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Varied Mechanisms of Oestradiol Mediated Regulation of Dopamine β-Hydroxylase Transcription

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Abstract

Experiments *in vivo* and in cell culture demonstrated that oestradiol induces dopamine β-hydroxylase (DBH) gene transcription. Here we examined oestrogen-responsive elements of the rat DBH gene promoter to characterize the mechanisms of oestradiol-induced DBH transcription. Various mutations and deletions of DBH promoter reporter constructs were tested for responsiveness to 17 β-oestradiol (E_2). Mutation of the half palindromic oestrogen response element (ERE) at position −759 reduced the response to E_2 in PC12 cells co-transfected with ERα indicating a functional role for this motif. In cells co-transfected with ERβ, mutations at the −759 site were unresponsive to E_2. To characterize the additional E_2 responsive elements, mediated by ERα, the DBH promoter was truncated to the proximal 249 or 200 nucleotides upstream of the transcription start site. Despite either truncation, 10 nM E_2 still elicited about a two-fold induction of DBH promoter activity. Mutation of a possible ERE like sequence at −59 had no effect. The lack of a functional ERE in the proximal region of the rat DBH promoter despite E_2-mediated DBH promoter activity, suggests regulation by a non-classical mechanism, such as a membrane-initiated signaling pathway. Moreover, the induction of DBH promoter activity and the rise in DBH mRNA levels were observed within hours. To determine whether membrane-initiated E_2 signaling is involved in rat DBH gene transcription, a membrane impermeable E_2 conjugate, E_2BSA, was used. Incubation with E_2BSA induced luciferase promoter activity and elicited a significant rise in DBH mRNA levels in the ERα transfected cells. The findings indicate two different mechanisms whereby DBH transcription is regulated by E_2 in the presence of ERα. The results implicate both genomic and membrane-initiated mechanisms, mediated by ERα, in E_2-induced DBH gene transcription.

Introduction

Oestrogens play a role in sex-specific differences observed in many disorders. For example, men have a greater risk of cardiovascular disease and hypertension than premenopausal women, with equal incidence between men and postmenopausal women, whereas women have a greater risk of depression, eating disorders and nicotine induced lung cancer (1–4). The role of oestrogens in the regulation of these sex-specific differences is likely by acting on numerous targets. The dopamine β-hydroxylase (DBH) gene has been implicated as one such target.

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The DBH gene encodes the catecholamine biosynthetic enzyme that catalyzes the hydroxylation of dopamine to form norepinephrine (NE). NE is an important hormone and neurotransmitter, controlling cardiovascular functions, such as blood pressure, as well as other functions including memory, learning, attention, mood, pain, and the stress response (5–9). Sex differences are found in the morphology of locus coeruleus (LC), the center of the majority of the brain's noradrenergic neurons in adult rats and humans (10). In female rats, the volume of the LC is greater and contains more DBH immunoreactive neurons than in male rats (11,12). Plasma DBH protein activity, which correlates with DBH protein levels (13), has been shown to be greater in female rats throughout the lifetime, as compared to males (14).

We previously demonstrated that injections of oestradiol benzoate (EB) to ovariectomized rats elevate DBH mRNA levels in the LC (15) as well as in the adrenal medulla (16). Furthermore, treatment of PC12 cells, exogenously expressing either oestrogen receptor (ER) α or β, with 17 β-oestradiol (E_2) induces DBH promoter activity, indicating that E_2 regulation of DBH occurs at the transcriptional level (15,17).

