A). In contrast, in the hippocampus, GFAP intron 1 levels are lower on proestrus evening (C) than on estrus (D), when E_2 levels are reduced. Boxes localize the areas where grain density was analyzed. Magnification, $\times 100$. High power micrographs show cellular localization for intron 1 (E) and mRNA (F) hybridization signals in arcuate nucleus. Magnification, $\times 1000$.

Statistics

Data were analyzed by ANOVA, and statistical significance in pairwise comparisons between means was assessed with Dunnett's *post-hoc* tests. All statistics were run on either StatView 4.5 or SuperANOVA (both from Abacus Concepts, Berkeley, CA).

Results

GFAP intron 1 and mRNA expression in hypothalamus and hippocampus during the estrous cycle

GFAP intron and mRNA levels were quantified by *in situ* hybridization (grains per cell for the 10 cells most clearly labeled above background per region) in the arcuate nucleus of the hypothalamus (Fig. 1, A and B), the outer molecular layer of the dentate gyrus (Fig. 1, C and D), and the hilus of the hippocampal formation. In the arcuate nucleus, both

GFAP intron (Fig. 2A) and mRNA (Fig. 2B) were increased (by 1.7- to 3-fold) on the afternoon of proestrus, after the LH surge. In contrast, in the outer molecular layer of the dentate gyrus, GFAP intron and mRNA were decreased (30–40%) on the afternoon of proestrus (Fig. 2, C and D); here, the ascending phase of mRNA levels lagged slightly behind intron levels. In the hilus, where GFAP protein levels respond to estrogen and progesterone (8) no changes in either intron or mRNA levels were detected during the estrous cycle (Fig. 2, E and F).

E₂-mediated responses of GFAP transcription and mRNA are influenced by the presence of neurons

Previously, we showed that cell-cell interactions can influence the direction and degree of astrocytic mRNA re-

