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Estrogen receptor-β regulates human tryptophan hydroxylase-2 through an estrogen response element in the 5′ untranslated region

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Abstract

In the dorsal raphe nucleus, 17β-estradiol (E2) increases the expression of the brain-specific, rate-limiting enzyme for serotonin biosynthesis, tryptophan hydroxylase-2 (Tph2). Although estrogen receptor beta (ERβ) has been localized to Tph2 neurons, little is known about the transcriptional regulation of the Tph2 gene by estrogen. Since the ERβ agonist, diarylpropionitrile (DPN) also increases Tph2 expression, we tested the hypothesis that E2 regulates the Tph2 promoter through direct interactions with ERβ. A serotonergic cell line, B14, which endogenously expresses ERβ was transiently transfected with a fragment of the human *TPH2* 5′-untranslated region (5′-UTR) cloned into a luciferase reporter vector (*TPH2-luc*). Treatment with E2 or DPN caused a dose-dependent increase of *TPH2-luc* activity. In contrast, E2 conjugated to bovine serum albumin, which is cell membrane impermeable, had no effect on *TPH2-luc* activity. An estrogen receptor (ER) antagonist blocked E2 or DPN-induced *TPH2-luc* activity suggesting a classical ER mechanism. *In silico* analysis revealed an estrogen-response element (ERE) half-site located within the *TPH2* 5′-UTR. Deletion and site-directed mutation of this site abolished ligand-induced *TPH2*-luc activity. These results support the concept that there is a direct and functional interaction between E2:ERβ and the ERE half-site of the *TPH2* promoter to regulate Tph2 expression.

Keywords

diarylpropionitrile; estradiol; estrogen receptor beta; estrogen response element; luciferase reporter; serotonin

Anxiety disorders are prevalent in today's society and women are twice as likely to suffer from affective disorders than men (Palanza 2001; Pigott 2003; Steiner *et al.* 2003). Fluctuations in circulating estrogens either across the menstrual cycle, or across their lifetime have been linked to this disproportionate rate (Best *et al.* 1992; Sichel *et al.* 1995; Arpels 1996; Gregoire *et al.* 1996). Reduced or changing levels of estrogen in perimenopausal women are also associated with anxiety and depression, and these may be

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effectively treated by hormone therapies (Best *et al.* 1992; Sichel *et al.* 1995; Arpels 1996; Gregoire *et al.* 1996). Correspondingly, preclinical studies in rodent models also show that 17β-estradiol (E2) can reduce anxiety- and depressive-like behaviors. Underlying these observations are differential roles for the two main forms of estrogen receptor (ER) in controlling anxiety- and depressive-like behaviors. In rats, selective agonists for ERβ are anxiolytic- and antidepressant-like, whereas activation of ERα increases anxiety- and depressive-like behaviors (Walf *et al.* 2004; Lund *et al.* 2005; Walf and Frye 2005). Thus, the possibility exists that ERβ may be an effective target for modulating affective disorders in humans.

Estrogen receptors are expressed in neurons located in many areas of the central nervous system (Shughrue *et al.* 1997; Alves *et al.* 1998; Lu *et al.* 2001), including the serotonin (5-HT) neurons in the dorsal raphe nucleus (DRN) in rats (Lu *et al.* 2001). These neurons are the major source of 5-HT in the forebrain and disruption of their function has been implicated in the etiology of affective disorders (Maes and Meltzer 1995; Sun *et al.* 2004; Nash *et al.* 2005; Zhang *et al.* 2005). Serotonin is synthesized in brain through the actions of a brain specific rate-limiting enzyme, tryptophan hydroxylase-2 (Tph2) and polymorphisms of the Tph2 have been associated with increased vulnerability to suicide and affective disorders (Sun *et al.* 2004; Nash *et al.* 2005; You *et al.* 2005; Zhang *et al.* 2005). Recent studies have shown that 17β-estradiol (E2) or an ERβ agonist, diarylpropionitrile (DPN) increases Tph2 mRNA in the DRN and this increase is important for the anxiolytic and antidepressant effects of E2 in rats (Hiroi *et al.* 2006; Donner and Handa 2009). Collectively, these studies suggest that E2 increases Tph2 mRNA in the rat DRN via ERβ; however, the molecular mechanisms for this effect remain unknown.

Little is known about the transcriptional regulation of the Tph2 gene. Previous studies have identified select regions of the 5′-untranslated region (UTR) of the human *TPH2* promoter that play a critical role in regulating Tph2 expression (Patel *et al.* 2007). Moreover, single nucleotide polymorphisms (SNPs) in *TPH2* have been shown to have significant association with major depressive disorder (Zill *et al.* 2004). However, to date, there have been no reports examining the *TPH2* promoter sequence for functional estrogen response elements (ERE) or other regulatory regions that may be sites of transcriptional control by ERs. Consequently, this study tested the hypothesis that E2 regulates *TPH2* expression through interaction with ERβ. To this end, we have identified a regulatory site in the *TPH2* promoter region that may be important for ER-induced *TPH2* transcriptional activity.

