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Glutamic Acid Decarboxylases (gad): Transcriptional Regulation by Estradiol and 2,3,7,8-Tetrachlorodibenzo-p-dioxin

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**GLUTAMIC ACID DECARBOXYLASES (GAD):
TRANSCRIPTIONAL REGULATION BY ESTRADIOL
AND 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN**

A Thesis Presented

by

EDWARD D. HUDGENS

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University of Massachusetts Amherst in partial fulfillment
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INTRODUCTION

The primary inhibitory neurotransmitter of the brain, gamma-aminobutyric acid (GABA), is a target of estrogen regulation in several of neural systems. For example, 17β -estradiol (E_2) mediates GABA signaling in the perirhinal cortex (Blurton-Jones 2006) and in the parabrachial nucleus (Saleh 2003) of adult rats. In addition, the rise in serum E_2 levels during late diestrous and early proestrous in cycling female rats is paralleled by an increase in GABA in the medial preoptic area (MPO) of the hypothalamus (Mitsushima 2002), and GABA turnover is higher in ovariectomized (OVX) rats treated with E_2 than in OVX control animals (Mansky 1982).

E_2 may exert its effects on GABA release and turnover through modulation of the rate-limiting enzymes for GABA production, GAD65 and GAD67. There is evidence to support the hypothesis that these two glutamic acid decarboxylases (GADs) are transcriptional targets of the steroid. A twenty-four hour exposure to E_2 in ten-day ovariectomized (OVX) adult rats results in an increase in the number neurons immunoreactive for GAD65 in the SP region of the hippocampus, and increases GAD65 messenger RNA levels in this region (Nakamura 2004). Conversely, the number of GAD-immunoreactive cells, and the intensity of GAD staining, decrease after a 24-hour exposure to E_2 in hippocampal cells taken from embryonic day 19 or 20 rats (Murphy 1998). In the hypothalamus, the two GAD enzymes can exhibit different responses to E_2 in different nuclei. The magnocellular preoptic area (mcPOA) responds to E_2 treatment with an increase in GAD65, but a decrease in GAD67 mRNA levels. However, GAD65 signal decreases, and GAD67 increases, in the dorsomedial nucleus

in response to E₂ (McCarthy 1995). Research in our laboratory has shown that GAD65 and GAD67 mRNA levels are differentially regulated by E₂ in the anteroventral periventricular nucleus (AVPV) of adult OVX rats. Specifically, GAD65 mRNA is tonically elevated on the day of the E₂-induced preovulatory surge in luteinizing hormone (LH), while GAD67 mRNA shows a morning rise, followed by an afternoon decrease (Curran-Rauhut 2002).

Although estrogenic regulation of the GADs may be indirect, studies demonstrating the presence of estrogen receptors (ERs) in GABAergic cells suggest that E₂ can affect GAD activity at a transcriptional level. ER α is present in GAD-positive hippocampal neurons (Hart 2001) and cultured embryonic hippocampal cells (Murphy 1998), and ER β is present in GABA-positive neocortical neurons (Blurton-Jones 2006). Colocalization of ER α and GAD also occurs in the hypothalamus of the ewe (Herbison 1993), and almost all cells positive for GAD mRNA in the AVPV also contain ER α mRNA in adult female rats (Ottem 2004).

Interestingly, GAD-positive AVPV cells have also been shown to contain the arylhydrocarbon receptor (AhR) (Hays 2002). Unliganded AhR forms a cytosolic complex with chaperone proteins (reviewed in Gu 2000). Several ligands, the most potent of which is 2,3,7,8-tetrachlorodibenzo-p-dioxin or TCDD (reviewed in Denison 2003), induce AhR translocation to the nucleus. Nuclear AhR, in association with arylhydrocarbon nuclear transporter (ARNT), can then bind to xenobiotic or dioxin response elements (XREs or DREs) in promoter sequences to initiate gene expression (Gu 2000). Our laboratory has shown that a prenatal exposure to TCDD results in decreased GAD67 mRNA levels in the rostral preoptic area (rPOA) of postnatal day 3

(PND3) female rats, and in the caudal POA of PND3 males (Hays 2002). Thus, not only are the GAD enzymes regulated by E₂, but they may also be potential targets of the environmental toxin TCDD. This could be important, as TCDD is a known endocrine disruptor (Birnbaum 1994), and can interfere with E₂ responses (Safe 1998).

Colocalization of ER and AhR with GAD suggests that E₂ and TCDD regulate the GADs through an intracellular mechanism. However it has not been determined whether these ligands induce their respective receptors to transactivate the GADs at a transcriptional level. In this study, I report that the activity of the rat GAD65 promoter is regulated by E₂ in an ER-dependent manner, and that mutation of either of two estrogen response elements (EREs) in the rat GAD65 promoter interferes with E₂ response. Additionally, I present preliminary evidence for a direct, physical interaction between ER α and the mouse GAD65 promoter. I also report that both GAD promoters respond to TCDD in the presence of AhR. Finally, I demonstrate that neither mutation nor deletion of the XREs in the GAD67 promoter blocks dioxin responsiveness.

CHAPTER 1

MATERIALS AND METHODS

Plasmids and Vectors

The estrogen receptor reporter vectors pRST7-ER α and pRST7-ER β were generously supplied by Dr. Julie Hall. The expression vectors pcDNA3- β AhR and pcDNA3- β ARNT were provided by Dr. Thomas Gasciewicz. The remaining vectors were generated in our laboratory.

GAD Promoter Fragments

The GAD65 promoter-reporter vector pGL3-GAD65 had been generated in our laboratory previously by Dr. Clifford Carpenter, but the protocol he used is described here. The GAD65 promoter fragment used to generate this vector was amplified from rat hypothalamus genomic DNA via polymerase chain reaction (PCR) using the sense primer 5'-CCGGTACCGAGATTTCTGGGTGGCGTGAA-3' and the antisense primer 5'-TAGTCTGGCGCTGGTCGC-3'. The resulting PCR product encompasses positions 14-2724 of the promoter sequence (NCBI accession number AF090195), or positions -2720 to -10 relative to the translation start site.

The GAD67 promoter was PCR-amplified from rat genomic DNA using the sense primer 5'-CCCAAACCGCCTTTTTATG-3' and the antisense primer 5'-CCGAAGCTTTTTGCTCTGCCACAGACGTA-3'. The product spans positions 38 to 1063 of the promoter sequence (acc # AF110132), or -920 to +106 with respect to the transcription start site. In addition, truncated versions of the GAD67 promoter were

generated with the following sense (or forward) primers, each used in conjunction with the antisense primer given above: 5'-ACCGCTCGAGTGGAGCGGCACTCGTG-3'; 5'-CCCCTCGAGCAGGTAGCTGTGTTCTGAACG-3'; 5'-CCCCTCGAGACCTTCTGGATTCGCCAATC-3'; and 5'-CCCCTCGAGAGAATGATTTTTTCCCTTGC-3'. The 5' end of the resulting promoter fragments are located at positions -742, -620, -460, and -320 relative to the transcription start site, respectively. All primers for this and subsequent protocols were purchased from Sigma-Genosys.

Genomic rat DNA was isolated from 100 mg rat tail tissue by digesting the tissue in 0.2 µg/ml proteinase K (Ambion)/SNET buffer overnight, followed by a phenol-chloroform extraction and two ethanol precipitations.