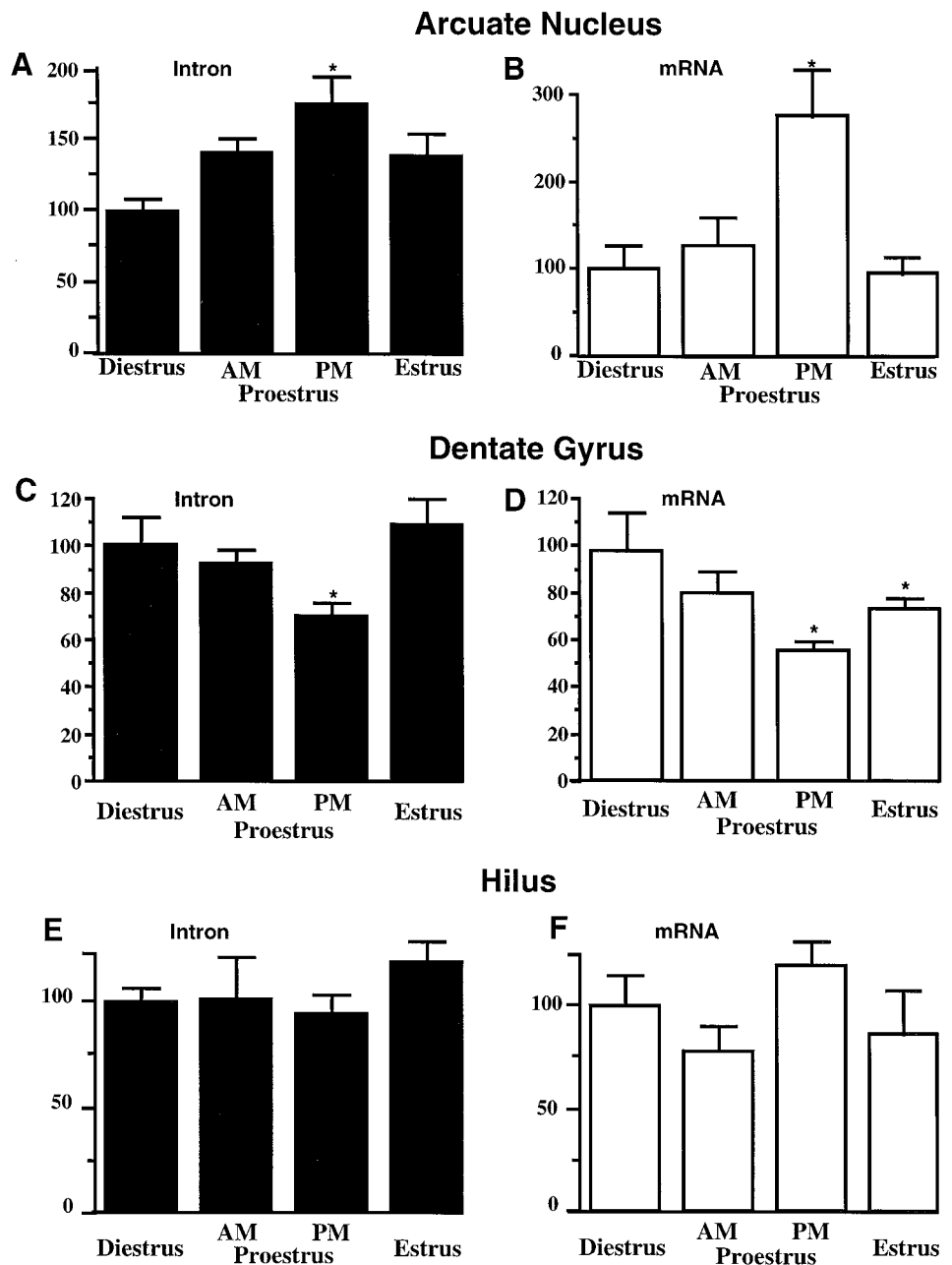


FIG. 2. *In situ* hybridization analysis of GFAP intron 1 (A, C, and E) and GFAP mRNA (B, D, and F) levels in the arcuate nucleus of the hypothalamus (A and B), the outer molecular layer of the dentate gyrus (C and D), and the hilus (E and F) during the rat estrous cycle. In all areas, grain density was calculated on the 10 cells most clearly labeled above background levels per region in each rat (n = 36; 9/time point). In the hypothalamus, both GFAP intron and mRNA levels were significantly higher on proestrus evening than on diestrus ($P < 0.05$; A and B), whereas in the outer molecular layer of the dentate gyrus, both GFAP intron and mRNA were significantly lower on proestrus evening than on diestrus ($P < 0.05$; C and D). However, in the hilar region of the hippocampus, neither GFAP intron nor mRNA levels changed during the estrous cycle (E and F). Data (mean \pm SEM) are expressed as a percentage of the value on diestrus.