Methods

Cell culture

A serotonergic cell line, RN46A-B14 (B14) that was derived from embryonic rat medullary raphe cells (Eaton and Whittemore 1996) (kindly provided by Dr. John Neumaier, University of Washington) was used for the transfection studies. The B-14 cell line was chosen because its phenotypic endocrine profile resembles that of 5-HT neurons *in vivo*, in that they express ERβ, but not ERα (Bethea *et al.* 2003). We confirmed this expression pattern in our B14 cells using RT-PCR to amplify ERβ mRNA. Undifferentiated B14 cells were maintained in Neurobasal-A (Invitrogen Inc., Carlsbad, CA, USA) with phenol red supplemented with

10% fetal bovine serum (FBS; Gemini Bioproducts, Woodland, CA, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, 250 μg/mL G418 (to select for large T antigens) and 10 μg/mL hygromycin (to select for brain-derived neurotrophic factor) in a 33°C incubator with 5% CO_2 at physiological pH 7.4. In addition, a mouse-derived hippocampal cell line, HT-22 (generously provided by Dr. Dave Schubert, Salk Institute, San Diego, CA, USA), was maintained as previously described (Pak *et al.* 2005). No differences were found when transfections were performed in media with or without phenol red supplements. Cells were grown to 70–80% confluency, and all transient transfection experiments were performed within 10 passages.

Reporter constructs and expression vectors

A 1036 bp fragment of the human *TPH2* promoter cloned into the luciferase reporter vector (*TPH2-luc*) was purchased from Switch-gear Genomics (Switchgear Genomics, Menlo Park, CA, USA). For some studies, constructs were made with successive deletions of the 5′-end by restriction enzyme digestion upstream from the transcription start site (TSS) at positions −637 and −158 yielding constructs with *TPH2* fragment spanning nucleotides +111 to −637 (−637 *TPH2*), +111 to −158 (−158 *TPH2)* from TSS, respectively. All constructs were ligated by T4 ligase, transformed and amplified. The deletion constructs were confirmed by DNA sequencing (Operon, Huntsville, AL, USA). The pRL-CMV renilla luciferase reporter construct (Promega, Madison, WI, USA) was used as an internal control for plasmid transfection efficiency. Because we used human *TPH2-luc* construct to examine interactions with ERβ expressed in a rodent cell line, to obviate issues concerning species difference, we also examined the responses to human ERβ. A full-length human ERβ cDNA, cloned into a pCMV6-XL4 vector, was purchased from Origene (Rockville, MD, USA). The ERE-luciferase reporter (generously provided by Dr. Paul Budworth, Case Western Reserve University, Cleveland, OH, USA) contained two copies of vitellogenin ERE sequence coupled to the minimal tk-luciferase promoter and subcloned into pGL2-Basic plasmid (Promega Corp.).

Transfection, hormone treatment, and luciferase assay

B-14 cells were transfected with *TPH2-luc* using a lipid-mediated reagent (Fugene6; Promega) according to manufacturer's protocol and allowed to express *TPH-2-luc* for 24 h. 0.3 μg of pCMV-renilla plasmid was used as an internal control for plasmid transfection efficiency and for normalization of data. Empty vector controls were used with each plasmid for negative control. Constructs were transfected in triplicate. Maintenance media were then replaced with treatment media containing hormones at a final concentration ranging from 10^{-15} to 10^{-6} M. Treatment media contained one of the following: vehicle, E2 (Steraloids, Newport, RI, USA), E2 conjugated with bovine serum albumin (E2BSA; Sigma, St. Louis, MO, USA), DPN or an ER antagonist, ICI182,780 (Tocris, Ellisville, MO, USA). The E2BSA is a membrane impermeable form of E2 that was used to test for membrane-associated effects of E2. It was filtered prior to use according to a previous protocol (Han *et al.* 2006) to remove unconjugated E2.

The ERβ agonist used in this study is the biologically active form of DPN, an ERβ agonist that is commonly used in its racemic form. Previously, it was separated into its two

enantiomers using chiral chromatography (Weiser *et al.* 2009) and the more selective and potent isomer was designated S-DPN based on the modeling studies of Sun and colleagues (Sun *et al.* 2003). Recently, it was found using enantiospecific synthesis that the more active and potent isomer is actually R-DPN (Carroll *et al.* 2012). Therefore, we now designate the more active form, R-DPN [previously labeled S-DPN, (Weiser *et al.* 2009; Oyola *et al.* 2012)] to be consistent with the current nomenclature. In these studies, we used the non-selective ER antagonist, ICI182,780 to block the actions of E2 and R-DPN and have not utilized an ERβ selective antagonist in our studies because we have not identified an appropriate antagonist that is selective and of sufficiently high affinity. Fetal Bovine Serum was charcoal stripped with dextran-coated charcoal to remove small molecules such as steroid hormones. All drugs were first diluted in 100% ethanol and then further diluted with media to achieve a final concentration of ethanol in the media of ≤ 0.001%. Control cells were treated with equivalent amounts of ethanol vehicle. One, six, twenty-four, or forty-eight hours later, cells were lysed and lysates were assayed for luciferase expression, using the Dual Luciferase assay according to manufacturer's protocol (Promega). Briefly, cells were lysed in passive lysis buffer for at least 15 min and luciferase activity of 10 μL cell lysate was measured by adding 100 μL luciferin substrate. Then, renilla activity was measured by adding 100 μL Stop and Glo solution. A 20/20 TD luminometer (Turner Designs, Sunnyvale, CA, USA) was used to measure the relative light units (RLUs).

