

The *gad2* Promoter Is a Transcriptional Target of Estrogen Receptor (ER) α and ER β : A Unifying Hypothesis to Explain Diverse Effects of Estradiol

Edward D. Hudgens, Lan Ji, Clifford D. Carpenter, and Sandra L. Petersen

Department of Biology, University of Massachusetts Amherst, Amherst, Massachusetts 01003

Estradiol (E_2) regulates a wide range of neural functions, many of which require activation of estrogen receptor α (ER α) and/or ER β , ligand-gated transcriptional regulators. Surprisingly, very few neural gene targets of ERs have been identified, and these cannot easily explain the myriad effects of E_2 . GABA regulates most of the same neural functions as E_2 , and GABAergic neurons throughout the brain contain ER. Therefore, we examined whether E_2 directly regulates expression of glutamic acid decarboxylase 2 (*gad2*), the enzyme primarily responsible for GABA synthesis for synaptic release. Using dual luciferase assays, we found that E_2 , but not other gonadal steroids, stimulated the activity of a 2691 bp rat *gad2* promoter reporter construct. Activation required either ER α or ER β , and ER β did not repress ER α -mediated transactivation. Site-directed mutagenesis studies identified three estrogen response elements (EREs) with cell-specific functions. An ERE at -711 upstream of the *gad2* translational start site was essential for transactivation in both MCF-7 breast cancer cells and SN56.B5.G4 neural cells, but an ERE at -546 enhanced transcription only in neural cells. A third ERE at -1958 was inactive in neural cells but exerted potent transcriptional repression in E_2 -treated MCF-7 cells. Chromatin immunoprecipitation assays in mouse GABAergic N42 cells confirmed that E_2 induced ER α binding to a DNA fragment containing sequences corresponding to the -546 and -711 EREs of the rat promoter. Based on these data, we propose that direct transcriptional regulation of *gad2* may explain, at least in part, the ability of E_2 to impact such a diverse array of neural functions.

Introduction

Estradiol (E_2) is best known as a regulator of the female reproductive tract and breast tissue, but it also affects neural functions. Some functions are directly related to reproduction (Mong and Pfaff, 2003; Petersen et al., 2003), but E_2 also modulates autonomic responses (Du et al., 1995), sensory processing (Papka and Mowa, 2003), arousal (Morgan et al., 2004), spatial learning (Fugger et al., 1998), and mood and cognition (Miller, 2003; Shively and Bethea, 2004). Many of these E_2 effects depend on activation of estrogen receptor α (ER α) or ER β , ligand-gated transcription factors, but few neural transcriptional targets have been identified. Therefore, our ability to use genetic tools to understand the etiology and pathology of neurological disease, particularly in women, is limited.

Evidence suggests that genes related to GABA synthesis may be E_2 targets in the nervous system. GABA is the most abundant inhibitory neurotransmitter in the brain, and it impacts most neural functions. In addition, GABAergic neurons throughout the brain contain ER α and/or ER β (Flügge et al., 1986; Herbison

et al., 1993; Blurton-Jones and Tuszyński, 2002; Ottem et al., 2004). Therefore, it is not surprising that E_2 increases GABA release (Elkind-Hirsch et al., 1992; Fabre-Nys et al., 1994; Jarry et al., 1995; Luine et al., 1999; Mitsushima et al., 2002; Saleh and Connell, 2003), possibly by regulating glutamic acid decarboxylase (GAD) levels.

There are two GAD isoforms, GAD65 (encoded by the *gad2* gene) and GAD67 (encoded by *gad1*) (Erlander et al., 1991). Both convert glutamate to GABA (Martin et al., 1991), but GAD65 is the isoform thought to regulate synaptic release of GABA (Kaufman et al., 1991; Martin and Rimmvall, 1993). The other isoform, GAD67, is distributed throughout the neuron (Kaufman et al., 1991) and may support nonvesicular GABA release (Soghomonian and Martin, 1998). Importantly, E_2 consistently elevates GAD65 (Weiland, 1992; McCarthy et al., 1995; Bosma et al., 2001; Curran-Rauhut and Petersen, 2002), but not GAD67 mRNA levels. In addition, the rat *gad1* proximal promoter region contains no likely estrogen response elements (EREs) (Pedersen et al., 2001), whereas the *gad2* promoter region has three putative EREs (Skak and Michelsen, 1999). It is unclear whether ERE sequences found in the *gad2* promoter region are functional or what roles ER α or ER β play in *gad2* gene expression.

To address these issues, we examined the effects of E_2 and other steroids on *gad2* proximal promoter activity. We also compared the abilities of ER α and ER β , alone or in combination, to mediate E_2 induction of *gad2* promoter activity. Finally, we used site-directed mutagenesis and chromatin immunoprecipitation (ChIP) to evaluate putative ERE sequences in the *gad2* promoter.

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Correspondence should be addressed to Dr. Sandra L. Petersen, Department of Biology, 611 North Pleasant Street, University of Massachusetts Amherst, Amherst, MA 01002. E-mail: sandyp@bio.umass.edu.

E. D. Hudgens's present address: Department of Veterinary and Animal Sciences, University of Massachusetts Amherst, Amherst, MA 01003.