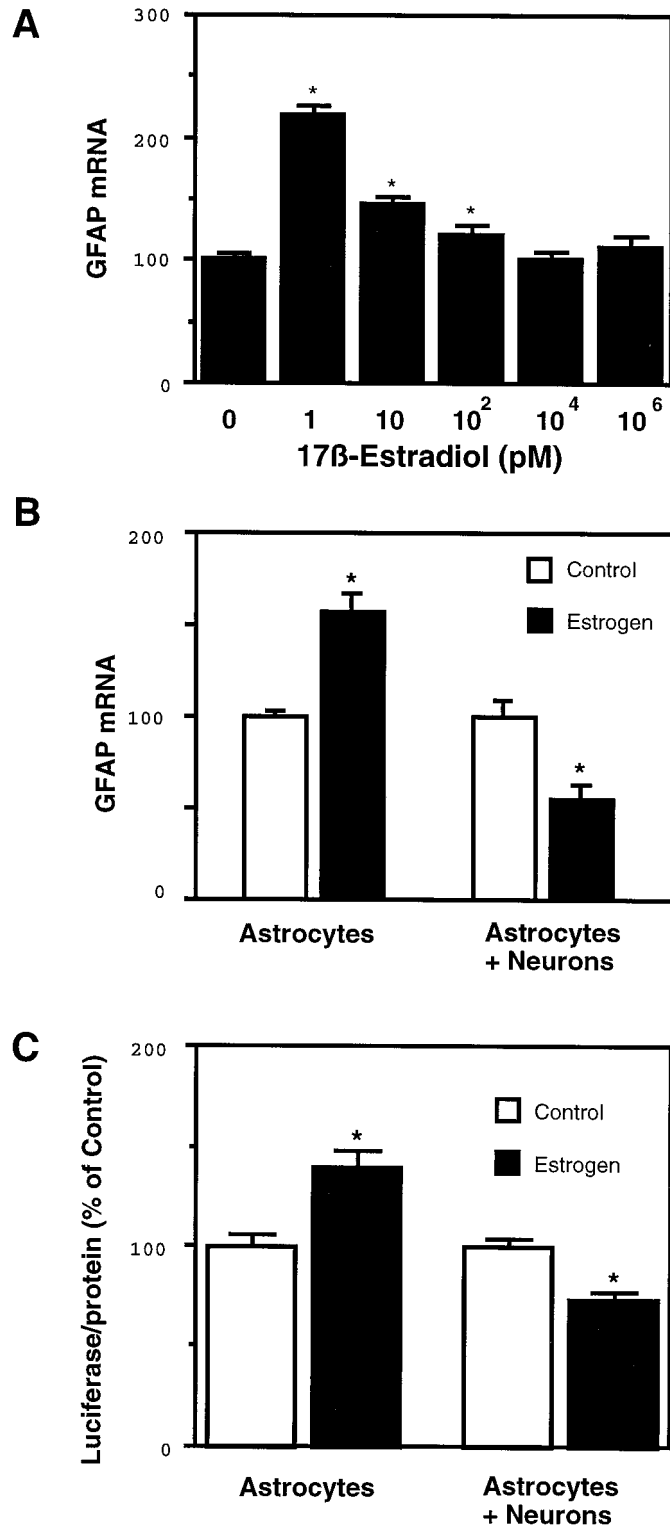


FIG. 3. E₂ effect on GFAP mRNA and transcription in pure astrocyte cultures and in astrocyte-neuron cocultures. A, GFAP mRNA 24 h after treatment of pure astrocyte cultures with various concentrations of E₂ in serum-free, phenol red-free medium. B, In contrast to the E₂-mediated increase in GFAP mRNA in pure astrocyte cultures, 24-h treatment of astrocyte-neuron cocultures with 1 pM E₂ resulted in a decrease in GFAP mRNA. Total cellular RNA was analyzed by Northern blot hybridization with the GFAP cRNA probe. Data (mean \pm SEM of three independent experiments) of integrated optical densities are

response to E₂ (11). We, therefore, examined the effect of estrogen on GFAP transcription in astrocytes both in monotypic culture and when cocultured with cerebral cortex neurons. In monotypic culture, astrocytes responded to E₂ with a 2-fold increase in GFAP mRNA levels at 24 h (Fig. 3A; dose-response curve), whereas in the presence of neurons GFAP mRNA was decreased by 50% when treated with 1 pM E₂ (Fig. 3B).

These findings were extended to GFAP promoter activity. Astrocytes in pure culture or cocultured with neurons were transfected with a 1.9-kb full-length GFAP promoter construct (Fig. 4A), which suffices for transcriptional regulation by glucocorticoids (9) and contains two putative EREs. Transfected cells were treated with 1 pM E₂ for 24 h. As was observed for mRNA levels, the full-length promoter showed an E₂-mediated increase in activity in pure astrocytic cultures. When cocultured with neurons, the direction of the response to E₂ was reversed from induction to inhibition of promoter activity (Fig. 3C). The sex of neonatal rats from which astrocytes were derived did not alter the direction of the GFAP response to E₂ (data not shown).

GFAP promoter construct analysis: role of ERE₁ in the rat GFAP promoter

Using sequential deletions in the GFAP promoter (see *Materials and Methods* and Fig. 4A), we examined the activity of putative EREs. ERE₁ is contained in construct A3, whereas ERE₂ is contained only within the full-length promoter and deleted in construct A7. Constructs A1 (minimal promoter) and A2 (-106 bp) that do not contain ERE sequence did not respond to E₂. However, in longer constructs (A3 to construct A7, which contain ERE₁), GFAP transcription was induced by E₂, which suggests that ERE₁ in the rat GFAP promoter is functional. Moreover, constructs A3–A7 showed consistently greater responses to E₂ (1.6- to 1.8-fold) than the full-length promoter (1.4-fold). Thus, ERE₂ and/or other sequences that are upstream of -1546 bp may inhibit the activity of ERE₁.

Site-directed mutagenesis of ERE₁ within the A3 construct (4 bp; see *Materials and Methods*) and ER α gel shift assays further show the functionality of ERE₁. The E₂-mediated activation of the GFAP promoter was abolished by mutation of ERE₁ in the A3 construct transfected into cultured astrocytes (Fig. 4B).