Site-directed mutagenesis

We scanned the *TPH2* promoter region for a classic consensus ERE using the nucleotide sequences, GGTCA or TGACC, and identified a putative ERE half-site located at −792 nucleotides upstream of the TSS of the human *TPH2* promoter. This site was then mutated using the QuikChange II XL kit according to manufacturer's instructions (Stratagene, La Jolla, CA, USA). The antisense primer sequence 5′-GAAAAAGCTTATTAACATAAAATGGAGTTGAGCCATGA GTAAAAAAAATATGCTGATGGGAGGG-3′ was designed to mutate three specific sites of the ERE half-site (GGTCA → G**AGC**C, with bold nucleotides denoting the mutation). The presence of the mutation was confirmed by DNA sequencing (Operon).

Data analysis

The RLUs for each treatment were normalized to the respective empty expression vector control and data were expressed as percent change compared with vehicle-treated, empty expression vector controls. A half maximal effective concentration (EC50) for each hormone was calculated from the dose-response curves to allow comparison between hormones. One-way (Hormone treatment) or Two-way (hormone treatment vs. treatment time, hormone treatment vs. antagonist, hormone treatment vs. deletion construct, or hormone treatment vs. mutation) ANOVA and Bonferroni *post hoc* test were used where appropriate. Differences were considered significant when $p < 0.05$.

Results

Time course of *TPH2-luc* activity with E2 and R-DPN treatments

The time course for *TPH2-luc* induction following treatment with 100 nM E2 or R-DPN for 1, 6, 24, or 48 h showed that optimal treatment duration was at 24 h (Fig. 1). Significant increases in *TPH2-luc* activity were found after 24 h of treatment in both treatment groups. Two-way ANOVA showed significant main effects for both Hormone Treatment: $F(2, 139) = 3.084$, $p = 0.0489$ and Treatment Time: $F(3,139) = 4.578$, $p = 0.0301$. *Post hoc* analysis revealed significant differences between vehicle versus E2 ($p < 0.05$) and R-DPN ($p < 0.05$) at 24 h, but not at any other time points. Undifferentiated B14 cells endogenously expressed ERβ mRNA (Bethea *et al.* 2003); however, we also examined the effects of over-expression of ERβ by transiently co-transfecting B14 cells with *TPH2-luc* and human ERβ and found similar results (data not shown).

Dose response for E2− and R-DPN− induced *TPH2-luc* activity

Using the optimal 24-h treatment duration, we next constructed a dose-response curve for E2 and R-DPN induced *TPH2-luc* activity to determine the EC50. Dose-response curves of the hormone treatments ranged from 1^{-15} to 1^{-6} M and revealed an EC50 for E2− and R-DPN-induced *TPH2-luc* activation of 5.15×10^{-15} and 1.53×10^{-12} M, respectively, when B14 cells were transfected with *TPH2-luc* (Fig. 1b). We also examined the effects of over-expression of ERβ by transiently co-transfecting *TPH2-luc* with human ERβ and found similar results to cells transfected with *TPH2-luc* alone (data not shown). Similar E2 and R-DPN induced luciferase activities were found when B14 cells were transfected with an ERE-driven luciferase reporter (ERE-luc), with calculated EC50s of 3.69×10^{-17} M for E2 and 3.31×10^{-13} M for R-DPN (Fig. 1c).

E2 and R-DPN induced increases in *TPH2-luc* activity are cell line specific

To assess whether E2-induced *TPH2* activity was cell-type specific, we compared the effects of E2 and R-DPN-induced *TPH-luc* activity in the B14 and the hippocampal derived cell line, HT22, which is a non-serotonergic cell line that reportedly lacks ER expression (Behl *et al.* 1995; Green *et al.* 1998; Kim *et al.* 2001; Pak *et al.* 2005). Using the highest dose of E2 and R-DPN from the previous study (100 nM) to induce maximal *TPH2-luc* activation with both ligands, we found significantly increased *TPH2-luc* activity when *TPH2-luc* alone or *TPH2-luc* and ERβ were co-transfected into the B14 cell line (Fig. 2a and b, respectively). For *TPH2-luc* alone (Fig. 2a), there was a significant main effect of treatment using one-way ANOVA: $F(2,49) = 6.012$, $p = 0.0046$. *Post hoc* test revealed significant differences between vehicle versus E2 and R-DPN. For *TPH2-luc* and ERβ (Fig. 2b), there was a significant main effect of treatment using one-way ANOVA: $F(2,39) = 12.51$, $p < 0.0001$. *Post hoc* test revealed significant differences between vehicle versus E2 and R-DPN. In contrast, there was no significant effect of E2 or R-DPN to induce *TPH2-luc* activity when *TPH2-luc* alone or *TPH2-luc* and ERβ were co-transfected into the HT-22 cell line (Fig. 2c and d, respectively).

E2 and R-DPN increase *TPH2-luc* activity via the classical ERβ signaling pathway

To determine whether ER mediates the E2-induced effects on *TPH2-luc* activity, cells were treated with ICI 182,780, an ER antagonist, at 100× the concentration of the hormone treatment. ICI 182,780 (10 μM) was sufficient to significantly block both 100 nM E2− and R-DPN-induced increases of *TPH2-luc* (Fig. 3a). Two-way ANOVA showed a significant main effect of antagonist: $F(1, 36) = 8.44$, $p = 0.0062$. *Post hoc* test showed significant differences between E2 versus E2 + ICI: $p = 0.0426$; and R-DPN versus R-DPN+ICI: $p = 0.0381$; Vehicle versus E2: $p = 0.0081$; and Vehicle versus R-DPN: $p = 0.003$. We also examined the effects of over-expression of ERβ by transiently co-transfecting *TPH2-luc* with human ERβ and found similar results (data not shown). To determine whether classical ERβ signaling pathways, or other membrane associated receptors are involved in the E2-induced *TPH2* activity, the effects of E2BSA, a cell membrane impermeable conjugate of E2, was compared to that of E2 and R-DPN (Fig. 3b). Despite robust and significant activation of *TPH2-luc* activity following E2 and R-DPN treatment, E2BSA had no significant effects on *TPH2-luc*. One-way ANOVA showed a significant main effect of treatment: $F(3,15) = 9.957$, $p = 0.0007$. *Post hoc* analysis revealed significant differences between vehicle versus E2 and R-DPN. These results support the hypothesis that a classical ERβ signaling pathway mediates the hormone-induced *TPH2* promoter activation.