C. D. Carpenter's present address: Department of Pathology, Northwestern University, Chicago, IL 60611.

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Figure 1. Diagram of relative locations of three putative EREs in rat *gad2* promoter sequence used in these studies. Site locations are mapped based on the location of the translational start site (Trl).

Materials and Methods

General methods

Cell lines and culturing methods. All cells were maintained at 37°C and 5% CO₂ in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (PS-Gln; Invitrogen, BRL). The medium for MCF-7 breast cancer cells (American Type Culture Collection) was further supplemented with 1 mg/ml insulin. Medium for SN56.B5.G4 cells derived from septal neurons (provided by Dr. Jan Krysztof Blusztajn, Boston University, Boston, MA) and N42 immortalized hypothalamic neurons (Cellutions Biosystems) was supplemented with 1 mM sodium pyruvate. COS-7 green monkey kidney cells (American Type Culture Collection) received no media supplements. In all cases, medium was changed every 48 h.

To verify that SN56.B5.G4 cells lose E₂ responsiveness after the tenth passage (Watters and Dorsa, 1998), we examined E₂ effects on the activity of a pGL3 ERE–Luc reporter vector (provided by Sallie Smith Schneider, University of Massachusetts Amherst, Amherst, MA) transiently transfected into SN56.B5.G4 cells of a passage number >20. We also used PCR and Western blots to verify ER status and E₂ responsiveness of MCF-7 and N42 cells.

***gad2* promoter construct.** We used reverse transcriptase-PCR and rat hypothalamic DNA to generate previously described promoter regions of the rat *gad2* gene (accession #AF090195; bases 18–2708) (Skak and Michelsen, 1999). The forward primer was 5′-CCGGTACCGAGATTTCTGGGTGGCGTGAA, and the reverse primer was 5′-TAGTCTGGCGCTGGTCGC. The resulting 2691 bp fragment of the proximal *gad2* promoter region was cloned into the pGL3 Basic vector (Promega).

Site-directed mutagenesis. To determine the sites through which ER α and ER β activate *gad2* promoter activity, we performed site-directed mutagenesis. We searched the sequences 3000 bp upstream from the transcriptional start site for known EREs or ERE half-sites and then confirmed our results using the online tools Dragon ERE (Bajic et al., 2003) and Match (Biobase Biological Databases) (Goessling et al., 2001). We found putative ERE sequences at positions 776, 2023, and 2190 [National Center for Biotechnology Information (NCBI) accession #AF090195]. The *gad2* promoter contains several transcriptional start sites (Skak and Michelsen, 1999), so to avoid ambiguity, we used the translational start site as the reference position (+1). Thus, the putative EREs were at –1958, –711, and –546 upstream from the translational start site (Fig. 1). We designed a series of oligonucleotide primers to specifically disrupt putative EREs at each of these positions. Mutations were as follows (mutations underlined): AGTTAAGGCGTAGGGATC-CGATCTCTACCTTCCCTCACTGC (for the –1958 site), CCTGGGCTCTTGAGGATCCAGAGATCTCCGCACGGGTTGG (–711 site), and GTGCCAGCTGCACCGAGGCCAGC (–546 site).

Each mutation introduced a new restriction site to aid in identification. Reagents and protocols from the GeneEditor *in vitro* Site-Directed Mutagenesis Kit (Promega) were used in conjunction with these oligonucleotides to introduce ERE mutations in the pGL3/*gad2* promoter construct. This was accomplished by annealing kinased mutagenic oligonucleotides to the plasmid along with a kit-supplied oligonucleotide designed to alter antibiotic resistance. After mutant strand synthesis (using T4 DNA polymerase) and ligation, resultant plasmids were introduced into *Escherichia coli* and transformants selected using the novel antibiotic resistance. Further screening involved restriction analysis to assay for the mutant sites.

Transient transfections. Using Superfect reagent (Qiagen), and following the manufacturer's protocols, we transiently transfected MCF-7, COS-7, and SN56.B5.G4 cells with pGL3 Basic vectors containing vari-

ous *gad2* promoter constructs. Twenty-four hours before transfection, cells were plated to produce a density of ~80% confluence. A ratio of 5 μ g pGL3 vector with promoter construct to 0.16 μ g cytomegalovirus (CMV) renilla luciferase internal control plasmid (pRL–CMV; Superfect) was optimal for transfecting MCF-7 and COS-7 cells grown in 48-well plates. For SN56.B5.G4 cells, the optimal ratio was 0.68 μ g reporter construct to 0.04 μ g pRL–CMV plasmid. Two to three hours later, cells were rinsed with PBS and transferred to phenol red-free DMEM with 10% FBS (charcoal-stripped) and PS-Gln. Transfected cells were incubated for 18–24 h before receiving steroid treatments as described below. All transfections and assays were performed in triplicate and replicated at least three times.

Dual-luciferase assays. Activity of the *gad2* promoter reporter constructs was determined in lysates and analyzed using the Dual Luciferase Assay kit (Promega) and a TD 20/20 luminometer (Turner Designs), according to manufacturer's instructions.