PCR-Mediated Mutagenesis

The pGL3Δ-Basic vector was generated from the pGL3-Basic vector (Promega) via PCR-mediated mutagenesis. The primers 5'-AGGTA~~CTTGGAGCGGCCGCA~~ATAAA-3' and 5'-CCC~~GGGCTAGGATCCTTAAGAGCTCG~~-3' were used to generate a subsequence of the vector in which the XRE in the multiple cloning site was mutated to a BamHI site; the underlined portions of the 3' primer indicate the points of mutation. The subsequence was then ligated into pCRII-TOPO (Invitrogen), and the ligated vector was cloned into DH5α chemically competent *E. coli* (Invitrogen) using the TOPO-TA Cloning Kit (Invitrogen). This construct and the pGL3-Basic vector were then restricted with NotI and SmaI (Promega) and run on a 1% gel. The sub-sequence and the restricted pGL3-Basic vector were eluted from the gel with the GenElute kit (Sigma). The open pGL3-Basic vector was then dephosphorylated with shrimp alkaline

phosphatase (Fermentas) to prevent re-ligation. Approximately 90 pmoles of insert and 30 pmoles of dephosphorylated pGL3-Basic vector were ligated overnight at room temperature with T4 DNA ligase (Fermentas). The final ligations were cloned into DH5 α or TOPO One-Shot cells (Invitrogen). The sequence of the resulting vector was then confirmed by restriction digest, and by sequencing through the Genomics and Bioinformatics facility at the University of Massachusetts Amherst.

Generation of Promoter-Reporter Vectors

Each promoter sequence was amplified from genomic DNA by PCR, using the primers listed above. The PCR product was then inserted into pGL3-Basic (for GAD65), or pGL3 Δ -Basic (for GAD67), using the cloning, excision, and ligation protocols described for PCR-mediated mutagenesis. The sequences were confirmed by restriction digest and by sequencing through the Genomics and Bioinformatics facility at the University of Massachusetts Amherst.

Site Directed Mutagenesis

Point mutations in the GAD65 and GAD67 promoters were introduced by Dr. Carpenter using the GeneEditor *in vitro* site-directed mutagenesis kit according to the manufacturer's protocol (Promega). Briefly, the reporter plasmid containing the promoter was denatured in an alkaline solution, and a primer introducing the mutation and an additional primer introducing a new antibiotic resistance were annealed to the denatured template. A mutant strand was synthesized from the primers with T4 DNA polymerase and T4 DNA ligase. The product was cloned into BMH71-18 mutS cells, which were then exposed to the GeneEditor antibiotic selection mix. Plasmid DNA was isolated from the surviving cells, and transformed into JM109 cells exposed to

ampicillin. The plasmid DNA isolated from JM109s was then transformed into DH5 α cells for long term storage.

Dr. Carpenter introduced the three GAD65 mutations with the primers 5'-AGTTAAGGCGTAGGGATCCGATCTCTACCTTCCCTCAACTGC-3', 5'-CCTGGGCTCTTGAGGATCCAGAGATCTCCGCACGGGTTTGG-3', and 5'-GTGCCCAGCTGCACCGAGGCCAGC-3'; the underlined portions represent the introduced mutations. These primers mutate the EREs located -1960, -713, and -549 relative to the translation start site, respectively. The first two primers replace the EREs with BamHI sites; the third introduces a PvuII site. The single GAD67 mutation was effected with the primer 5'CAAAAACAGAGCTCGCTGAGTGCATTC-3', and mutated the XRE located at position -246 to produce a SacI site. Mutated sequences were verified by restriction enzyme digest and sequencing through the Genomics and Bioinformatics facility.

Cell Culture

SN56.B5.G4 cells (SN56) were generously provided by Dr. Jan Blusztajn. N42 cells were purchased from Cellutions Biosystems (see also Belsham 2004). The cells were maintained in growth media consisting of Dulbecco's Modified Eagle Medium (1X DMEM; Cellgro), supplemented with 10% v/v characterized fetal bovine serum (FBS; Hyclone) and 1% v/v penicillin-streptomycin-glutamine (PS-Gln; Invitrogen). Media for SN56 cells was further supplemented with 1 mM sodium pyruvate (GIBCO). All cells were grown and maintained at 5% CO₂ and 37C in 100-mm tissue culture plates (Costar). When the cells reached 50-70% confluence, they were rinsed with phosphate-

buffered saline (1X PBS; Hyclone), after which the cells were dissociated from the plates with 0.05% Trypsin-EDTA (Invitrogen). A volume of growth media equivalent to the volume of trypsin was added after dissociation, after which the cells were triturated with repeated pipetting and passaged into fresh culture plates at ratios varying with cell type.

Transfection

SN56 cells were passaged into 48-well plates (Costar) and allowed to grow to 70% confluence before transfection. Each well was transfected with 0.8 μg DNA using the Superfect transfection reagent and manufacturer's protocols (Qiagen). The DNA consisted of 0.68 μg reporter vector, 0.04 μg pRL-CMV, and 0.04 μg of each of two expression vectors (or empty control vectors), as appropriate. After 2-3 hours of incubation in transfection mixture, the media was changed to steroid-free media consisting of 1X phenol-red free DMEM (Cellgro) supplemented with 10% v/v dextran-coated charcoal treated FBS (Hyclone), 1% v/v PS-Gln, and supplemented with 1 mM sodium pyruvate. Transfected cells were incubated in 5% CO₂ at a temperature of 37C in steroid-free media overnight.

Treatment

E₂ treatments consisted of steroid-free media supplemented with 10 nM 17 β -estradiol (Sigma), or 1 ppm ethanol as a vehicle control. TCDD treatments consisted of steroid-free media supplemented with 10 nM TCDD (Accustandards), or 1:5000 v/v DMSO (Sigma). Cells were incubated in treatment media 24 hours, unless stated otherwise. Transfected SN56 cells were rinsed in 1X PBS and lysed with 1X passive lysis buffer included in the Dual Luciferase Assay kit (DLA; Promega), and the lysates

were either assayed immediately, or were stored at -20C. N42 cells were fixed in 1% formaldehyde (Fisher) after treatment for use in chromatin immunoprecipitation.

Chromatin Immunoprecipitation Assay

N42 cells were grown to approximately 50% confluence in normal growth media in 100-mm tissue culture plates. On the day of treatment, the cells were rinsed with 4 ml PBS per plate, and covered with 10 ml steroid-free media supplemented with either 10 nM E₂ or 1 ppm EtOH. The cells were incubated under normal cell growth conditions for 24 hours before fixation. Formaldehyde (Fisher Scientific) was added directly to the media for a final concentration of 1% by volume, and the cells were incubated at room temperature for 10 minutes on a platform shaker. The fixed cells were rinsed twice with ice-cold PBS supplemented with 2 µl Protease Inhibitor Cocktail III (Calbiochem) per ml, then scraped and transferred to 15-ml centrifuge tubes (BD Sciences). After centrifugation at 3,300 rpm (1,300 g) for 3 minutes, the supernatant was discarded, and the pelleted cells were resuspended in 1 ml lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.0) and incubated on ice for 10 minutes. Cell nuclei were then pelleted by centrifugation at 5,000 rpm (1,677 g) for 5 minutes, the supernatant discarded, and the pellets resuspended in 375 µl lysis buffer. After another 10-minute incubation on ice, 625 µl dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.0, 167mM NaCl) was added. The samples were then sonicated at 15% amplitude with 10 pulses of 10 seconds in 1.7 ml microcentrifuge tubes with a Branson digital sonifier. After centrifugation for 5 minutes at 14,000 rpm (13,000 g), the supernatant was transferred to new tubes, and centrifuged again for 10

minutes. The supernatant was precleared with 60 μ l 50% salmon sperm DNA/protein G slurry (Upstate) for 30 minutes at 4C on a shaking platform. Samples were then centrifuged for 1 minute at 1000 rpm (67 g), and the supernatant transferred to a new tube. Sixty microliters of sample was stored separately as total input (TI). The remaining portion of each sample was then divided into three equal volumes. One portion received 5 μ g of ER alpha-specific antibody (Abcam ab13538), one received 5 μ g of ER beta-specific antibody (Abcam ab16813), and the third portion received no antibody as a negative control. The samples were then incubated at 4C overnight on a shaking platform.