Oestrogens can regulate gene transcription by binding to ERs, which function as ligand-activated transcription factors. Oestrogen-induced modulation of transcription through ERs occurs via several different mechanisms (reviewed in (18,19)). In the classical mechanism, oestrogen bound to ERs induces dimerization and subsequent binding to oestrogen response elements (EREs) of target gene promoters. ERs can also regulate transcription by an ERE-independent mechanism, whereby ERs tether to transcription factors via protein-protein interactions for binding to target gene promoters via cognate *cis*-regulatory elements. ERs can also be activated by a ligand-independent mechanism via phosphorylation by growth factor-activated protein kinase cascades, leading to binding to EREs of target gene promoters. Whereas the effects of these genomic mechanisms are often observed on the time scale of hours to days, oestrogens can also act by a more rapid non-genomic mechanism initiated by ERs localized at the plasma membrane [reviewed in (20,21)]. These membrane ERs activate signaling cascades leading to second messenger production and subsequent transcription factor phosphorylation, and therefore modulate gene expression. The genomic and non-genomic mechanisms may thus converge to potentiate transcriptional regulation by oestrogens (19,22).

Although we have previously shown that E_2 induces DBH transcription both *in vivo* and in cell culture (15,17), the underlying mechanisms are not yet known. In this study, we aimed to identify the oestrogen-responsive elements of the rat DBH gene promoter and to characterize the mechanisms of E_2-induced DBH transcription. The results indicate the importance of both classical and non-classical mechanisms in ERα-mediated induction of DBH gene transcription in response to E_2.

Materials and Methods

PC12 cell culture and maintenance

PC12 cells (23) were grown in 100 mm tissue culture dishes (Falcon, Lincoln Park, NJ) with Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), 5% heat inactivated horse serum (Gemini Bio-Products, Woodland, CA) and 0.5% penicillin-streptomycin (Gibco BRL, Grand Island, NY) at 37°C in a humidified incubator at 7% CO_2 with the media changed every other day as previously described (15,24).

To reduce oestrogens or oestrogenic compounds in the media, cells were pretreated for at least one day at approximately 50% density in phenol red free DMEM (Gibco BRL, Grand

Island, NY) supplemented with 10% fetal bovine serum, 5% heat inactivated horse serum, and 0.5% penicillin-streptomycin in 6-well tissue culture plates (Falcon, Lincoln Park, NJ). This media was replaced with stripped media, containing phenol red free DMEM supplemented with 10% charcoal stripped fetal bovine serum (Atlanta Biologicals, Norcross, GA), 5% dialyzed donor horse serum (Gibco BRL, Grand Island, NY), and 0.5% penicillin-streptomycin one day prior to transfection.

DBH promoter constructs and site direct mutagenesis

The rDBH(−1624/+21)/Luc plasmid, containing the first 1624 bp of the rat DBH promoter relative to the transcriptional start site (McMahon and Sabban 1992), fused to the firefly luciferase (Luc) gene in the pGL3-basic vector (Promega, Madison, WI), was constructed by digesting the rDBH(−2236/+21)/Luc plasmid (Serova et al. 2002) with KpnI, followed by religation. The rDBH(−249/+21)/Luc construct was made as previously described (25).

The mutant (mut) reporter constructs −759ERE/Mut1 and −759ERE/Mut2 were generated using the wild-type plasmid rDBH(−1624/+21)/Luc as the template for PCR using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The deletion (del), mutation, and truncation reporter constructs −56ERE/Del, −56ERE/LeftMut, −56ERE/RightMut, rDBH(−200/+21)/Luc, and rDBH(−100/+21)/Luc were generated using the wild-type plasmid rDBH(−249/+21)/Luc as the template. The forward and reverse oligonucleotides used to generate the different constructs are shown in Table 1. All plasmids were isolated using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA) and dissolved in TE buffer. All deletions, mutations, and truncations were confirmed by sequencing (Davis Sequencing, Davis, CA).

Transient transfection of PC12 Cells

Transient co-transfection of PC12 cells with rDBH/Luc reporters and expression vectors for mouse ERα (pcDNA3/mERα) or ERβ (pSG5/mERβ), kindly provided by Dr. E. R. Levin (University of California, Irvine, CA), was performed as previously described (15,17). In some experiments, cells were additionally co-transfected with β-galactosidase expression vector (pSV β-gal; Promega, Madison, WI) to normalize the transfection efficiencies.