Specific studies

Steroid regulation of *gad2* promoter activity. We performed initial studies of steroid effects on *gad2* promoter activity in MCF-7 breast cancer cells. These cells contain androgen receptors (Andò et al., 2002), progesterin receptors (Horwitz et al., 1978), and ER α but little or no ER β (Chang et al., 2008). We performed a dose–response study of E₂ effects on *gad2* promoter activity using vehicle (ethanol), 0.01, 0.1, 1.0, and 10 nM E₂ and lysing cells 24 h after steroid addition. We then performed a time course study wherein we collected lysates at 0, 4, 8, 12, 16, 24, and 48 h after treatment with 10 nM E₂. Next, we compared effects of E₂ on *gad2* promoter activity with those of other gonadal steroids using 10 nM E₂, testosterone (T), progesterone (P₄), or combined E₂ and P₄. Similar studies were done in COS-7 cells as negative controls because these cells have no steroid receptors. MCF-7 cells containing the *gad2* promoter construct were treated also with the pure antiestrogen, ICI 162,780 (100 nM) or vehicle. Six hours later, 10 nM E₂ or vehicle was added, and cultures were incubated 42 h before lysates were collected.

ER α and ER β regulation of *gad2* promoter activity. The *gad2* gene is most commonly expressed in the nervous system; therefore, we followed up positive findings from studies of MCF-7 cells with studies in a mouse neural cell line, SN56.B5.G4 cells (described above). E₂ did not activate a pGL3 ERE–Luc reporter construct in these cells without cotransfection with ER expression vectors. This allowed us to use SN56.B5.G4 cells to assess the roles of ER α and ER β in *gad2* promoter regulation. We performed a series of dose–response (1 nM through 100 nM) studies of *gad2* promoter activity in cells cotransfected with pRST7–hER α and/or pRST7–hER β expression vectors (provided by Julie Hall, Duke University Medical Center, Durham, NC).

Identification of functional EREs in *gad2* promoter. In MCF-7 cells, we compared the effects of 10 nM E₂ on the reporter activity of wild-type and mutated *gad2* promoter sequences prepared as described above. Mutations were at putative EREs found at –1958, –711, and –546 upstream from the translational start site. We performed similar studies in SN56.B5.G4 cells cotransfected with pRST7–hER α and/or pRST7–hER β expression vectors.

We also investigated whether the three ERE sequences in the rat *gad2* promoter were found in the *gad2* promoters of other animal species for which there was data in the NCBI database. For these studies, we used BLAST (NCBI) (Altschul et al., 1990) to find the ERE sequences, then aligned identified ERE sequences with the rat promoter using CLUSTALW (1.81) (<http://align.genome.jp>) (Larkin et al., 2007).

ChIP assays. To determine whether ER α binds to the ERE identified as a binding target in mutagenesis studies, we used N42 hypothalamic cells after verifying with PCR that they contain endogenous GAD2 mRNA (our unpublished observations). We focused on ER α binding to verify the importance of the ERE, because at the time of the studies, we were unable to find appropriate ER β antibodies validated for ChIP, a problem described previously (Weitsman et al., 2006). Cells were plated at 1.90 \times 10⁶ density in 10 cm tissue culture dishes in DMEM without phenol red and supplemented with PS-Gln and 10% FBS overnight at 37°C with 5% CO₂. The following day, N42 cells were treated with 10 nM E₂ or vehicle and incubated for 2 h, after which they were treated with 1% formalde-

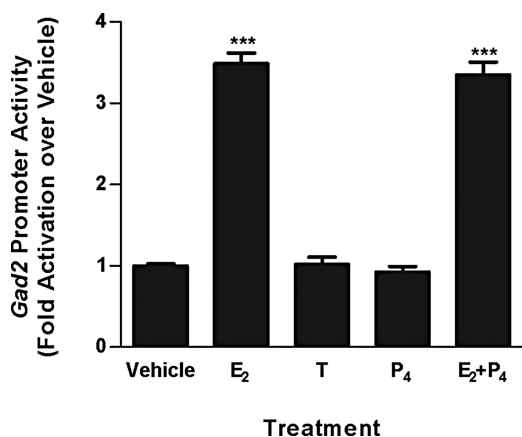


Figure 2. Effects of 10 nM E₂, T, P₄, or E₂ plus P₄ (E₂ pretreatment followed 6 h later with P₄) on rat *gad2* promoter activity. *gad2* promoter reporter construct was transiently transfected into MCF-7 cells, and cells were treated for 24 h with steroids after 18 h incubation in steroid-free media. Reporter activity was measured using a dual-luciferase assay with normalization to a renilla luciferase control. Each bar represents treatment mean \pm SEM of at least nine samples. ***Significantly different from vehicle; $p < 0.0001$.

hyde in PBS for 10 min at room temperature on a platform shaker. The crosslinking reaction was stopped by adding glycine at a final concentration of 125 mM. Cells were rinsed twice with cold PBS before harvesting then resuspended in cell lysis buffer (5 mM Tris-HCl, pH 8.0, and 85 mM KCl) with 0.5% NP-40 and Protease Inhibitor Cocktail III (Calbiochem). After a 10 min incubation on ice, we collected nuclei by centrifugation and resuspended them in nuclear lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS and Protease Inhibitor Cocktail III). Cell lysates were incubated on ice for 10 min then sonicated seven times for 10 s at 15% amplitude (Branson Ultrasonic Corporation) followed by centrifugation at 4°C. Sonicated cell lysate (30 μ l) was used to verify that the average size of DNA fragments was in the range of 100–500 bp. DNA/protein complex (4 μ g) was diluted in 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, and 167 mM NaCl to a final volume of 400 μ l. The samples were next precleared with 40 μ l 50% salmon sperm DNA/protein G bead slurry (Millipore Bioscience Research Reagents) for 30 min at 4°C. Supernatant (30 μ l) was saved for total input control after preclearing. Immunoprecipitation was performed by adding 1 μ g ER α antibody (Abcam) and then incubating at 4°C overnight with rotation. Samples with no antibody were run in parallel as negative controls.