An ERE half-site on the promoter region is important for the E2 and R-DPN-induced *TPH2* activity

To identify the regulatory region important for the ligand-induced *TPH2* transcriptional activity, two deletion constructs, −637 *TPH2* and −158 *TPH2*, of the promoter fragment were made and transfected into the B14 cells (Fig. 4a). Both E2 and R-DPN treatment significantly increased luciferase activity of the full length *TPH2-luc*. In contrast, E2− and R-DPN-induced luciferase activity was abolished by the deletion constructs (Fig. 4b). Two-way ANOVA showed a significant interaction effect: $F(4, 43) = 3.251$, $p = 0.0204$; treatment effect: $F(2,43) = 4.118$, $p = 0.0231$; and deletion construct effect: $F(2,43) = 9.622$, $p = 0.0004$. *Post hoc* test revealed significant differences between vehicle versus E2 or R-DPN when using full length *TPH2*. Next, we scanned the TPH2 5′UTR for classical ERE sites and identified an ERE half site beginning at −792 nt upstream of the TSS. Because the putative ERE half-site was eliminated in both of the deletion constructs, we performed site-directed mutagenesis of the ERE half-site in the full length *TPH2* promoter to determine whether this region of the promoter was required for the ERβ-induced *TPH2* transcription activity (Fig. 4c). Mutation of the ERE half-site abolished the effect of E2 and dramatically reduced the effect of R-DPN on *TPH2-luc* activity. Two-way ANOVA showed a significant main effect of interaction: $F(2,34) = 5.842$, $p = 0.0066$; mutation: $F(1,34) = 8.170$, $p = 0.0072$. *Post hoc* test revealed significant differences between vehicle versus E2 in Full length *TPH2*. To our knowledge, this is the first report identifying a functional ERE half-site on the *TPH2* promoter region.

Discussion

In this study, we examined the functional regulation of the human *TPH2* gene by E2 to identify the region within the *TPH2* promoter that ERβ may use for this interaction. We

found that both E2 and R-DPN increased *TPH2* promoter activity, as measured by increases in *TPH2-luc* activity in the serotonergic B14 cell line, *in vitro*. This E2-induced *TPH2-luc* activity was blocked by co-treatment of the B14 cells with the ER antagonist, ICI182,780. While E2 and R-DPN treatment produced robust *TPH2-luc* activity, the cell membrane impermeable conjugate, E2BSA, was unable to affect luciferase activity. As B14 cells solely expressed ERβ, and not ERα (Bethea *et al.* 2003) these data support the concept that E2 induced *TPH2* transcriptional activity is through classical ERβ signaling pathways. Moreover, our studies identified a classical ERE half-site located at nucleotides −792 to −787 from the TSS on the *TPH2* promoter, and showed that this site is functional in that the deletion and mutation of this sequence blocked the E2-induced *TPH2-luc* activity. This finding confirms that the ERE half-site plays an important role in the ER mediated regulation of *TPH2* transcriptional activity.

Previous studies examining the regulation of the TPH2 promoter have identified select regions of the 5′UTR that play a critical role in gene expression via interaction with multiple transcriptional factors. Such studies have also identified numerous putative DNA elements in the 5′-UTR of the *TPH2* promoter, including a cAMP-response element (CRE), Sp1, AP-1, AP-2, CCAAT/enhancer binding protein (C/EBP), and TATA box, GREF/PRE, STRE, Sox-5, SBPF, GFI1, GATA, NEUROD1, Brn-2, IA-1, Myf-3, RU49, VDR/RXR (Remes Lenicov *et al.* 2007; Chen *et al.* 2008). In addition, Patel and colleagues confirmed a binding site for the bipartite neural restrictive silencing element (NRSE) (Patel *et al.* 2007). Examination of human *TPH2* polymorphisms also revealed 5′-UTR regions of *TPH2* that modulate gene expression (Scheuch *et al.* 2007; Chen and Miller 2009). The results of these studies suggest that there are a number of distinct transcriptional regulatory elements within the *TPH2* 5′-UTR that regulate transcriptional activity. However, to our knowledge, this study is the first to describe a functional site in the *TPH2* promoter that provides E2 sensitivity.

To characterize the effects of E2-induced activation of the *TPH2* gene, we first examined the time course of the effects of hormone treatment on the *TPH2* promoter activity. The maximal increase in *TPH2-luc* activity occurred after 24 h of E2 treatment. Luciferase activity was also significantly increased after 24 h of R-DPN, and although further elevations occurred after 48 h, these were not statistically significant. At this point, the ½ life of R-DPN or E2 in B14 cells has not been explored. An examination of a later time point beyond 48 h may reveal distinct kinetic profiles between E2 and R-DPN in their interaction with ERβ to induce *TPH2* promoter activity.