For transfection, 1.5 μg of luciferase reporter plasmid DNA, 1.5 μg expression vector plasmid DNA (ERα or ERβ) and 0.75 μg pSV β-gal DNA were diluted to 150 μl in phenol red-free DMEM, and mixed with SuperFect Transfection Reagent (Qiagen, Valencia, CA) in the ratio of 1:2 (7.5 μl SuperFect for 3.75 μg total DNA), according to the manufacturer's protocol. After 10 min the transfection complexes were diluted with 600 μl of stripped media and the total volume was gently added to the cells and incubated at 37°C in a humidified incubator at 5% CO_2. After 3 h the complexes were replaced with fresh stripped media and the cells were incubated for an additional 24 h.

Preparation of stock E_2BSA

β-estradiol-6-(O-carboxy-methyl) oxime-bovine serum albumin (E_2BSA) (Sigma, St. Louis, MO) was carefully prepared just prior to use to remove any free, contaminating oestradiol, as previously described (26,27). Briefly, a 10 μM E_2BSA stock solution was prepared in 50 mM Tris-HCl, pH 8.5 and filtered through a Microcon YM-3 (membrane molecular weight cut-off of 3,000) centrifugal filter device, according to conditions described previously (27).

Pharmacological treatments and luciferase assay

After transfection for 24 h, the cells were treated with either 17 β-oestradiol (E_2; Sigma, Saint Louis, MO) dissolved in ethanol, not exceeding 0.01% of the final concentration, or purified E_2BSA. Controls for E_2 and E_2BSA treatments were the vehicle or BSA in the

vehicle, respectively. The cells were then further incubated under the same conditions for various times from 1–48 h (see figure legends). After incubation, the cells were harvested in phosphate buffered saline and collected by centrifugation. The lysates were prepared by re-suspending the cell pellet in Passive Lysis Buffer (Promega, Madison, WI). For luciferase reporter assays, an aliquot of each lysate was mixed with five volumes of luciferase assay reagent (Promega, Madison, WI) and assayed for firefly luciferase activity within the linear range by immediately measuring in a luminometer (TD-20/20 Turner, Sunnyvale, CA). β-galactosidase activity was assayed using the β-Galactosidase Enzyme Assay System by Promega (Madison, WI) according to the manufacturer's protocol. Otherwise, the concentration of protein in the lysates was determined with Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA) using Synergy HT plate reader (BioTek, Winooski, VT). Luciferase activity was normalized to the β-galactosidase activity or protein levels in each lysate. Each experimental group contained 5–6 replicate culture wells and each experiment was repeated at least twice.

Isolation of RNA from PC12 cells, northern blot analysis, and quantitative RT-PCR

For RNA isolation, the cells were homogenized in RNA-Stat-60 (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's protocol. Northern blot analysis was performed as previously described (16). Briefly, RNA was fractionated on 1.2% agarose gels and transferred to Gene-Screen Plus membranes (New England Nuclear, Boston, MA). Hybridization was performed with a [^{32}P]α-dCTP rat DBH cDNA probe and DNA for 18S rRNA as a control in ULTRA-Hyb solution (Ambion, Austin, TX) at 42°C. The blots were washed and exposed to BioMax film (Kodak, Rochester, NY) within the linear signal range. The blots were subsequently re-probed using a probe for 18S rRNA. Autoradiograms were analyzed by Image-Pro-Analysis software (Media Cybernetics). DBH mRNA levels were normalized to 18S rRNA levels.

For quantitative RT-PCR, total RNA (800 ng), quantitated by spectrophotometric analysis, was reverse transcribed in 5 μl total volume using AMV reverse transcriptase (Roche, Indianapolis, IN). The 5 μl RT mixture contained 1× RT buffer (Roche, Indianapolis, IN), 1 mM dNTP mix, 5 units of RNAse inhibitor (Roche, Indianapolis, IN), 1 μM specific reverse transcription primer (5'-AGGCTGCAAGGCTTCTGTGATGGC-3') and 5 units of AMV reverse transcriptase (Roche, Indianapolis, IN).