The following day, samples were incubated with 80 μ l 50% salmon sperm DNA/Protein G agarose slurry for 90 minutes at 4C with agitation. The samples were then pelleted at 1000 rpm for 1 minute at 4C, and the immunoprecipitate (IP) was then washed with the following solutions for 5 minutes each at room temperature: once with low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl); once with high salt (similar to low salt, but with 500 mM NaCl); once with LiCl wash buffer (0.25 M LiCl (EMD Biosciences), 1% IGEPAL-CA630 (MP Biomedicals), 1% deoxycholate sodium salt (MP Biomedicals), 1 mM EDTA, 10 mM Tris-HCl pH 8.0); and twice with 1X TE pH 8.0. Two hundred fifty microliters of elution buffer (1% SDS, 0.1 M NaHCO₃, freshly prepared) was added to each pellet, and the samples were vortexed at room temperature for 15 minutes. Samples were centrifuged for 3 minutes at 14,000 rpm RT, the supernatants transferred to new tubes, and the elution repeated. Formaldehyde crosslinking was reversed in TI and IP samples by incubating the samples at 65C overnight in NaCl (0.2 M final concentration).

All samples were brought to 16.7 mM EDTA and 66.7 mM Tris-HCl pH 6.8 final concentrations. IP samples were treated with 2 μ l of 25 mg/ml Proteinase K (Ambion), while TI samples received 1 μ l Proteinase K. Samples were incubated for 1 hr at 45C, after which the samples were purified by phenol/chloroform extraction and two rounds of EtOH precipitation using 2.5 μ g brewer's yeast tRNA (Calbiochem) per 100 μ l TI or IP as a carrier.

Polymerase Chain Reaction

The EREs in the mouse and rat GAD65 promoters are highly homologous, and the ERE that corresponds to the -713 ERE in the rat promoter is located at position -695 in the mouse GAD65 promoter (accession # AB032757). The mouse -695 ERE is nearly identical to the rat -713 ERE. PCR primers were designed to flank this ERE site, as follows: 5'-ATGAGTTCGTTGGTGTGGAAG-3' (forward primer); 5'-AGTGCTGAGGTCGCTGTG-3' (reverse primer). In addition, negative control primers were designed for a part of the coding region of mouse GAD65 that does not contain an identifiable ERE (accession # NM_008078, bases 2402-2610). The sequences of these primers are 5'-TCAAAACCAACAGGAAACATCA-3' and 5'-TTCAAGGGTGATTTGGCACT-3'.

Polymerase chain reactions (PCR) were used to validate the chromatin immunoprecipitation assay (ChIP). For each test template, two 25 μ l PCRs were prepared, one with the -695 ERE primer pair, and the other with the negative control primers. The test templates included the TI and each antibody IP samples derived from the E₂-treated cells, 5 μ g tRNA, and a no template control. The reactions were prepared on ice with 5 μ l 5X PCR Buffer, 1.5 μ l 25 mM MgCl₂ (both reagents from Promega),

0.5 μ l 10 mM dNTPs (Invitrogen), 0.25 μ l 20 μ M of each of the appropriate primers, 1 μ l template (except as noted above), 0.125 μ l GoTaq (Promega), and were brought to volume with nuclease-free H₂O (nf H₂O). The reactions were performed in a Mastercycler thermocycler (Eppendorf). An initial 2 minute step at 95C was followed with 40 cycles: 1 min. 95C; 1 min. 60C; 1 min. 72C. After the cycles were completed, the samples were brought to 72C for 10 minutes, then 4C until removed. The samples were run on a 2% agarose gel stained with 0.01% v/v GelRed (Biotium) and visualized under UV light. Samples that yielded the expected bands in the TI groups, and that yielded positives in the -695 IP groups, but not in the tRNA, no template, or negatively primed IPs, were then used for real-time PCR.

Real-Time PCR

Real-time PCRs were performed in 96-well plates using the MX3005P thermocycler (Stratagene). A 25- μ l reaction was prepared in each well, with the following reagents: 12.5 μ l SYBR Green Master Mix (Stratagene); 0.375 μ l 0.2% ROX reference dye (Stratagene); 0.25 μ l 20mM forward primer; 0.25 μ l 20mM reverse primer; 1 μ l template (or nf H₂O for the no template controls); 10.625 μ l nf H₂O. For each ChIP DNA sample, 4 reactions were prepared; 2 reactions received the -695 primer pair, while the other 2 received the negative control primers. In addition, 2 no-template reactions were prepared for each primer pair. The cycling conditions were identical to those used in PCR. The $2^{(-\Delta\Delta Ct)}$ method (Livak 2001) was used to quantitate the results.

CHAPTER 2

RESULTS

The GAD Promoters Contain EREs and XREs

The GAD65 promoter contains several transcription start sites (Skak 1999); thus, to avoid ambiguity, I use the translation start site as the reference (+1) position. To determine whether the rat GAD promoters contain EREs, Dr. Carpenter and I searched the sequences for the ER half-sites AGCTT and AAGCT. We confirmed our scans with the online tools Dragon ERE (Bajic 2003) and MATCHTM, created by Biobase Biological Databases (Goessling 2001).

The GAD65 promoter contains three potential EREs at positions -1960, -713, and -549 (Fig. 1). Dragon ERE did not confirm any strong EREs in the rat GAD67 promoter. However, MATCHTM detected two EREs when we chose the option to minimize the rate of false negatives. These EREs are located 489 and 213 bases upstream of the GAD67 transcription start site (Fig. 1).

Similarly, I searched the promoter sequences for the concensus AhR/ARNT site GCGTG, and confirmed the results with MATCHTM. I detected a single XRE in the GAD65 promoter, at position -2710 relative to the translation start site (Fig. 1). Dr. Carpenter and I also found two XREs in the GAD67 promoter, at positions -746 and -246 relative to the transcription start site (Fig. 1). However, MATCHTM only confirmed the latter XRE, and only when the cut-off was selected to minimize for false negatives.

The GAD65 Promoter, but not the GAD67 Promoter, Responds to E₂

Results of studies examining the effect of E₂ on GAD65 and GAD67 promoter activity are shown in figure 2. Cells that were cotransfected with empty pRST7 vector did not exhibit any response to E₂, regardless of reporter vector. Cotransfection with both ER expression vectors was sufficient to increase luciferase signal in cells transfected with pGL3-GAD65, and treatment of ER-transfected cells with E₂ significantly increased the pGL3-GAD65 signal further (fig. 2A). In contrast, cells transfected with pGL3Δ-GAD67 exhibited a small but significant decrease in luciferase signal with ER cotransfection. E₂ did not significantly alter luciferase activity either with or without exogenous ER (fig. 2B).

I further characterized the E₂ response of the GAD65 promoter with different ER complements (fig. 3). GAD65 promoter signal significantly increased with E₂ in the presence of either ER, and the response is not significantly different between the ERα or ERβ transfected groups. However, the E₂ response significantly increases when both ERs are present. As previously, there was no response to E₂ in the absence of ER.

I also determined E₂ dose response curves for pGL3-GAD65 for each combination of cotransfected estrogen receptors, and the results are shown in figure 4. Although I had applied doses of 1 μM in the initial trials, SN56 cells exposed to this dose died before lysis whether or not ERs were cotransfected. For each dose, I normalized the luciferase signal for the ER-transfected cells to the signal for blank pRST7 transfectants.

pGL3-GAD65 transfected cells that received pRST7-ERα, with or without pRST7-ERβ, showed significant responses to E₂ at doses of 100 pM or higher. Cells

transfected with pRST7-ER β alone responded significantly to 1 nM and higher doses of E₂. Also, the ER β -transfected cells exhibited significantly lower responses to E₂ than cells that received both ERs for doses ranging between 10 pM to 10 nM. No other significant differences were detected.