In addition to differences in the kinetic profile of *TPH2* induction, E2 and R-DPN also differ in their relative potencies to activate the *TPH2* promoter. Estradiol had a much greater potency than R-DPN in activating *TPH2-luc*, as measured by a lower EC50 value for E2 compared to R-DPN. This higher potency of E2 in *TPH2-luc* induction may be in part because of the greater binding affinity of E2 over R-DPN for the ERβ (Meyers *et al.* 2001; Weiser *et al.* 2009; Carroll *et al.* 2012), but this approximately 300 fold difference does not completely explain the much higher potency that E2 possesses for transcriptional regulation of the TPH2 promoter.

In these studies, we observed that the EC50 for both E2 and R-DPN in B14 cells is well below the typical range for binding the receptor (Paech *et al.* 1997; An *et al.* 1999; Kulakosky *et al.* 2002). This extremely high potency of E2 in driving the *TPH2* promoter may be because of one of several factors including the artificial condition created by the *in vitro* system herein, or a unique property of the B14 cell line. Despite the robust E2 and R-DPN-induced *TPH2-luc* activation in B14 cells, the same hormone treatment, when tested in a separate cell line, HT22, transfected with *TPH2-luc* did not have any effect on the *TPH2* promoter activity. As HT22 cells originated from embryonic mouse hippocampal cells and are non-serotonergic (Behl *et al.* 1995; Green *et al.* 1998; Kim *et al.* 2001; Pak *et al.* 2005), this cell line may not possess the appropriate cellular machinery for E2-induced activation of tph2. On the other hand, B14 cells are derived from embryonic rat serotonergic cells and also express Tph1 and 5-HT, despite the low levels of expression in the undifferentiated state (White *et al.* 1994; Eaton *et al.* 1995; Bethea *et al.* 2003). Therefore, these cells are most likely equipped with the necessary constellation of co-regulators and transcription factors involved in *TPH2* transcription.

Cell-type specific induction of human (and rat) *TPH2* transcription has been previously reported. Remes Lenicov and colleagues (Remes Lenicov *et al.* 2007) have shown that calcium mobilization induced *TPH2* transcription in the B14 cells, but not in the Tph2-negative non-neuronal L6 cell line, or in the Tph2-expressing pituitary GH4C1 cell line. Thus, these results suggest that there is a distinct milieu of transcriptional factors and co-regulators that uniquely impact *TPH2* transcription, even within cell lines that are capable of producing 5-HT. These studies underscore the importance of cell-type specific effects on *TPH2* transcription and suggest that ERβ-bound estrogens may require recruitment of a cell-specific set of co-regulators and transcriptional factors for *TPH2* gene activation.

Indeed, ERβ plays an important role in estrogen-induced *TPH2* promoter activity. We found that E2 and R-DPN-induced *TPH2-luc* activity was blocked when cells were co-treated with a non-selective ER antagonist. Given that our B14 cell line solely express ERβ, and not ERα, these results support the hypothesis that ERβ mediates the estrogen-induced *TPH2* promoter activity. Moreover, despite a robust induction of *TPH2* promoter following E2 and R-DPN, the membrane impermeable form of E2, E2BSA, was not able to induce *TPH2-luc* activity. Collectively, these studies suggest that there is likely a direct interaction of E2 with the *TPH2* promoter utilizing classical ERβ signaling pathways to increase transcriptional activity.

Thus far, the regulatory regions involved in estrogen-induced *TPH2* promoter activity have not been identified. Therefore, we scanned the promoter region for a consensus palindromic ERE sequence and found that the *TPH2* promoter does not contain a classical ERE; however, a putative ERE half-site was found at −792 nucleotides upstream of the TSS of the human *TPH2* promoter. Our studies now demonstrate that this ERE half-site on the *TPH2* promoter is important for the E2 and R-DPN-induced *TPH2* promoter activity, as deletion and site-directed mutation of the ERE half-site blocked E2 and R-DPN-induced *TPH2-luc* activity. In addition, E2 and R-DPN treatment of these B14 cells also induced luciferase activity of transiently transfected ERE-luc, suggesting that the environment of the cells is also sufficient for hormone interaction with the ERE to induce transcriptional activation.

Future studies are warranted to determine the specific environment essential for the ERβ-bound E2 to interact with this ERE half-site on the *TPH2* promoter, as the identification of co-regulatory proteins and alternative transcription factors involved in controlling Tph2 expression is of important biological relevance.

The regulation of the Tph2 gene by estrogens has been shown to have important roles in neurobiology, notably related to the regulation of anxiety-like and depressive-like behaviors. Recent studies have shown that E2 treatment of ovariectomized rats increases Tph2 mRNA in the DRN (Hiroi *et al.* 2006), a major source of 5-HT neurotransmitter in the forebrain. This increase in Tph2 is important for the anxiolytic effects of E2, as over-expression or knockdown of Tph2 mRNA in the DRN of the ovariectomized rats mimicked and reversed the anxiolytic effects of E2, respectively (Hiroi *et al.* 2011). Furthermore, the direct bilateral stereotaxic implantation of racemic DPN-containing wax pellets flanking the DRN in ovariectomized rats also increased Tph2 mRNA expression in the DRN and induced anti-depressant like effects (Donner and Handa 2009). Collectively, these studies suggest that the anxiolytic and antidepressant effects of E2 may, in part, be mediated by increases in Tph2 expression via the ERβ selective activation of Tph2 expression within neurons of the DRN. This in turn may result in changes in the forebrain 5-HT neurotrans-mission that have important ramifications for regulating behaviors.