The binding of ER α to the ERE₁ site in the GFAP promoter was assayed by gel shift. Oligonucleotide (32 bp) containing the putative ERE₁ site in the GFAP promoter was incubated with human recombinant ER α or BSA for a control. Complexes of ER α protein and DNA were resolved on a poly-

expressed as a percentage of the untreated control value. *, $P < 0.05$. C, Pure astrocytes or astrocyte-neuron cocultures were transfected with full-length GFAP promoter; 48 h later, cells were treated with 1 pM E₂ for 24 h in serum-free, phenol red-free medium. Luciferase activity was normalized to total cellular protein and β -galactosidase to control for transfection efficiency. GFAP promoter was not active when transfected into neurons. Data (mean \pm SEM of three independent experiments) are expressed as a percentage of the untreated control value. *, $P < 0.05$, by ANOVA (Abacus Concepts).

acrylamide gel (Fig. 4C). Unlabeled 32-bp DNA containing wild-type ERE₁ or mutated oligonucleotide DNA (mERE₁; containing a 4-bp mutation in the putative ERE₁-binding site; see *Materials and Methods*) was used as a competitor in the binding reaction to demonstrate that ER α binds DNA specifically. Wild-type DNA at equimolar and higher concen-

trations effectively competed for the labeled ER α protein/DNA complex (Fig. 4C, lanes 3–6). mERE₁ competed less effectively for binding of ER α (Fig. 4C, lanes 7–10). Titration of the competition in a concentration range from 2- to 100-fold revealed a 11.5-fold higher affinity of the wild-type DNA for ER α . These results show that ER α protein binds specif-