The following day, 40 μ l 50% salmon sperm DNA/protein G beads slurry was added and incubated for 2 h at 4°C to capture the immune complexes. Proteins and DNA nonspecifically associated with the beads were removed by sequentially washing with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), and LiCl washing buffer (0.25 M LiCl; 1% deoxycholate sodium salt, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 1% IGEPAL-CA630; MP Biomedicals), and twice with Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 4°C for 12 min each wash. The immunoprecipitated DNA–protein complexes were eluted from beads with 1% SDS in 0.1 M NaHCO₃. Then the DNA–protein complexes were incubated in 0.2 M NaCl at 65°C overnight followed by proteinase K digestion for 2 h at 45°C to reverse cross linking. After protein removal, DNA was purified by phenol/chloroform extraction followed by EtOH precipitation.

Quantitative PCR analysis of ChIP assays. To quantify ChIP DNA, we used a MX3500P real-time quantitative PCR (QPCR) system (Stratagene) and designed to flank the putative ERE of the mouse *gad2* promoter (accession #AB032757) at –693 that align with the rat –711 ERE. The primers were 5'-ATGAGTTCGTTGGTGTGGAAG (forward) and 5'-AGTGCTGAGGTCGCTGTG (reverse). A second set of primers was designed to flank the putative ERE at –532 of the mouse promoter that aligns with the rat –546 ERE. The sequences of those primers were

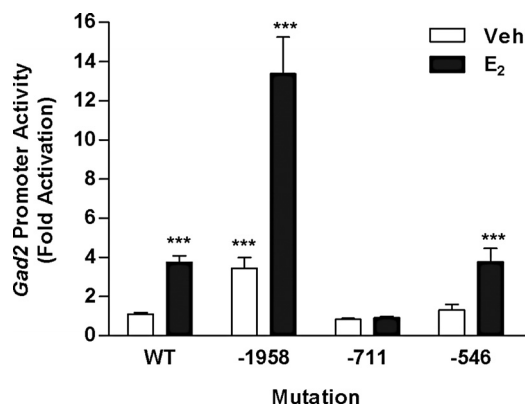


Figure 3. E₂ activation of *gad2* wild-type (WT) promoter and promoter constructs with mutated EREs. Activation was assessed using dual-luciferase assays. Designation of constructs is based on the positions of the mutated ERE upstream of the translational start site (see Materials and Methods). Constructs were transiently transfected into MCF-7 cells and treated with ethanol vehicle (Veh) or 10 nM E₂ for 24 h. Values are expressed relative to the wild-type construct in cells treated with vehicle. Each bar represents the mean \pm SEM of values from at least nine independent samples. ***Significantly different from WT, vehicle control ($p < 0.001$).

5'-GCCAAGGTCTCCTGTCC (forward) and 5'-TGCCTGCGTAGTGTGAC. In addition, negative control primers were designed for a part of the coding region of the mouse *gad2* gene devoid of an identifiable ERE (accession #NM_008078, bases 2402–2610). The sequences of these primers were 5'-TCAAAACCAACAGGAAACATCA (forward) and 5'-TTCAAGGGTGATTTGGCACT (reverse).

Each 15 μ l DNA amplification reaction contained 5 mM Tris HCl, 5 mM Tris-base, 50 mM KCl, 3 mM MgCl₂, 0.01% Tween 20, 0.8% glycerol, 40,000-fold dilution of SYBR Green I (Invitrogen), 200 μ M dNTPs, 50 mM ROX reference dye (Stratagene), 5 pmol of each primer, 2 μ l of DNA, and 1.0 U Platinum TaqDNA polymerase (Invitrogen). The QPCR conditions were 95°C (5 min), followed by 40 cycles of 95°C (15 s), 55–67°C (30 s) for annealing and 72°C (30 s) for extension. We included one sample without cDNA template to establish the specificity of target cDNA amplification, and we verified identity of products by electrophoresis on 2% agarose gels and sequence confirmation. In these studies, we ran three separate ChIP reactions in duplicate for each of the three targets (negative control, –693 and –532), as well as a sample with no antibody and one for total input for each target.

Statistical analyses. We first obtained means for each treatment from at least three replicates of three or more independent runs for each promoter reporter assay. Treatment effects were evaluated using one-way ANOVA or two-way ANOVA, depending on the number of independent variables. We used Bonferroni *t* tests for *post hoc* analyses to probe treatment effects when a significant interaction of main effects was detected by two-way ANOVA.

We analyzed QPCR data for ChIP studies using the $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001). For these analyses, duplicates for each sample were averaged and corrected for background by subtracting values of the no antibody controls. Fold enrichment was determined for each sample by comparing with total input controls. To facilitate comparisons, data were expressed as fold induction over the vehicle controls. Finally, means of the vehicle group and E₂-treated group for each DNA target were compared using Student's *t* tests.