PCR reactions, with a total volume of 20 μl, were set up with a final concentration of 1× LightCycler DNA Master SYBR Green I, 0.5 μM of each the forward (5'-CCACGCCATGCAGTTCTTCACCA-3') and reverse (5'-AGGCTGCAAGGCTTCT-GTGATGGC-3') primers, 3 mM $MgCl_2$, and 2 μl of the standard DBH cDNA or cDNA with unknown concentration. A standard curve plotted using serial dilutions from 2 ng to 0.2 pg of DBH cDNA was used for quantification by the Fit Points method. The specificity of the amplified target sequences was confirmed with melting curve analysis by comparing its melting temperature with the melting temperatures of the standards as a positive control. The values for DBH mRNA were normalized to levels of total RNA.

Statistical analysis

Data are presented as mean ± SEM of representative experiments. Statistical significance was evaluated by Student's *t*-test for two experimental groups or by one way ANOVA followed by Fisher's post-hoc comparisons for more than two experimental groups. A value of $p \leq 0.05$ was considered significant.

Results

Mapping oestrogen responsive elements in the rat DBH promoter

We have previously shown that treatment of PC12 cells with E_2 induces reporter activity under the control of the proximal 2236 bases of the rat DBH promoter [rDBH(−2236/+21)/Luc] (15) Analysis of the *cis*-regulatory elements in this promoter region identified perfect half palindromic EREs (GGTCA or TGACC) at positions −1968, −759 and −739, relative to the transcriptional start site [MatInspector and Transcription Element Search Software (TESS)]. Truncation of this construct to contain the proximal 1624 bases of the rat DBH promoter [rDBH(−1624/+21)/Luc] demonstrated a similar ability to induce luciferase activity after 2.5 or 10 nM E_2 treatment in cells co-transfected with ERα (Fig. 1B) or ERβ (data not shown).

To determine whether the half ERE at −759 is functional, the GGTCA sequence at −759 of rDBH(−1624/+21)/Luc was mutated to either GGTCT or GTACA (Fig. 1A). In PC12 cells co-transfected with ERα expression vector, treatment with 2.5 and 10 nM E_2 were able to induce luciferase activity under the control of both mutated DBH promoter constructs, but the induction was significantly reduced compared with the wild-type DBH promoter construct (Fig. 1B). This indicates that the site at −759 is functional and plays a role in the response to E_2 in the presence of ERα. In cells co-transfected with ERβ, mutants of the −759 site in the rDBH(−1624/+21)/Luc vector, in contrast to the wild type promoter, were unresponsive to treatment with 2.5 or 10 nM E_2 (data not shown), indicating that this site is sufficient for the response to E_2 with ERβ. However, at least one additional oestrogen responsive motif exists downstream of the −759 position of the rat DBH promoter for the response with ERα. Therefore, we focused on identifying the additional ERα-dependent oestrogen responsive motifs of the rat DBH promoter.

The rDBH(−1624/+21)/Luc plasmid was truncated to determine the additional ERα-dependent oestrogen responsive motif(s) of the rat DBH promoter (Fig. 2A). The constructs with the proximal 249 or 200 nucleotides upstream of the transcription start site display about two-fold elevation in reporter activity in response to 10 nM E_2. With rDBH(−100/+21)/Luc reporter construct the response to E_2, while still significant, was greatly reduced (Fig. 2B).

Further analysis of the rat DBH promoter identified a motif with some homology to an ERE (28) at position −56 to −43 (Fig. 3A). To determine the functionality of this element, site-directed mutagenesis was used to mutate or delete this region of the rDBH(−249/+21)/Luc plasmid. Upon treatment with 10 nM E_2, luciferase activity was induced by about two-fold with all three mutants or with the wild type plasmid (Fig. 3B). Therefore, the −56 to −43 site is not a functional ERE.

Time course of the changes with oestradiol

The time course of the response of DBH mRNA and DBH promoter activity with the short rDBH(−200/+21)/Luc plasmid to E_2 was examined in PC12 cells transfected with ERα expression vector and treated with 10 nM E_2. DBH mRNA levels are elevated approximately two-fold after 6, 12, and 24 h of treatment with 10 nM E_2 (Fig. 4A). By 48 h the levels were no longer increased.