Mutations in the GAD65 Promoter EREs Alter E₂ Response

The E₂ responses of the mutant GAD65 promoters, in which the putative EREs were individually mutated, are shown in figure 5. I normalized the dual-luciferase assay results to the average signal of the wild-type GAD65 promoter vector in cells treated with ethanol. Two-way ANOVA reveals that the wild-type and m-1960 promoters responded significantly to E₂ treatment, whereas no significant E₂ response was observed for the m-713 or m-549 mutant promoters. In addition, the average E₂ responses of the wild-type and m-1960 promoters did not significantly differ. Finally, the basal (EtOH-treated) activity of the m-549 mutant was significantly reduced.

GAD65 Promoter Mutant Responses to E₂ Vary with ER Subtypes

The E₂ responses of the GAD65 promoter mutants were tested across different ER subtypes, and the results are presented in figure 6. I normalized promoter activities to the signal generated by the wild-type GAD65 promoter treated with ethanol in the absence of ERs. In each experiment, the wild-type GAD65 promoter significantly responded to E₂ in the presence of either estrogen receptor subtype. Similarly, the GAD65m-1960 promoter responded to E₂ across different estrogen receptor combinations, and these responses were not significantly different from wild-type (fig.

6A). However, mutation of the -713 ERE abrogated the E₂ response regardless of ER complement, when compared with the intact GAD65 promoter signal (fig. 6B). The -549 ERE mutant did show significant E₂ responses across ERs; however, these responses were significantly lower than the intact promoter responses (fig. 6C).

To correct for changes in the luciferase signal in the ethanol-treated cells across promoter and ER transfections, I then normalized the same data to the average signal of the ethanol-treated cells for each promoter/ER combination individually. When I analyzed these relative E₂ responses, I found that the -713 ERE mutant did yield a significant response to E₂ in the presence of ER α , either with or without ER β , but not when ER β was transfected alone. However, the ER α mediated responses of this mutant promoter were still significantly lower than the GAD65 wild-type promoter responses (fig. 7A). The -549 ERE mutant also exhibited significant E₂ responses across all ER complements, as expected, but these relative responses were significantly lower than the wild-type responses (fig. 7B).

ER α May Interact with the GAD65 Promoter

The results of the preliminary ChIP assay are presented in figure 8. I normalized the resulting values to the no-antibody controls by treatment group. Considering that the results are based on a single trial, I have not performed any statistical analyses on the data. However, it appears that immunoprecipitation with the ER α -specific antibody enriches the sample for the mouse GAD65 promoter region containing the -695 ERE. The ER β -specific antibody, however, does not seem to yield enrichment compared to

the no-antibody controls. E₂ treatment does not seem to affect enrichment in either the ER α or the ER β IP groups.

Both GAD Promoters Respond to TCDD

The TCDD responses of both GAD promoters are presented in figure 9. In the presence of the empty pcDNA3 vector, neither promoter exhibited a response to TCDD. However, when cotransfected with the AhR and ARNT expression vectors, both promoter vectors exhibited a significantly higher signal in the presence of TCDD, compared with DMSO.

I also determined TCDD dose response curves for pGL3 Δ -GAD67; the results are shown in figure 10. I normalized the luciferase signals to the signal generated in the cells with no treatment. I observed a significant increase in promoter activity in response to TCDD doses of 1 nM or higher. Lower doses elicited no significant response, and the responses generated at 1, 10, and 100 nM were not significantly different from each other.

Mutations in the GAD67 XREs do not Reduce TCDD Response

I tested the TCDD response of the GAD67 promoters in which the XREs were either deleted or mutated, or both. The results are shown in figure 11. I first normalized the luciferase signals to the average activity of the GAD67 wild-type promoter in cells treated with DMSO. In this case, all mutated GAD67 promoter vectors exhibited significant responses to TCDD, compared to DMSO treatment. In addition, the responses were not significantly different between the wild-type, d-742, and d-742m-

246 promoter vectors. The TCDD response of the m-246 promoter vector was, however, significantly lower than the responses of the other GAD67 promoter vectors (fig. 11A).

I then corrected for variations in basal signals by normalizing signals to the DMSO controls for each promoter type to determine relative promoter responsiveness to TCDD. In this analysis, the resulting fold inductions did not vary across GAD67 promoter types (fig. 11B).

The TCDD Response is Mediated by a Proximal GAD67 Promoter

Truncated GAD67 promoters were assayed for TCDD response, and the results are presented in figure 12. As shown in Figure 12A, all truncated promoters display significant responses to TCDD when the signals are normalized to GAD67 promoter responses in cells treated with DMSO. In addition, the truncation vectors yielded significantly higher TCDD responses than the wild-type GAD67 promoter vector. Interestingly, the shortest GAD67 promoter, d-320, exhibited a significantly higher basal signal than the other vectors.

When I normalized the different GAD67 promoter signals to their respective controls, I still observed variation in the promoter responses. Specifically, the d-620 and d-460 truncations yielded higher relative responses than the GAD67 wild type promoter. However, the d-320 TCDD response was not significantly different from the responses of the GAD67 wild-type or the d-620 truncation promoters (fig. 12B).

CHAPTER 3

DISCUSSION

The results presented in this study are consistent with the hypothesis that GAD65 is transcriptionally upregulated by E₂, and that this regulation is ER-dependent. The rat GAD65 promoter exhibits a strong response to E₂ in SN56 cells if and only if some form of ER is present. Additionally, mutations in two of the three putative EREs (the -549 and -713 EREs) significantly reduced the response of the promoter to E₂; mutation of the -713 ERE, specifically, results in an E₂ response that appears biologically insignificant when compared to the response of the wild-type promoter. Finally, a preliminary ChIP assay suggests that ER α interacts directly with the region of the ERE in the mouse GAD65 promoter that is homologous to the -713 ERE in the rat promoter, although the ChIP will require validation with further trials.

Both of the above mentioned ERE mutations reduced the E₂ regulation of GAD65; however, they did not individually abolish the response of the promoter to the steroid. Mutation of the -549 ERE allowed a response in the presence of ER α or ER β , while the promoter mutated at the -713 ERE responded to E₂ when ER α was present, either with or without ER β , but not in the presence of ER β alone. Although the E₂ response in this mutation was very low compared to the wild type, the pattern of response, along with the responsiveness of the -549 mutation, suggest a synergistic mechanism for ER action. Liganded ER binding to both EREs is required for the full estrogen responsiveness of the promoter, but the actual interaction between the two EREs is not clear. One possibility is that the -713 ERE may be bound by ER α or ER β ,

either as homo- or heterodimers, while the -549 ERE response may require ER α binding. Alternatively, ER β may bind to the -549 ERE, but, in the absence of ER binding to the -713 ERE, may act as a repressor of GAD65 promoter activity. The latter hypothesis may be more consistent with my observations, as the former would imply that the presence of ER β alone would result in a lower E₂ response from the wild type GAD65 promoter and it does not.

Conversely, GAD67 promoter activity did not appear to be affected by E₂, either in the presence or absence of ERs, which suggests that the GAD67 responses to E₂ observed *in vivo* are due to indirect, possibly trans-synaptic mechanisms. This may explain why the EB response of GAD67 mRNA is delayed in adult OVX rats compared to the GAD65 response (Nakamura 2004). Additionally, this may explain the phasic response in GAD67 mRNA levels to E₂ in the AVPV of ovariectomized female rats, as opposed to the tonic increase observed in GAD65 message (Curran-Rauhut, 2002); GAD67 may be responding to some phasic trans-synaptic signal that is in turn modulated by E₂, whereas GAD65 mRNA levels remain elevated in the presence of E₂.