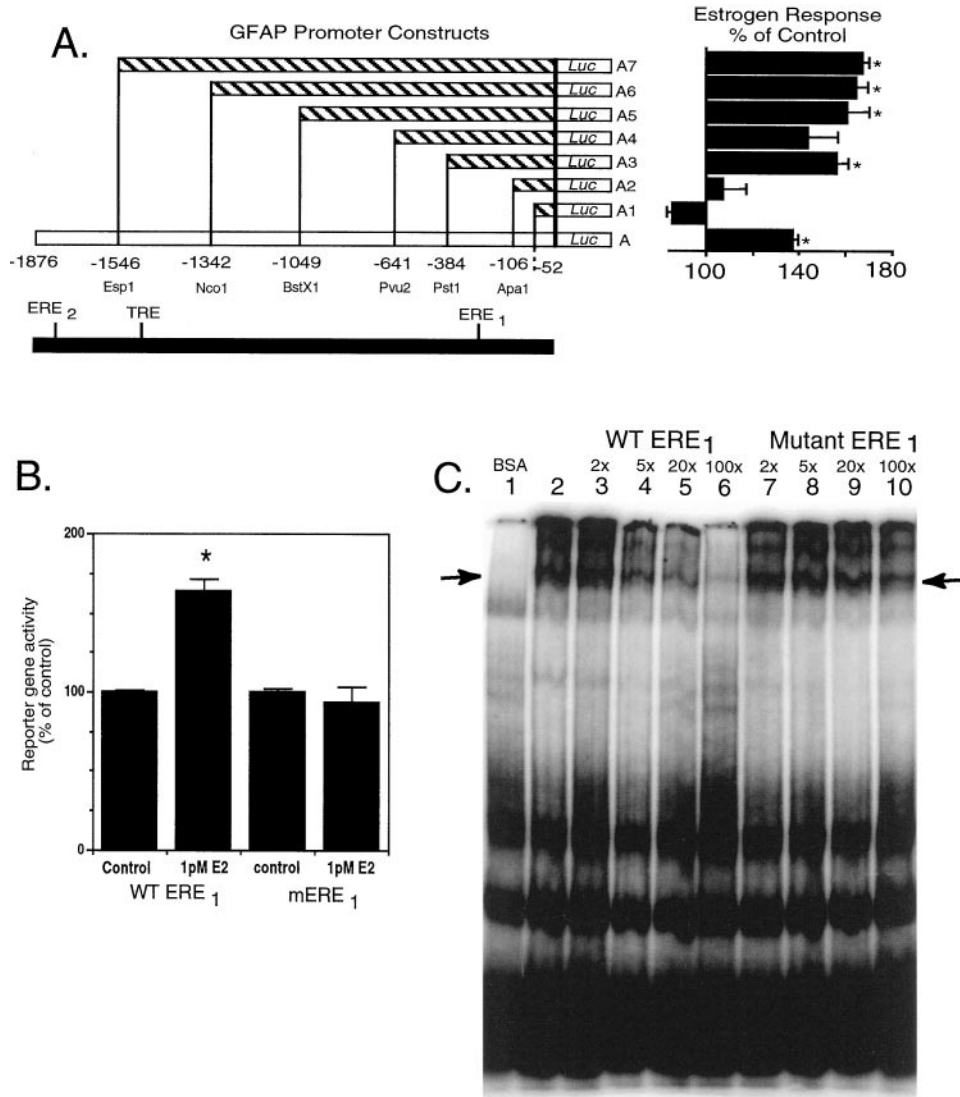


FIG. 4. GFAP transcription is controlled by E₂. Role of ERE₁ in the rat GFAP promoter. A, E₂ responses of the full-length promoter and several deleted constructs (A1–A7). Construct A contains the full 1.9-kb 5'-upstream sequence. All other constructs result from the consecutive deletions of construct A (numbering according to the transcription start site). These fragments were cut out using internal restriction sites in combination with *Sma*I and *Hind*III sites in the PGL-3 basic vector, blunt ended using T4 DNA polymerase, and religated. Primary astrocyte cultures were transfected with full-length and deleted constructs of the rat GFAP promoter; 48 h later, cells were treated with 1 pM E₂ for 24 h in serum-free, phenol red-free medium. Luciferase activity was normalized to total cellular protein and β -galactosidase to control for transfection efficiency. Data (mean \pm SEM of three independent experiments) are expressed as a percentage of the untreated control value. *, $P < 0.05$, by ANOVA. B, The ERE₁ sequence in the A3 construct of the rat GFAP promoter was mutated by site-directed mutagenesis (see *Materials and Methods*). This mutation abolished the E₂-mediated increase in GFAP transcription. WT ERE₁, Astrocytes transfected with wild-type A3; mERE₁, astrocytes transfected with mutated A3. Luciferase activity was normalized to total cellular protein and β -galactosidase to control for transfection efficiency. Data (mean \pm SEM) are expressed as a percentage of the value in untreated cells transfected with WT or mERE A3 constructs, respectively. *, $P < 0.05$, by ANOVA. C, Labeled 32-bp DNA containing the putative ERE₁ site in the GFAP promoter was incubated with human recombinant ER α or BSA as a control. Complexes of ER α protein and DNA were resolved on a polyacrylamide gel. Unlabeled 32-bp DNA containing wild-type ERE₁ or mERE₁ was used as a competitor in the binding reaction to demonstrate that ER α binds DNA specifically. Lane 1, BSA control; lane 2, no competitor; lanes 3–6, wild-type DNA that at equal molar and higher concentrations effectively reduced the labeled ER protein/DNA complex; lanes 7–10, mERE₁. In contrast to the wild-type DNA, the mutated oligonucleotide was a much less effective competitor for binding of ER α . Competitors were used in a concentration range from 2- to 100-fold.

ically to the wild-type ERE₁-binding site in the GFAP promoter and that the 4-bp mutations in the ERE₁ reduced the binding of ER α in this competition assay.

Discussion

These data demonstrate that changes in GFAP mRNA expression in response to E₂ are the result of changes in transcription, in which GFAP intron and mRNA levels follow parallel trends. However, this coordination does not extend to GFAP protein levels. In the arcuate nucleus, GFAP immunoreactivity is maximal on estrus (4), 16 h after the peak we observed in GFAP intron mRNA. GFAP immunoreactivity is increased on the afternoon of proestrus in the hilus (8), a region in which no changes in GFAP intron or mRNA were found during the estrous cycle. These effects of estrogen on GFAP transcription are region specific, with rates being high in the hypothalamus but low in the hippocampus on proestrus, when circulating E₂ levels are highest. These results are consistent with regional differences in sex steroid regulation of GFAP levels in male rats, in which castration caused GFAP mRNA to increase in the hippocampus but decrease in the hypothalamus (15). Both E₂ and testosterone were shown to reverse this effect.

These results give further insight into the effect of estrogen on GFAP expression in response to brain lesions in the cerebral cortex and hippocampus. GFAP levels in the hippocampus are increased after deafferenting entorhinal cortex lesioning (ECL) (16, 17). Castration also increased GFAP mRNA, and this effect is additive with the effect of ECL and is reversed by the administration of E₂ or testosterone (15, 18). Moreover, after a brain stab wound, gonadal steroids (E₂, progesterone, or testosterone) decrease GFAP immunoreactivity at 72 h postlesion up to 2 mm away from the wound (19). E₂ also increases reactive synaptogenesis in response to ECL (20, 21). Because inhibition of GFAP expression supports dendritic outgrowth (22), we suggest that decreased GFAP expression may be one mechanism by which E₂ increases reactive synaptogenesis to the dentate gyrus in response to ECL. How these diverse steroids similarly enhance responses to brain lesions is very puzzling.