The promoter activity was determined at various times after addition of E_2 to PC12 cells co-transfected with rDBH(−200/+21)/Luc and ERα. A rise in luciferase activity was evident by 3 h and remained significant during 24 h (Fig. 4B).

Involvement of a membrane-initiated oestradiol signaling pathway

The lack of a functional ERE in the proximal region of the rat DBH promoter despite E_2-mediated DBH promoter activity, and the relatively rapid induction of promoter activity suggests regulation by a non-classical mechanism, such as a membrane-initiated signaling pathway.

To determine whether membrane-initiated E_2 signaling is involved in rat DBH gene transcription, a membrane impermeable E_2 conjugate, E_2BSA, was used. As in our previous study (27), even 100 nM E_2BSA did not induce a luciferase reporter construct driven by a promoter containing three canonical EREs in tandem, showing that there was no dissociated free E_2 (data not shown). PC12 cells were transfected with ERα expression vector then treated with 100 nM E_2BSA or BSA for 24 h. DBH mRNA levels were elevated compared to BSA-treated controls (Fig. 5A) demonstrating that E_2, acting by an ERα-mediated membrane-initiated signaling pathway can induce endogenous DBH mRNA levels.

Next the effects of various concentrations of E_2BSA, from 0.1 to 100 nM, were examined for their effect on DBH promoter activity. Both 10 and 100 nM E_2BSA elevated luciferase promoter activity in PC12 cells co-transfected with ERα expression vector and rDBH(−249/+21)/Luc (Fig. 5B). These results demonstrate that DBH gene transcription is regulated by a membrane-initiated E_2 signaling pathway via an element within the proximal 249 bases of the rat DBH promoter in PC12 cells exogenously expressing ERα. The time course of the 10 nM E_2BSA triggered induction of DBH promoter activity shows a small, but significant elevation after 3 h with induction of nearly 2 and 3 fold after 6 and 24 h respectively (Fig. 5C).

Discussion

The findings of this study indicate two different mechanisms whereby DBH transcription is regulated by oestradiol in the presence of ERα. Mutational analysis of the rat DBH promoter reveals that E_2-induced DBH transcription is mediated by at least one functional ERE half-site at −759 as well as additional element(s) within the proximal 249 bp of the rat DBH promoter. The latter is mediated by membrane impermeable E_2-initiated signalling. Together, these results implicate both genomic and membrane-initiated mechanisms, mediated by ERα, in the modulation of E_2-induced DBH gene transcription.

The findings pinpoint the importance of the half palindromic ERE at −759 in the DBH promoter. Although half EREs generally confer weak oestrogen responsiveness, there are examples for which transcription is induced by two or more ERE-half palindromic elements, for example corticotrophin releasing hormone, platelet activating receptor transcript 2 and prothymosin α [reviewed in (28)]. Chromatin immunoprecipitation in genome wide analysis revealed diverse ERα binding sites, with about 25% of them identified as ERE half sites (29). While constructs with a mutated half palindromic ERE at −759 in the DBH promoter still responded to E_2 in cells transfected with ERα, they were unresponsive in cells transfected with expression vector for ERβ. These results suggest differences in the response of the DBH promoter to oestrogens in the presence of ERα or ERβ. We have previously shown that treatment with E_2 induces DBH promoter activity and this induction is greater and more sensitive to E_2 with ERα than ERβ (17). In this regard, our recent experiments showed that injections of rats with an ERα agonist (PPT), but not an ERβ agonist, effectively elevated DBH mRNA levels in the LC and in the rostral medial and caudal nucleus of the solitary tract (NTS) (30). This is despite the fact that both ERα and ERβ are expressed in these noradrenergic neurons (31).