The responsiveness of GAD65 to TCDD in AhR-transfected SN56 cells was surprising, as previous studies in our lab had demonstrated that the GAD65 promoter does not respond to dioxin in MCF-7 cells (Carpenter, unpublished). This human breast cancer cell line is known for its robust, AhR-dependent TCDD responsiveness (reviewed in Safe 2000), which raises the question as to why the GAD65 promoter does not respond to TCDD in MCF-7 cells, but does so in SN56 cells. It is possible that the neural hybridoma cells express neural-specific factors required for TCDD induction of

GAD65. However, more work is needed to elucidate the interaction between TCDD, AhR, and the GAD65 promoter in neural cells.

While my data support the hypothesis that the GAD67 promoter responds to TCDD in an AhR-dependent manner, the interaction between AhR and this promoter did not seem to involve a consensus XRE. The relative TCDD responses of the -742 DRE deletion construct and the -246 DRE mutation, either separately or together, were not statistically different from the dioxin response of the intact GAD67 promoter. I observed a decrease in TCDD responsiveness in the -246 mutant when compared directly with the wild-type signal, but I also observed a decrease in basal promoter activity for this mutation. When the signal was adjusted for this change in basal signal, the relative response of this mutant was similar to the relative responses of the other mutants. This suggests that the apparent decrease in TCDD response was an artifact of the lower basal activity of the mutated promoter. The change in basal signal in the -246 XRE mutant bears further study, especially considering that the combined deletion/mutation construct, d-742m-246, does not exhibit a change in basal activity.

Although I did not find a functional XRE in the mutation experiments, truncation experiments demonstrated that the site mediating the dioxin response is located within 320 bases of the transcription start site. This truncated GAD67 promoter contains binding sites for transcription factors that are known to interact with, or be regulated by, the ligand activated AhR/ARNT complex. Specifically, MATCHTM reveals an AP-1 site 214 bases upstream of the transcription start site, and it has been shown that TCDD, acting through the AhR, can modulate AP-1 activity (Suh et al., 2002), possibly through regulation of AP-1 dimerization partners such as *c-jun* or *junD*

(Hoffer et al., 1996). Additionally, this region of the promoter contains three SP1 sites which are critical for GAD67 transactivation (Pedersen et al., 2001), and SP1 is known to interact with liganded AhR to control gene expression from the cathepsin D promoter (Wang et al., 1999) and the CYP1B1 promoter (Tsuchiya et al., 2003). However, this direct interaction between AhR and SP1 seems to require an XRE in proximity to the SP1 binding site (Wang 1999, Tsuchiya 2003). More work is required to determine the mechanism through which dioxin induces GAD67 promoter activity.

As the rate-limiting enzymes for GABA production, GAD65 and GAD67 are targets for regulation of GABAergic systems, and previous research suggests that these two enzymes are differentially regulated. Although the two enzymes are commonly (but not always) found in the same neurons, their intracellular distributions differ. GAD65 is generally located in nerve terminals, while GAD67 is distributed more evenly throughout the cell (Kaufman 1991). Additionally, the two GADs are differentially associated with the common obligatory cofactor pyridoxal-5'-phosphate (pyridoxal-P) in that almost all GAD67 is conjugated to the cofactor, while approximately half of GAD65 is not (Kaufman 1991). Another difference between the enzymes is that, of those neurons which express only one of the two isoforms, GAD65-positive neurons tend to be phasically or transiently active, while GAD67-positive cells are thought to be tonically active (Feldblum 1993). These facts together suggest that GAD65 plays a role in transient production of vesicular GABA, while GAD67 may play a more important role in either tonic GABA release or cellular metabolism.

It is therefore interesting to note that the GAD65 promoter is responsive to E_2 , while the GAD67 promoter is not. In the context of the AVPV neurons, E_2 may

increase the availability of vesicular GABA by raising levels of GAD65 apoenzyme during early proestrous. The phasic E₂ response of GAD67 may, in contrast, reflect E₂ induced metabolic or phenotypic changes in of the cells. The peak of this response occurs at noon on the day of the LH surge (Curran-Rauhut 2002), and it has been suggested that AVPV neurons projecting to GnRH cells switch from primarily GABA output to mixed GABA and glutamate output by this time (Ottem 2004). It will be interesting to determine whether the E₂-induced changes in GAD67 mRNA in the AVPV is linked to the phenotypic change in the GABAergic neurons in this nucleus.

The effects of TCDD on GAD mRNA expression have not been well characterized. GAD67 mRNA levels are decreased in the rPOA/AVPV of post natal day 3 (PND 3) female pups of rats that had been treated with TCDD, compared with DMSO treated controls, but GAD67 message is increased in the caudal medial preoptic nucleus (MPN) of male pups under the same treatment (Hayes 2002). It is particularly interesting to note that both these effects abolish sex differences in GAD67 mRNA levels in these two regions. However, it is not known whether GAD67 is a direct target of TCDD, or whether the changes in GAD67 mRNA reflect TCDD-induced changes in the cell density or morphology of these nuclei.

My observation that the GAD67 promoter can be transactivated (albeit indirectly) by TCDD suggests that the changes in GAD67 mRNA in rat pups exposed to TCDD *in utero* is, indeed, transcriptional. Dioxin regulation of the GAD67 promoter may have implications for neural development. Before the maturation of inhibitory synapses in mice, the GAD67 gene acts bicistronically to produce two smaller proteins, GAD25 and GAD44, the latter of which is enzymatically active (Szabo 1994). This

suggests that GAD44 may play a role in neural development, and the results I present here suggest that this embryonic GAD may be a target of dioxin. This is important, as TCDD administered *in utero* can demasculinize and feminize reproductive behavior and physiology in male rats (Mably 1 and 2, 1992), and it is intriguing to speculate that this dysregulation of reproductive development may be caused by inappropriate expression of GAD44 via the GAD67 promoter. It will be interesting to determine whether the observed dioxin responses of GAD67 mRNA in male and female rat pups in sexually dimorphic nuclei (Hayes 2002) involves changes in GAD44 expression, and whether these changes are linked to the dioxin induced dysregulation of sexual development (Mably 1 and 2, 1992).

FIGURES

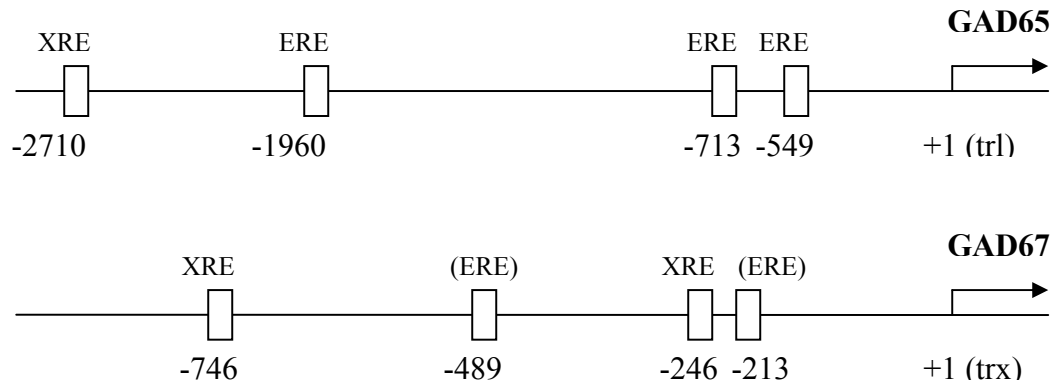


Fig. 1: Rat GAD promoter structure. ERE: Estrogen response element; XRE: Xenobiotic response element (or AhR/ARNT transcription factor binding site); trl: translation start site; trx: transcription start site.