Results

E₂, but not other gonadal steroids, stimulated *gad2* promoter activity *in vitro*

MCF-7 cells

E₂, but not T or P₄, increased *gad2* promoter reporter activity when the reporter construct was expressed in MCF-7 cells (Fig. 2). Results of dose–response studies showed that E₂ increased *gad2* promoter activity in a dose-dependent manner, reaching saturation at 10^{–8} M (data not shown). Time course studies

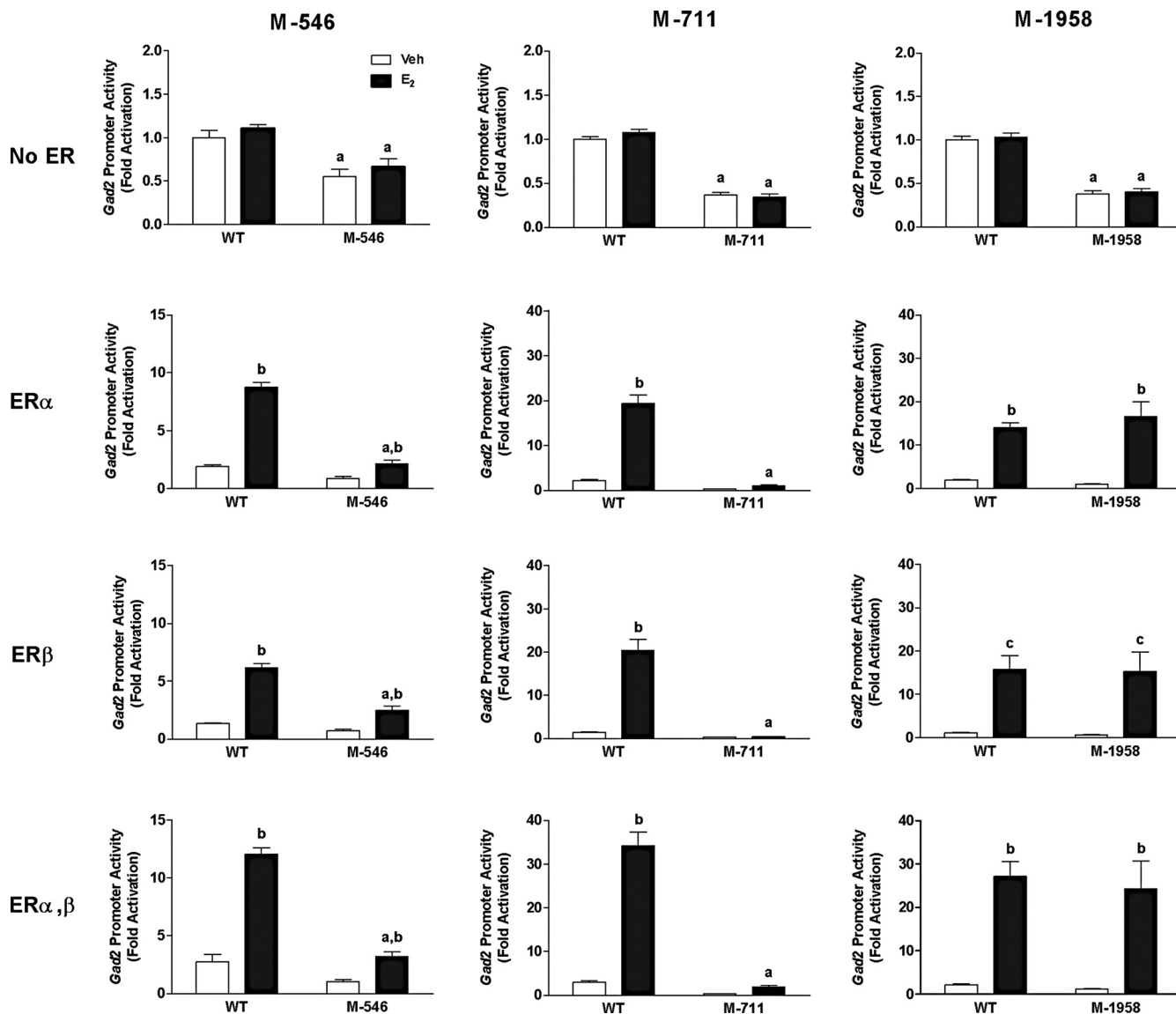


Figure 4. Effects of E₂ on reporter activity of a wild-type (WT) *gad2* promoter construct or constructs with mutations of ERE sequences at -549 , -711 , and -1958 upstream of the *gad2* translational start site (M-549, left column; M-711, middle column; and -1958 , right column; for details, see Materials and Methods). Constructs were expressed in SN56.B5.G4 neural cells that were transiently transfected with empty vector (No ER, top row), hER α (second row), hER β (third row), or both hER α and hER β (ER $\alpha\beta$, bottom row). Cells were treated with 10 nM E₂ or ethanol vehicle (Veh) for 24 h before assaying reporter activity using dual-luciferase assays. The induction of promoter activity was expressed as fold activation compared with vehicle control. Each bar represents the mean \pm SEM of values from at least nine independent samples. ^aSignificantly different from corresponding WT ($p < 0.001$); ^bsignificantly different from corresponding Veh control ($p < 0.001$); ^csignificantly different from corresponding Veh control ($p < 0.01$).

showed that promoter activity peaked 12 h after initiation of E₂ treatment and remained elevated for at least 48 h (data not shown). The stimulatory effect of E₂ on *gad2* promoter activity was blocked by pretreatment with the ER antagonist, ICI 182,780 (data not shown).