Although it is feasible that ERα may have effects on the *TPH2* promoter, we did not test it here as B14 cells used in this study were derived from rats and do not express ERα. However, it would be important to test the effects of both ERα and ERβ on *Tph2* promoters of different species, as distribution of ERα and ERβ on 5-HT neurons differs in distinct species and there is a marked species specific regulation of Tph2 expression by E2. Chronic E2 treatment increases Tph2 mRNA in the DRN in rats (Hiroi *et al.* 2006; Donner and Handa 2009) and macaques (Sanchez *et al.*, 2005), but not in mice (Clark *et al.*, 2005). This may, in part, be because of the differential distribution of the two ERs within the DRN of each species. For instance, like rats, non-human primates express ERβ, but not ERα, in 5-HT neurons (Bethea 2002), but ERα and ERβ are co-expressed in some 5-HT neurons of mice (Mitra *et al.*, 2003). Therefore, it is possible that species specific regulation of the brain serotonergic system by the estrogens may result in distinct biological functions, allowing adaptive responses to differential environment encountered by each species.

Conclusions

The results from this study add to the growing body of evidence showing that ERβ mediates the E2-induced increases in Tph2 activity. Specifically, a newly identified ERE half-site located within the *TPH2* promoter region plays an essential role in activating E2 induced *TPH2* activity. Further analysis of the co-regulators and transcription factors involved in mediating the interaction among ERβ, E2, and *TPH2* may reveal novel pharmacological targets that could be used alone or in conjunction with current treatment options available for women suffering from anxiety and affective disorders.

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Abbreviations used

5-HT	serotonin
B14	RN46A-B14
DPN	diarylpropionitrile
DRN	dorsal raphe nucleus
E2	17β-estradiol
E2BSA	17β-estradiol conjugated with bovine serum albumin
EC50	half maximal effective concentration
ERE	estrogen response elements
ERE-luc	estrogen response element driven luciferase reporter
ERβ	estrogen receptor-beta
RLUs	relative light units
TPH2-luc	tryptophan hydroxylase-2 promoter cloned into the luciferase reporter vector
Tph2	tryptophan hydroxylase-2
TSS	transcription start site
UTR	untranslated region

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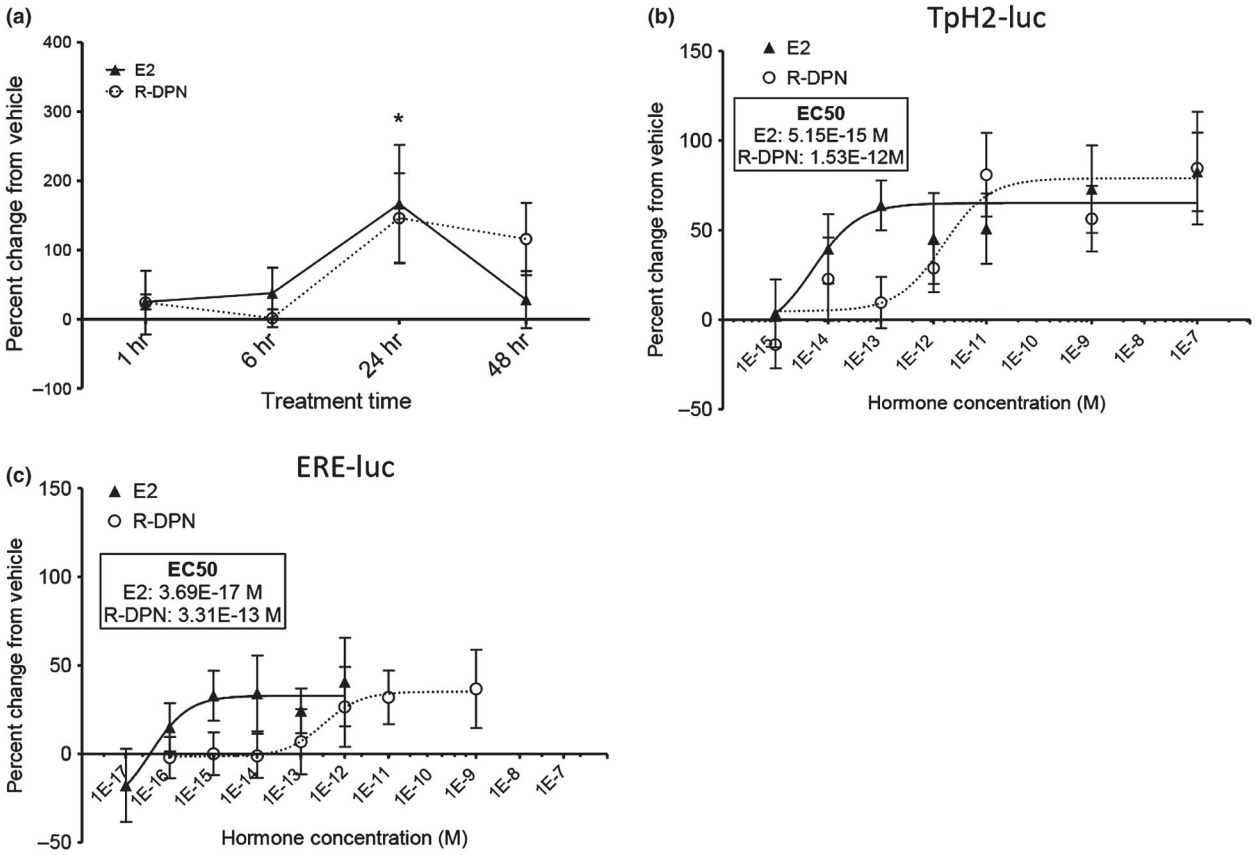

Fig. 1.