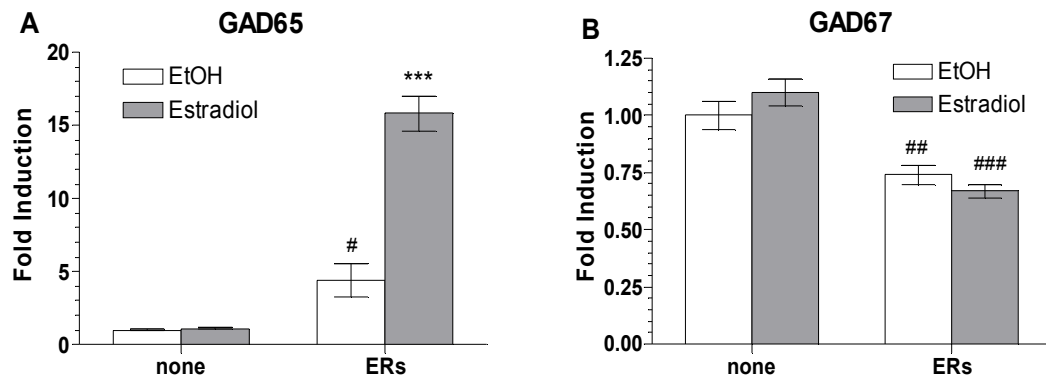


Fig. 2: GAD promoter responses to estradiol. Bars represent averages \pm SEM. #, ##, ### p < 0.05, 0.01, or 0.001 relative to EtOH, no ER control. *** p < 0.001 compared with EtOH treated ER cotransfectants. n = 6 for GAD65. n = 9 for GAD67.

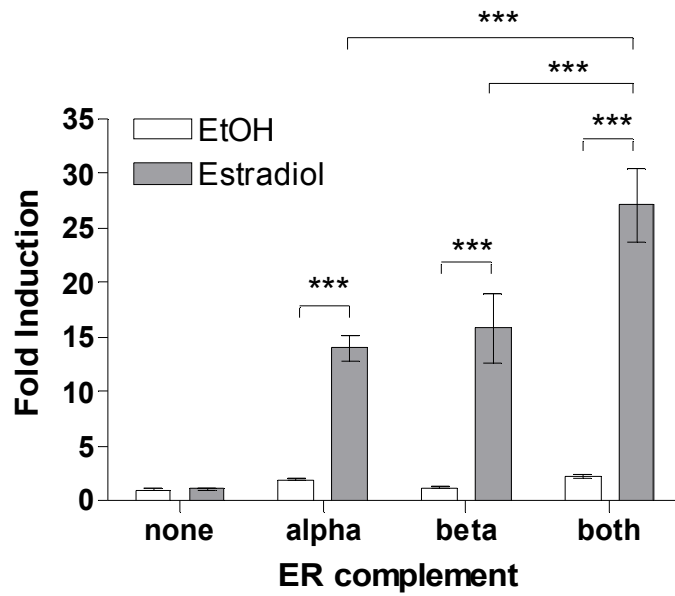


Fig. 3: GAD65 responses to estradiol across ER subtypes. Bars represent averages \pm SEM. Luciferase activity of 1 represents GAD65 promoter driven luciferase signal in the absence of ER and treatment with ethanol. *** $p < 0.001$. $n = 9$.

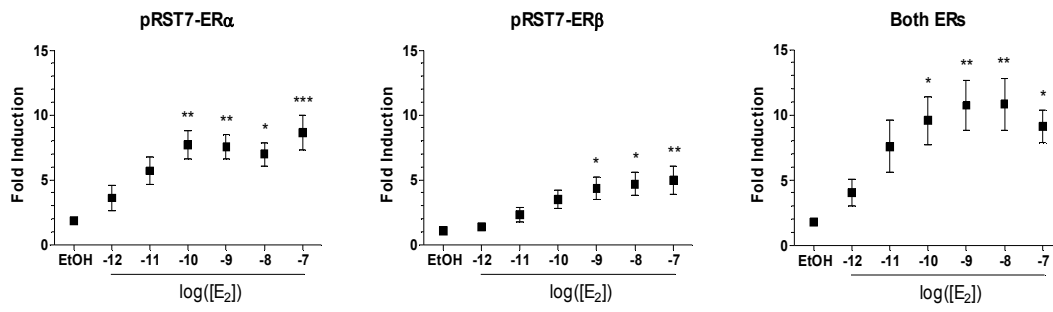


Fig. 4: Estradiol dose response of the GAD65 promoter. Luciferase signals are normalized to the E₂ dose response of the GAD65 promoter in the absence of cotransfected ER (not significant with respect to EtOH treated controls). Significance calculated with respect to ethanol treated groups. * p < 0.05; ** p < 0.01; *** p < 0.001. n = 9.

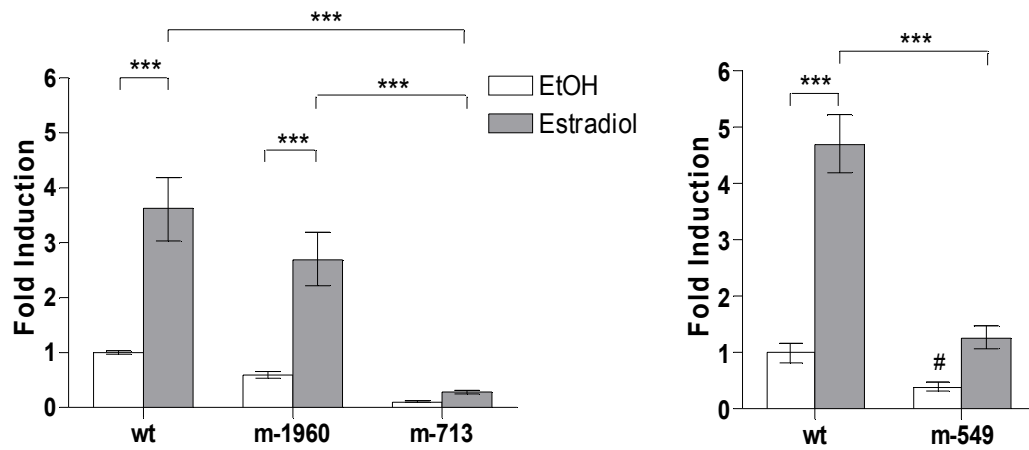


Fig. 5: Estradiol responses of mutated GAD65 promoters. Responses are normalized to wild-type EtOH control. Bars represent averages \pm SEM. *** $p < 0.001$; # $p < 0.05$ compared to wild-type/EtOH. $n = 15$ (left panels) or $n = 9$ (right panels).