SN56.B5.G4 cells

As reported previously (Watters and Dorsa, 1998), SN56.B5.G4 cells were unresponsive to E₂ unless transfected with ER expression vectors. E₂ had no effect on reporter activity in the absence of ER, and the stimulatory effect of E₂ was completely blocked by pretreatment with ICI 182,780 (data not shown). In log dose-response studies (10^{-12} to 10^{-7} M; data not shown), we found that E₂-induced *gad2* promoter reporter activity peaked at 10^{-8} M, regardless of whether cells were transfected with hER α , hER β , or both expression vectors; however, activity was generally higher when both receptors were present.

EREs are important for cell-specific regulation of the *gad2* promoter

Mutations of putative EREs in the *gad2* promoter resulted in different responses to E₂ in MCF-7 breast cancer cells compared with SN56.B5.G4 neural cells. In MCF-7 cells, only mutation of the -711 site abrogated transactivational effects of E₂ (Fig. 3). Importantly, mutation of the -1958 ERE increased basal transcriptional levels significantly and also increased E₂ responsiveness from less than fourfold to over 13-fold.

Unlike responses seen in MCF-7 cells, we found that in SN56.B5.G4 cells, mutation of the -546 site repressed E₂ transactivation when hER α , hER β , or both expression vectors were present (Fig. 4, left column). However, mutation of the -711 site completely abolished E₂ responsiveness of the *gad2* promoter when the construct was expressed in SN56.B5.G4

Table 1. Structures of imperfect EREs found in the rat *gad2* DNA sequence between +1 and –3000 bp upstream of the translational start site

		–5	–4	–3	–2	–1	N	N	N	+1	+2	+3	+4	+5
–546	5'	G	G	T	C	A	C	C	G	A	G	G	C	C
–711		G	G	T	C	A	C	A	G	C	G	A	C	C
–1958		G	G	T	C	A	A	G	A	T	G	A	C	T
Vitellogenin A2		G	G	T	C	A	C	A	G	T	G	A	C	C
Consensus ERE		G	G	T	C	A	N	N	N	T	G	A	C	C

The first column denotes the site of the ERE in the promoter region. The consensus and vitellogenin A2 ERE sequences are shown in the last two rows for comparison. Bold indicates differences between *gad2* and consensus EREs. N, Any nucleotide.

cells (Fig. 4, middle column), as it did when tested in MCF-7 cells (Fig. 3). Unlike the dramatic stimulatory effect seen in MCF-7 cells when the –1958 site was mutated in MCF7 cells, the same mutation of the *gad2* promoter had no effect on E₂ transactivation in SN56.B5.G4 neural cells (Fig. 4, right column). Finally, in contrast to the responses observed in MCF-7 cells, basal promoter activity (no ER) decreased in SN56.B5.G4 cells when any of the three EREs was mutated (Fig. 4, top row).

Rat *gad2*–711 ERE is similar to the consensus ERE and aligns with similar sites in several species

The structures of the various imperfect EREs found in the *gad2* promoter, as well as the vitellogenin and consensus EREs, are shown in Table 1. Database analysis of the rat *gad2* promoter (AF090195) showed that the sequence of the ERE at –711 differs from the consensus ERE at the +1 position, the –546 site differs at the +3 position, and the –1958 site at the –5 position.

Alignment of rat, mouse (*Rattus norvegicus*, *Mus musculus*; AB032757), dog (*Canis lupus familiaris*; NW_876290), horse (*Equus caballus*; NW_001867401), rhesus monkey (*Macaca mulatta*; NW_001124107), chimpanzee (*Pan troglodytes*; NW_001220649), and human (*Homo sapiens*; NT_008705) *gad2* promoter regions are shown in Figure 5. Numbering of sites is relative to the known translational start sites for rat, mouse, and human and to predicted translational start sites for dog, horse, rhesus monkey, and chimpanzee. The site we found most important for E₂ induction of *gad2* promoter activity, the rat –711 site, is identical with sites at –693 in mouse, –752 in dog, and –730 in horse. A site at –713 of the rhesus monkey differs from the rat sequence by one base (A to G at –1 position). Sites at –702 of the chimpanzee and –708 in human were identical to one another and differed from the rat sequence by two bases (A to G at –1; C to T at +1). The reverse palindrome sequences of the rat –546 and –1958 EREs are similar to sites at –532 and –1838 of the mouse *gad2* promoter, and each differs by only one base in the intervening 3-base sequence. Sequences of dog and horse that align with the rat –546 site may be ERE half-sites, but the sites in monkey, chimpanzee, and human do not appear to be either EREs or ERE half-sites. BLAST analysis showed that the region containing the rat –1958 ERE was not sufficiently homologous to nonrodent species to allow sequence alignment.