17β-estradiol (E2) and R-diarylpropionitrile (R-DPN) time- and dose-dependently increase tryptophan hydroxylase-2 promoter cloned into the luciferase reporter vector (*TPH2-luc*) activity. (a) The effects of E2 and R-DPN treatment duration on *TPH2-luc* activity. B14 cells were transiently transfected with *TPH2-luc*. Twenty-four hours later, cells were treated with vehicle, 100 nM E2 or 100 nM R-DPN for either 1, 6, 24, or 48 h. Cells were then trypsinized and assayed for *TPH2*-luc activity. $n = 4–9$. (b, c) Dose response curves for ligand regulation of *TPH2-luc* (b) and ERE-luc (c) activity. B14 cells were treated for 24 h with vehicle or with increasing concentrations of E2 or R-DPN. B14 cells were co-transfected with *TPH2-luc* only (b, $n = 9–21$), or ERE-luc only (c, $n = 9–18$). Twenty-four hours later, cells were lysed and assayed for luciferase activity. All values are expressed as mean percent change of *TPH2-luc* activity from vehicle treatment ± SEM. *Significantly different from vehicle group, $p < 0.05$.

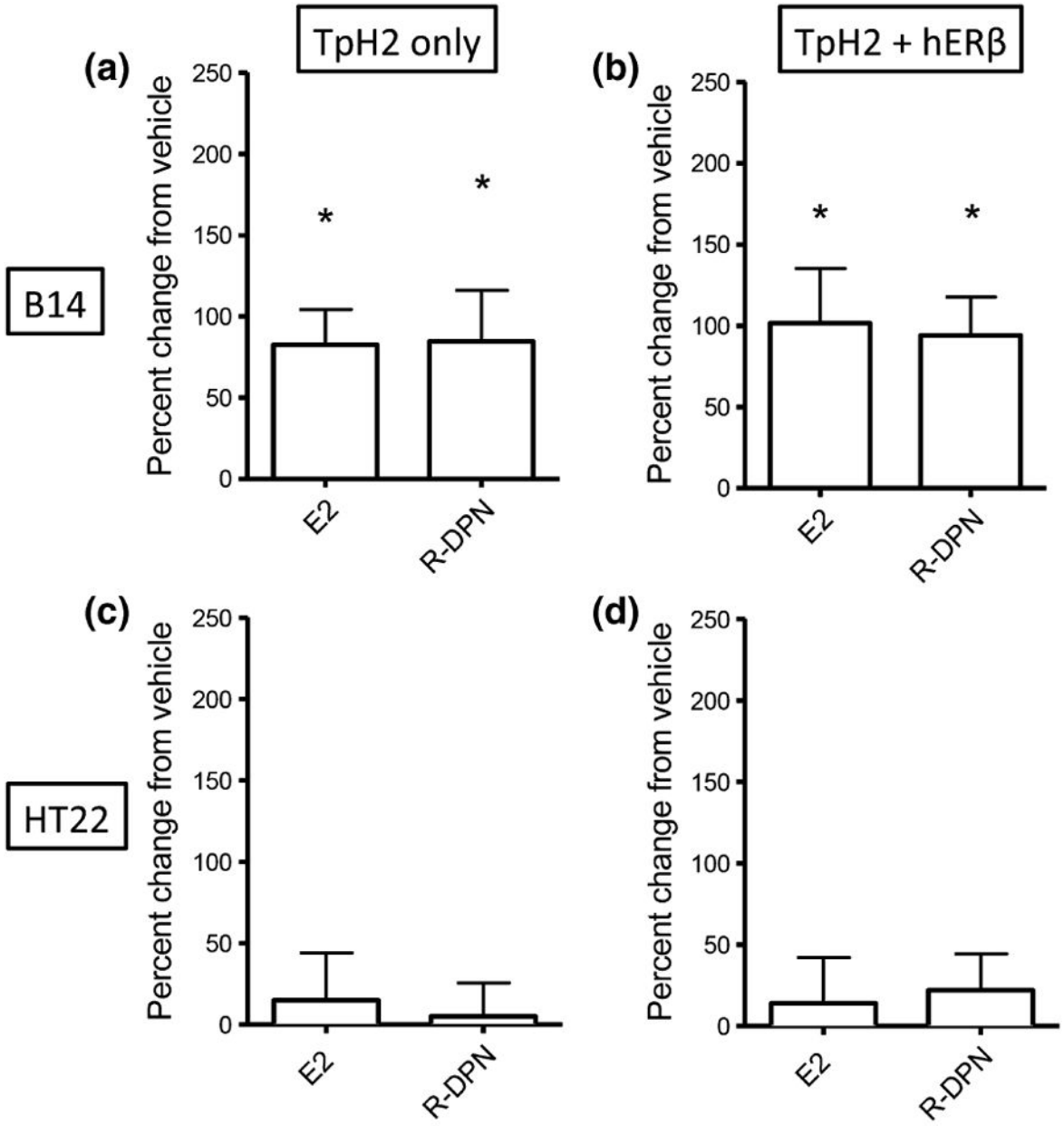

Fig. 2.