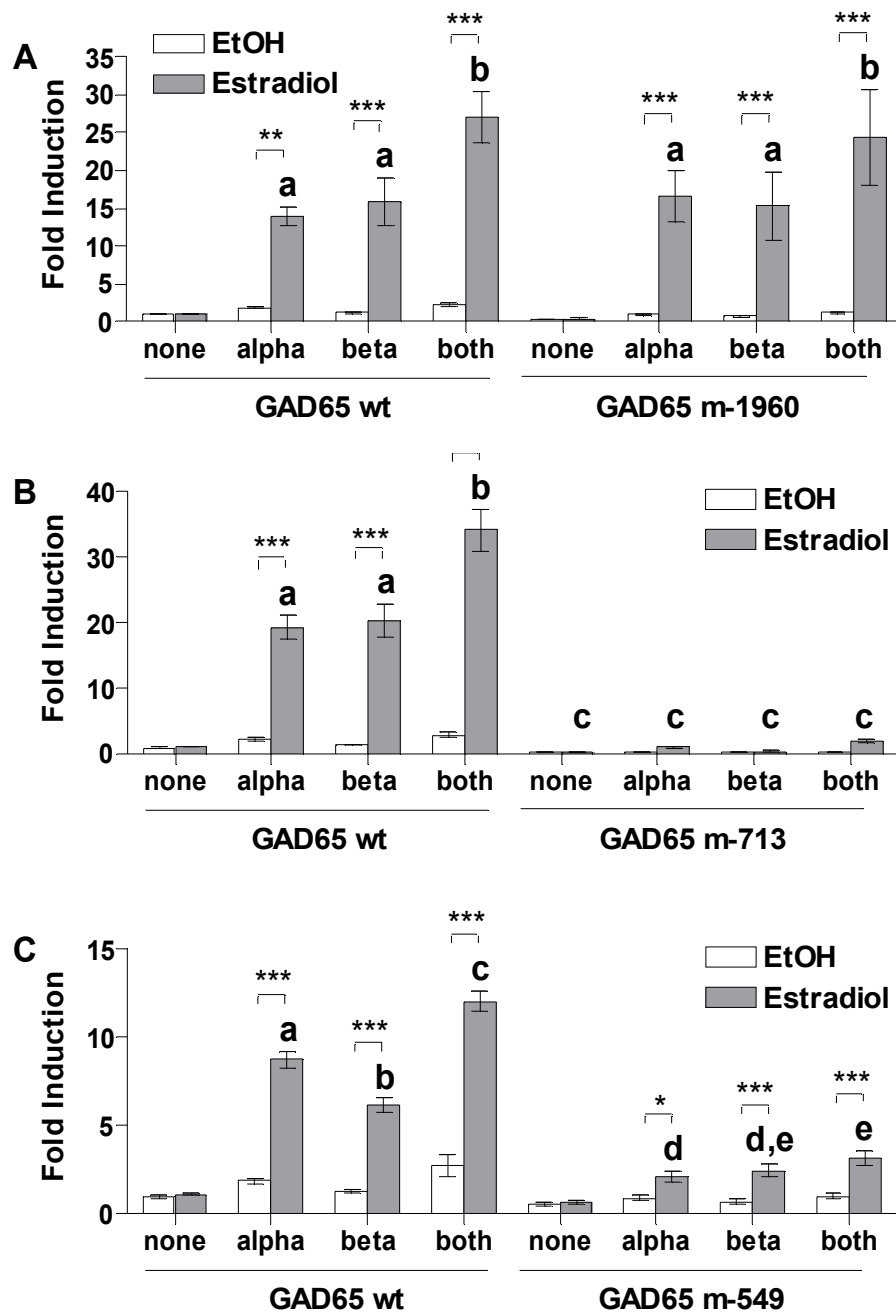


Fig. 6: Mutated GAD65 promoter estradiol responses across ERs. Comparison of estradiol responses of wild-type and mutated GAD65 promoters across different estrogen receptor complements, normalized to the wt, no ER, EtOH group. A) Wt vs. m-1960 (n = 9); B) wt vs. m-713 (n = 12); C) wt vs. m-549 (n = 9). * p < 0.05; ** p < 0.01; *** p < 0.001.

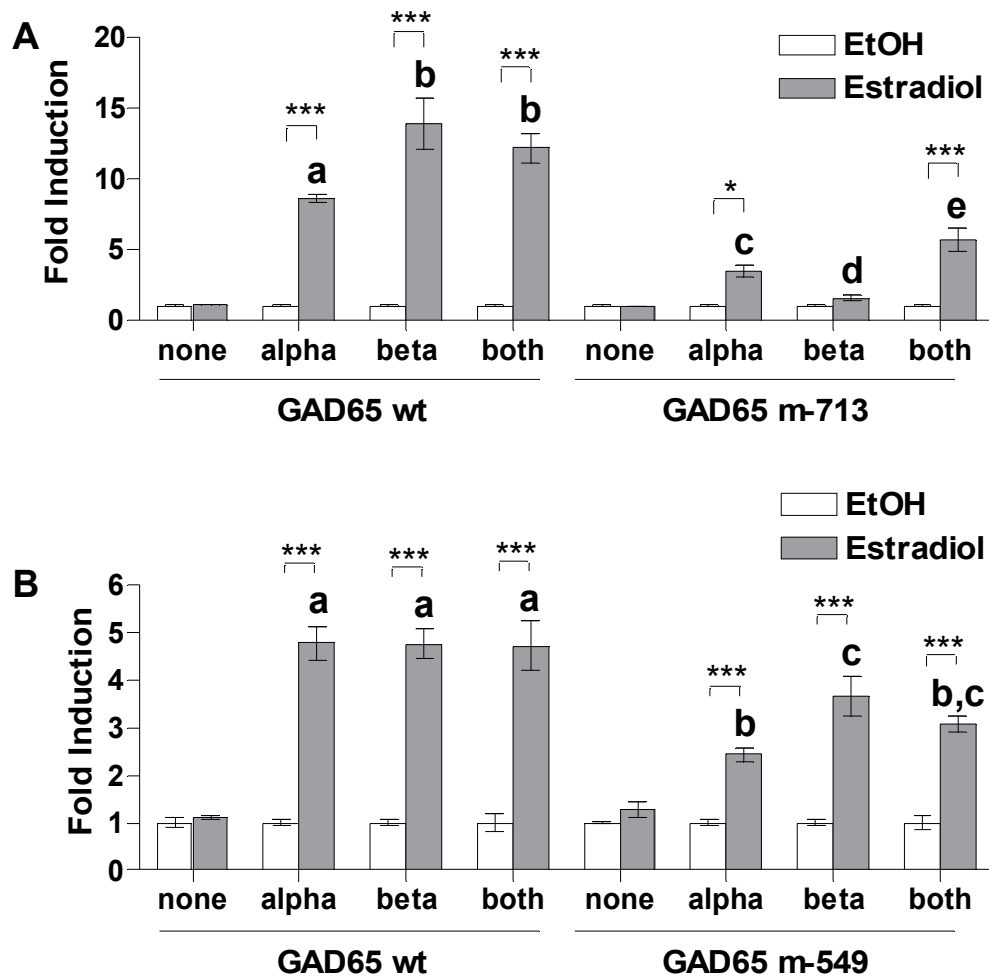


Fig. 7: Relative estradiol responses of mutated GAD65 promoters. Fold induction normalized to EtOH treated groups for each transfection set. A) wt vs. m-713 (n=12); B) wt vs. m-549 (n=9). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

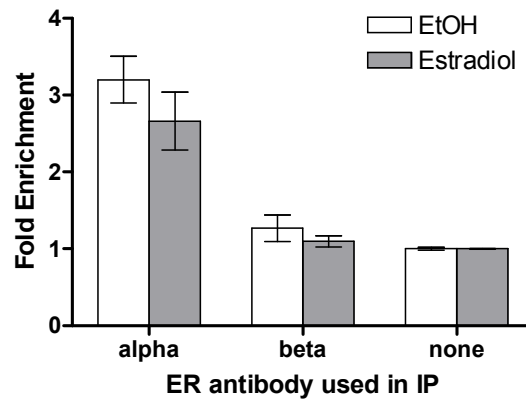


Fig. 8: ChIP assay targeting the mouse GAD65 -695 ERE. Bars represent fold enrichment over no-antibody controls, calculated by treatment.