Figure 5. Promoter sequence alignment for *gad2* promoters of rat (AF090195), mouse (AB032757), dog (NW_876290), horse (NW_001867401), rhesus monkey (NW_001124107), chimpanzee (NW_001220649), and human (NT_008705AD2) using CLUSTALW (1.81) multiple alignment software. Designations of –1958, –711, and –546 refer to positions of putative EREs of rat *GAD2* promoter relative to the translational start site. Shading of bases denotes identity with bases of the rat sequence, and asterisks indicate sites conserved among all species compared. BLAST analysis found insufficient homology to allow alignment between rodent and nonrodent species in the region containing the rat –1958 site.

E₂ increases ERα binding to DNA fragments containing ERE sequences

Results of ChIP assays using ERα antibody showed that fragments containing the –532 and –693 ERE sequences were significantly enriched in DNA samples from E₂-treated N42 cells compared with DNA from vehicle-treated cells (Fig. 6). No enrichment was seen using negative control primers.

Discussion

Our findings show that the *gad2* promoter is a direct target of E₂ and activated through the canonical pathway by ERα and ERβ. We identified three novel EREs within the first 3000 bp upstream of the translational start site of the *gad2* promoter. Mutational and ChIP analyses showed that two of these sites were important for promoter activation by E₂ in neural cells. Importantly, a third ERE was a strong repressor of E₂ transactivation in breast cancer cells. Although either ERα or ERβ could mediate transactivation of the *gad2* promoter, we found no evidence for ERβ repression of ERα-mediated transcription. Together, these findings show that the *gad2* gene is a direct transcriptional target of E₂ regulated, at least in part, by the canonical ER pathway in a tissue-specific manner. In view of the widespread colocalization of ER and GAD2, our findings may explain, at least in part, the effects of E₂ on diverse neural functions.

Consistent with our *in vitro* findings, previous studies showed that E₂ increases GAD2 mRNA levels *in vivo*, specifically in neu-

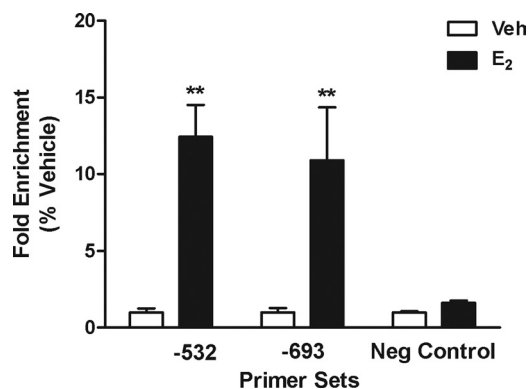


Figure 6. Results of ChIP using ER α antibody and DNA isolated from N42 cells. Cells were treated with ethanol vehicle (Veh) or E₂ for 2 h before they were harvested for ChIP assays. Samples were assayed using primers flanking the -532 ERE and the -693 ERE or a site with no ERE consensus sequences (for primer sequences, see Materials and Methods). Each data point represents the mean \pm SEM of three independent samples, each analyzed in duplicate. **Significantly different from corresponding vehicle control; $p < 0.01$. Neg, Negative.

ronal populations that contain ER. For example, in the rat CA1, an ER-containing region of the hippocampus, GAD2 mRNA levels decline after ovariectomy and then increase after E₂ replacement (Nakamura et al., 2004). Another intriguing report suggests that estrogen synthesized locally also upregulates *gad2* gene expression in the hippocampus (Ikeda et al., 2006). Similarly, in the rat anteroventral periventricular nucleus (AVPV), nearly all GABAergic neurons contain ER α mRNA and many also contain ER β mRNA (Ottem et al., 2004). E₂ consistently elevates GAD2 mRNA levels in the ER-rich AVPV but not in a more rostral preoptic region where ER expression is found only in scattered GABAergic cells (Curran-Rauhut and Petersen, 2002). These *in vivo* findings support our contention that the rat *gad2* promoter region is a direct target of ERs.

Results of our mutational and ChIP analyses indicate that E₂ regulates the *gad2* promoter through the classical pathway wherein ER binds to ERE sequences (Matthews and Gustafsson, 2003; Dietz and Carroll, 2008), although nonclassical mechanisms also may be involved in the expression of the gene *in vivo*. The ERE most important for regulating *gad2* promoter activity was -711 upstream of the translational start site of the rat promoter and identical to ones in the mouse, as well as the predicted promoters of dog and horse. Alignment studies also detected a similar site in the predicted *gad2* promoters of rhesus monkey, chimpanzee, and in the human promoter. The ERE in the rhesus monkey promoter differed from the rat sequence by one base and the sites in chimpanzee and human by two bases. Notably, differences among species were at either the -1 or $+1$ position. We detected no likely ERE sequences that aligned with either the -546 or -1958 sites in any of the nonrodent animals. These findings provide further evidence that the highly conserved site at -711 of the rat is critical to E₂ regulation of the *gad2* promoter.

The other ERE active in the rat *gad2* promoter was at -546 and identical to an ERE at -532 in the mouse gene. ChIP assays in mouse N42 cells showed E₂ induction of ER α binding to DNA fragments quantified using QPCR and primers flanking either the -532 or the more highly conserved -693 ERE. Considering the proximity of these sequences and the size of fragments used in the ChIP assays, it is not possible to determine whether binding differs between the -693 and -532 sites. However, EREs separated by >2000 base pairs can synergistically activate a promoter (Sathya et al., 1997), so both sites may contribute to activation of the native rodent *gad2* promoter.