These data also give insight into the mechanism of neuroendocrine control of the LH surge. Because synaptic retraction in the arcuate nucleus precedes the LH surge, and because blocking the synaptic retraction on proestrus also blocks the LH surge (2), astrocyte process reorganization in the arcuate nucleus could be an early trigger of the cascade that leads to the LH surge. Estrogenic control of the LH surge, and thus ovulation, would be mediated by astrocytes through GFAP transcription as a primary locus of control.

The region-dependent effect of estrogen on GFAP expression parallels the distribution of ER α and ER β . In the arcuate nucleus, where estrogen induces GFAP expression, ER α is the prominent ER (23), whereas in the hippocampus, where estrogen inhibits GFAP expression, ER β is predominant (24). The number of astrocytes expressing these receptors in these regions, however, is not known, and it is not clear which nuclear ER mediates the E₂ responses observed *in vivo* or *in vitro*.

E₂ regulation of GFAP transcription *in vitro*

The induction of GFAP transcription by E₂ is consistent with the presence of two palindromic, ERE sequences in the upstream region of the rat GFAP promoter: ERE₁ (at -149 bp) and ERE₂ (at -1830 bp; Fig. 4A). These EREs differ in 1–2 bases from the perfect palindrome consensus ERE in the vitellogenin promoter of frog and chick (14).

We demonstrated the functional significance of ERE₁ (at -149) in the rat GFAP promoter. A 4-bp mutation in ERE₁ abolished the E₂-mediated increase in GFAP expression. We also showed that human recombinant ER α protein binds to the wild-type ERE₁-binding site in the GFAP promoter and that the 4-bp mutation in this putative binding site reduces the binding of ER α by more than 10-fold. The ERE in the near upstream region of the human GFAP promoter (at -150 bp) was also cited in an abstract as functional on the basis of site-directed mutagenesis and ER α gel shift assays (25). Note that this ERE in the human GFAP promoter is identical to ERE₁ in the rat GFAP promoter (26). As mentioned previously, ER α has been localized to glial cells *in vitro* (12), but ER β may also be involved. Currently, we are examining the distribution of ER α and ER β in rat brain astrocytes.

Treatment with E₂ induced the greatest increase in GFAP mRNA at a very low physiological concentration of 1 pM, which approximates plasma E₂ levels after ovariectomy. Others also found that 1 pM E₂ is sufficient to induce elaboration of astrocytic processes (27). The concentration of E₂ in the microenvironment of astrocytes is not known; however, because E₂ levels in CSF are less than 1/10th of blood levels (28), it is likely that E₂ levels within the brain are lower than those in the periphery.

Cell-cell interactions and transcriptional inversion

Cell-cell interactions are involved in the regulation of GFAP transcription by estrogen. Monotypic astrocytes responded to E₂ by induction of both mRNA levels and rates of transcription as assayed by exogenous rat GFAP promoter activity after transient transfection. In contrast, coculture of astrocytes with neurons reversed these responses to inhibition. Similarly, the transcriptional response of GFAP to glucocorticoids was inverted from positive to negative by the presence of neurons in cocultures (9), the latter of which was the direction of *in vivo* glucocorticoid-induced responses throughout the brain (29). The ability of neurons to alter the estrogen response in astrocytes is also region dependent. Hypothalamic glial cells in culture responded to E₂ treatment with increases in GFAP-immunoreactive processes when cocultured with hypothalamic neurons, but not when in monotypic culture. However, coculture with cerebellar neurons failed to change the estrogen response of hypothalamic astrocytes from that of monotypic cultures (30).

In vivo, GFAP transcription is subject to both negative and positive physiological influences by hormones and cytokines (this report and Ref. 26). The ability of neurons *in vitro* to switch the direction of E₂- and glucocorticoid-mediated GFAP expression suggests that local changes in neuronal activities and/or astrocyte-neuron interactions *in vivo* could accomplish the same result. Ongoing studies in the lab implicate a tetra-phorbolacetate response element sequence

(AP-1-binding site) in the transcriptional inversion induced by both E₂ and glucocorticoids (Rozovsky, I., and C. E. Finch, in preparation). These results are not surprising in light of recent findings of differential transcriptional activation by the ER α and ER β at AP-1 sites (31), in which ER α activates transcription and ER β inhibits transcription. The effect of induced mutation of the AP-1 site in the GFAP promoter on GFAP expression is currently under investigation in this lab.

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