17β-estradiol (E2) and R-diarylpropionitrile (R-DPN)-induced tryptophan hydroxylase-2 promoter cloned into the luciferase reporter vector (*TPH2-luc*) activity is cell type specific. B14 (a and b, $n = 12–16$) or HT22 (c and d, $n = 4–5$) cells were either transfected with *TPH2-luc* only (a and c) or co-transfected with *TPH2-luc* and human ERβ expression vectors (b and d). Twenty-four hours later, cells were lysed and assayed for luciferase activity. Values are expressed as mean percent change of *TPH2-luc* activity from vehicle treatment ± SEM. *Significantly different from vehicle group, $p < 0.05$.

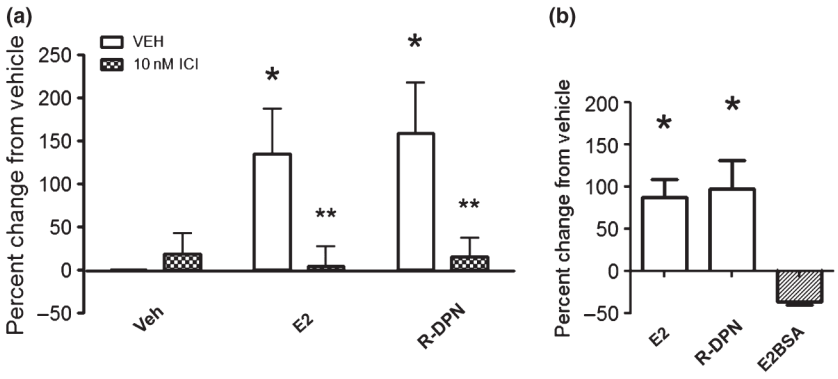

Fig. 3.

Estradiol-induced *TPH2* promoter activity is mediated by the classical estrogen receptor-beta (ERβ) signaling pathway. (a) ER antagonist, ICI182,780 treatment blocks 17β-estradiol (E2) and R-diarylpropionitrile (DPN)-induced tryptophan hydroxylase-2 promoter cloned into the luciferase reporter vector (*TPH2-luc*) activity. B14 cells were transfected with *TPH2-luc*. B14 cells were then co-treated 24 h later with 100 nM E2 or R-DPN and 10 uM ICI 182,780. Twenty-four hours later, cells were lysed and assayed for luciferase activity. n = 6–7. (b) A membrane impermeable form of E2 has no significant effect on *TPH2-luc* activity. B14 cells were transfected with *TPH2-luc* and treated with 100 nM E2, R-DPN, or E2 conjugated with BSA (E2BSA), which is incapable of crossing the cell membrane. Twenty-four hours later, cells were lysed and assayed for luciferase activity. n = 4. Values are expressed as mean percent change of *TPH2-luc* activity from vehicle treatment ± SEM. Values are expressed as mean percent change of *TPH2-luc* activity from vehicle treatment ± SEM. *Significantly different from vehicle group, **Significantly different from vehicle-treatment group, $p < 0.05$.

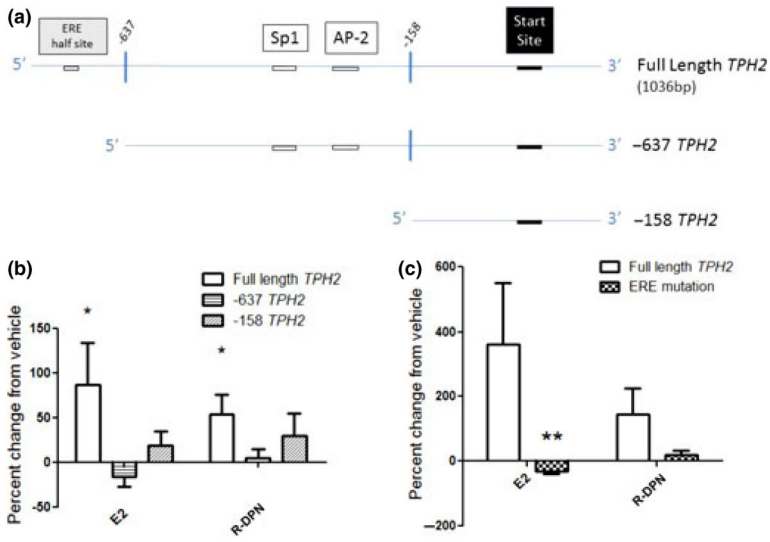

Fig. 4.

Estrogen response element (ERE) half-site plays an important role in the hormone regulation of the tryptophan hydroxylase-2 promoter cloned into the luciferase reporter vector (*TPH2-luc*) promoter. (a) Schematic diagram of the 5′ end deletion constructs. Full length (1036 bp) *TPH2-luc* construct was progressively deleted from the 5′ end of the promoter using restriction enzyme digests. Note that the ERE half-site of the *TPH2* promoter is not present in the −637 or the −158 *TPH2* constructs. (b) B14 cells were transfected with the *TPH2-luc* constructs, 24 h prior to hormone treatment (Veh or 100 nM E2, 100 nM R-DPN) and luciferase activity was measured 24 h later. $n = 5–6$. (c) B14 cells were transfected with Full-length *TPH2-luc* or *TPH2-luc* construct with site directed mutagenesis of the ERE half-site. Cells were treated 24 h later with vehicle, 100 nM E2, or 100 nM R-DPN. Cells were then lysed and assayed for dual luciferase activity, 24 h following hormone treatment. $n = 5–7$. Values are expressed as percent change of *TPH2-luc* activity from vehicle treatment ± SEM. *Significantly different from vehicle group, **Significantly different from Full length-*TPH2* group, $p < 0.05$.