Despite the extensive colocalization of ER α and ER β , little is known about the relative contributions of the two isoforms in regulating neural gene expression. Some have suggested that ER β acts primarily as a dominant repressor of ER α transactivation (Pettersson et al., 2000; Liu et al., 2002), particularly when E₂ levels are not saturating (Hall and McDonnell, 1999). In contrast, we found that ER β stimulated, rather than repressed, *gad2* gene expression. Consistent with our findings, microarray studies from other laboratories identified genes activated solely by ER β and others in which ER β enhances ER α -induced expression (Monroe et al., 2005; Chang et al., 2006). These data support our findings that ER β can potentially stimulate gene transcription independent of ER α .

E₂ induction of *gad2* promoter activity was significantly lower in MCF-7 breast cancer cells than in SN56.B5.G4 neural cells. MCF-7 cells lack ER β , but the differences persist even when we compare MCF-7 cells with SN56.B5.G4 cells lacking ER β . It may be that the tissue-specific responses are attributable to differential utilization of EREs or to the complement of ER α in the two cell lines. Interestingly, the ERE at -1958 was inactive in SN56.B5.G4 cells but served as a potent repressor in MCF-7 cells. In fact, after mutating the -1958 site, E₂ stimulated *gad2* promoter activity in MCF-7 cells to nearly the same extent as wild-type promoter activation in SN56.B5.G4 neural cells. Future studies will evaluate possible mechanisms underlying the ability of the -1958 site to repress *gad2* gene transcription in MCF-7 cells.

E₂ alters expression of many neural genes, but few are known to be regulated directly through EREs in the promoter region. Until recently, it was assumed that such genes had not yet been identified, but this view is changing, as investigators interrogate the entire genome for ER binding sites. Researchers using whole-genome ChIP-based strategies made the surprising discovery that only 4% of ER α binding sites map to the proximal promoter region 0–1 kb upstream of the transcriptional start site (Carroll et al., 2006) or 5% to the 0–5 kb upstream region (Lin et al., 2007). Interestingly, EREs adjacent to E₂-upregulated, but not E₂-downregulated genes, are enriched in the proximal promoter region, particularly around the transcriptional start site (Lin et al., 2007). If the distribution of EREs in the neural transcriptome is similar, our finding that the *gad2* proximal promoter has two stimulatory EREs would suggest that the *gad2* gene is an unusual and important neural target of E₂.

The finding that E₂ directly targeted the *gad2* gene is notable, because GAD65 maintains levels of GABA used in vesicular release (Soghomonian and Martin, 1998). Considering the ubiquitous distribution and myriad functions of GABA, regulation of *gad2* gene expression may explain how E₂ is able to regulate such a wide array of neural functions. Two regions in particular, the AVPV and bed nucleus of the stria terminalis (BNST), have among the highest concentrations of ER α - and ER β -containing neurons in the brain (Simerly et al., 1990; Shughrue et al., 1997). In the AVPV, virtually all ER expression is in GABAergic neurons (Ottem et al., 2004), and E₂ tonically elevates *gad2* gene expression in these cells (Curran-Rauhut and Petersen, 2002). In turn, tonic elevation of GABA release plays a key role in regulating E₂-dependent gonadotropin release during the estrous cycle (Herbison, 1998; Petersen et al., 2003). E₂ also regulates *gad2* gene expression (Pompolo et al., 2002) in the BNST, a nucleus important for stress responses. Disruption of *gad2* expression results in anxiety-like behavior (Stork et al., 2000), whereas E₂ attenuates stress responses (Gerrits et al., 2005). The underlying mechanism may be through E₂ activation of GABA release from BNST neurons that project to corticotrophin-releasing hormone neurons

in the medial parvocellular subdivision of the paraventricular nucleus (Miklos and Kovacs, 2002).

In addition to the AVPV and BNST, ER and GAD2 are coexpressed in neurons throughout the hypothalamus, as well as in regions of the hippocampus, amygdala, and brainstem. In these regions, GABA and E₂ modulate such diverse functions as feeding behavior (Butera et al., 1990; Meister, 2007), spatial memory (Spencer et al., 2008), emotional responses (Krezel et al., 2001), and autonomic functions (Saleh and Connell, 2003). Finally, *gad2* gene expression is found in ER-containing cells of non-neural tissues, including β -cells of the pancreas (Franklin and Wollheim, 2004; Alonso-Magdalena et al., 2008), epiphyseal chondrocytes (Nilsson et al., 1999; Tamayama et al., 2001), and oviductal epithelial cells (Tillakaratne et al., 1992; Wang et al., 2000). Thus, it is tempting to speculate that the relationship between ER and GAD2 is widespread and important both inside and outside the nervous system.

In summary, these findings show that E₂ directly stimulates *gad2* promoter activity through the canonical ER pathway. Transcriptional activation in neural cells required ER α or ER β , as well as two EREs located within the proximal promoter region. Importantly, a third ERE was inactive in neural cells but had potent repressor activity in breast cancer cell lines. Considering the co-distribution patterns of GAD2 and ERs in the brain, direct regulation of the *gad2* gene by E₂ may explain the wide range of neural functions modulated by this steroid.

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