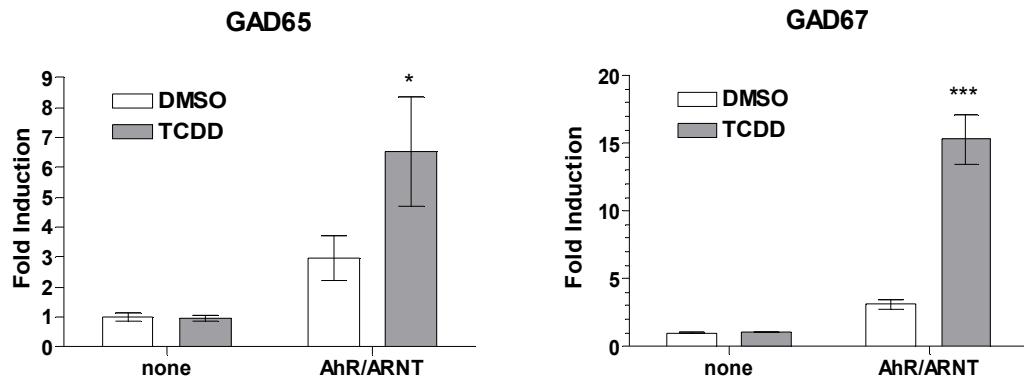


Fig. 9: GAD promoter responses to TCDD. Bars represent averages \pm SEM. *** $p < 0.001$, * $p < 0.05$ compared with DMSO treated AhR/ARNT cotransfectants. $n = 9$ for GAD65. $n = 6$ for GAD67.

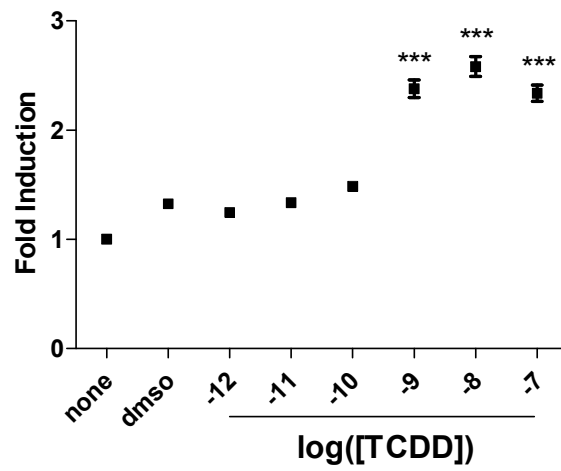


Fig. 10: TCDD dose response of the GAD67 promoter. Luciferase signals are normalized to the signal yielded in the no treatment groups. *** $p < 0.001$. $n = 9$.

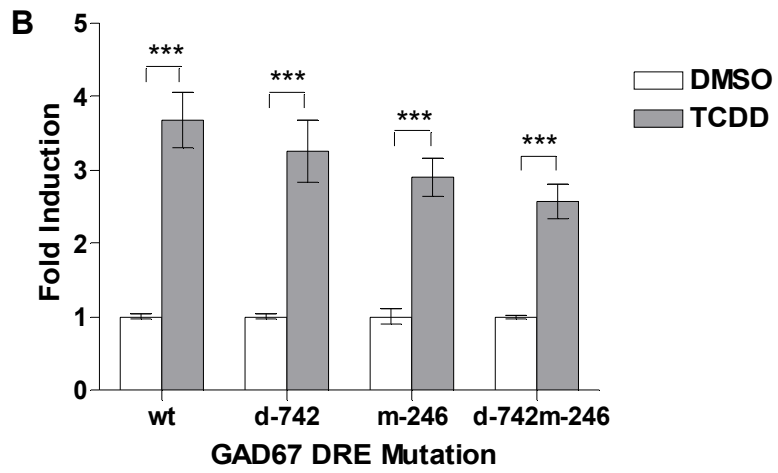
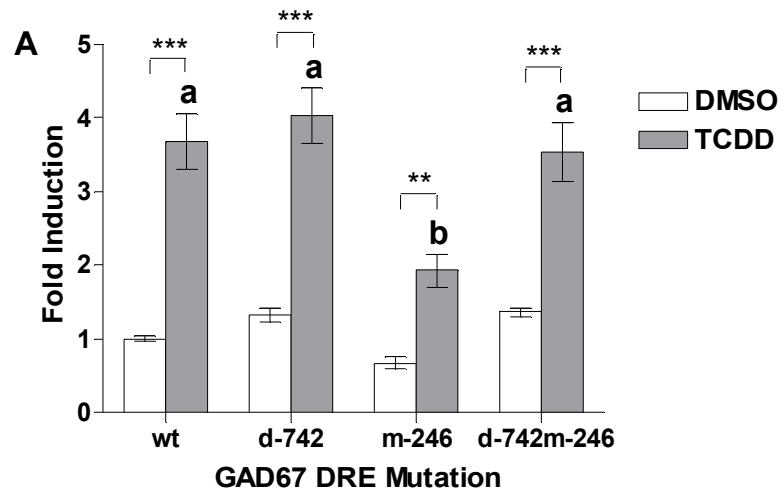


Fig. 11: Mutated GAD67 promoter responses to TCDD. Fold induction normalized to A) the wild-type DMSO control, or B) DMSO controls for the respective promoters. Bars represent averages \pm SEM. ** $p < 0.01$; *** $p < 0.001$. $n = 12$.

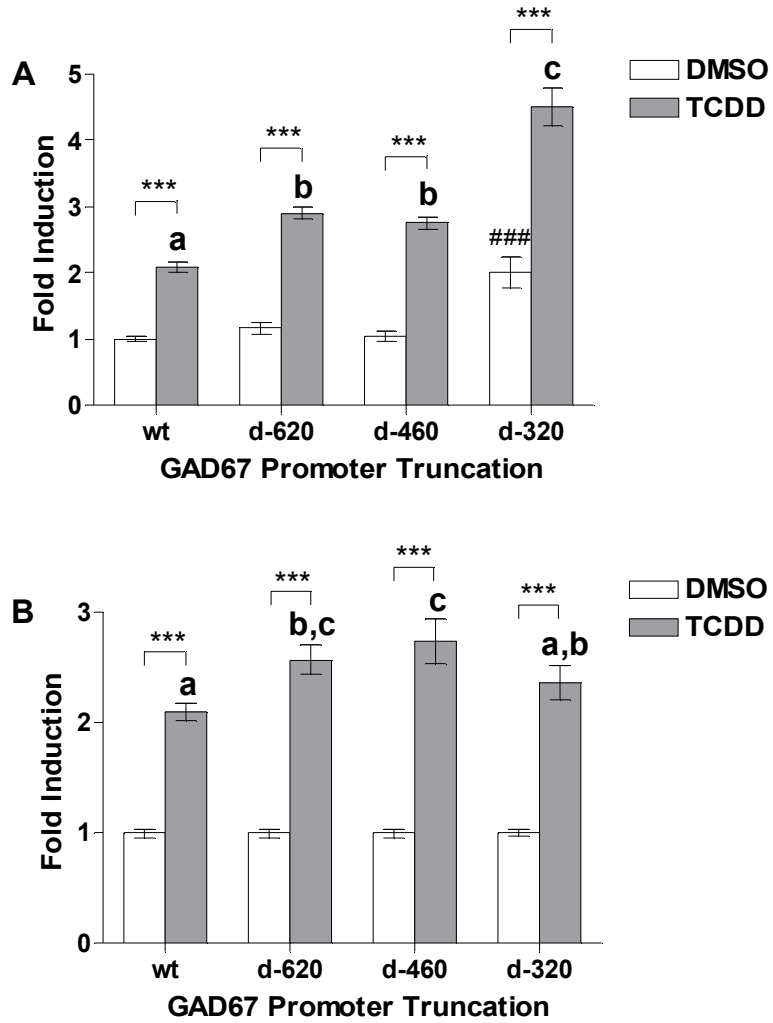


Fig. 12: Truncated GAD67 promoter responses to TCDD. Fold induction normalized to A) the wild-type DMSO control, or B) DMSO control for the respective promoter. Bars represent averages \pm SEM. *** $p < 0.001$; ### $p < 0.001$ with respect to wild-type DMSO control.

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