Although the −759 half palindromic ERE of the rat DBH promoter is functional, our results indicate that the GGTCA site at −1968 is unimportant for the response to oestrogen since a similar response to E_2 was observed with the construct containing 2236 or 1624 bp of the promoter (15). Further upstream GGTCA sequences in the rat, such as those at −2268, −2304, −2538 and −2670, as well as any additional upstream sites, were not examined. The promoter region containing the −759 half ERE in the rat is not homologous to any region of the human DBH promoter. There are, however, numerous sites in the human DBH promoter with the same GGTCA half palindromic ERE sequence. For example, within the proximal 5 kb of the human DBH promoter, these sites are located at −413, −3192, −3800, −4406, and −4924, with additional sites located further upstream. Similarly, this sequence is also found in the proximal 5 kb of the mouse DBH promoter (−1113, −1820, −2381, −2654, −3308, −3840, −4217, −4525, and −4633). Which, if any, of these GGTCA half palindromic ERE sequences are functional remains to be determined.

In addition to the functional role of the −759 half ERE in the rat, the results of this study show that truncation to the proximal −249 bp of the rat DBH promoter is still responsive to E_2 or E_2BSA in the presence of ERα. There is a great deal of homology between rat, mouse and human DBH genes in the approximately 300 bp upstream of the transcription start site. Functional elements in this region of the rat DBH promoter include CRE/AP1, Egr1, homeodomain, and E box motifs, many of which are highly conserved in the human DBH promoter [reviewed in (7)]. Although the element(s) responsible for mediating E_2-induced DBH gene transcription via membrane initiated signaling in the rat is currently unknown, the high degree of homology between the proximal region of the rat and human DBH promoter support the likelihood of similar mechanisms.

The findings reveal the importance of membrane initiated signalling in the oestrogen triggered regulation of DBH transcription with ERα. It is likely that ERα is acting as the membrane E_2 receptor, as exogenously expressed ERα has been reported to localize to the plasma membrane of PC12 cells (32,33). While these findings are so far restricted to PC12 cells and need to be confirmed in normal noradrenergic and adrenergic cells, they emphasize the importance of both classical genomic and membrane initiated mechanisms. The physiological importance of membrane initiated oestrogen signalling is increasingly apparent, for example in preventing bone loss or by providing cardioprotection in mice (34,35). The *in vivo* effects on catecholaminergic systems remain to be determined. However, the actions at the membrane are likely integrated with classical effects. In neuroblastoma cells, membrane initiated actions of oestrogen, by E_2BSA, were found to potentiate subsequent transcriptional activity at a classical ERE motif (22). Since our findings display that DBH possesses both types of responses with ERα, this may enable DBH to manifest a potentiated response to oestrogens in ERα expressing cells, and may explain our finding that an ERα agonist is especially effective in inducing DBH mRNA levels in rat LC and NTS (30).

Oestrogens however have paradoxical effects on catecholaminergic systems. While they enhance basal DBH transcription they reduce or attenuate the response of DBH gene expression to stress (16,30). Selective ERα agonists were especially effective in attenuating the response of DBH in the LC and NTS to even repeated immobilization stress (30). The finding of membrane initiated and classical responses in regulation of DBH may help explain this paradox. Long term exposure to estrogens, especially via membrane bound ERα may modulate the signalling pathways responsible for the elevation of DBH gene expression upon exposure to stress, and may be responsible for the attenuated autonomic response to stress in women between puberty and menopause [reviewed in (36)].

Our findings are consistent with a membrane-initiated E_2 signaling pathway via ERα in PC12 cells which regulates transcription of the tyrosine hydroxylase (TH) gene, which encodes the first and major rate-limiting enzyme in catecholamine biosynthesis (27). E_2 or E_2BSA elicited rapid phosphorylation of CREB and of extracellular signal regulated kinase (ERK), especially ERK1. Specific inhibitors indicated that both protein kinase A and MEK signaling pathways are required for the response of the TH gene to membrane impermeant E_2.

DBH transcription and gene expression are also regulated by activation of protein kinase A activity (37–40). An adjacent CRE/AP1 motif slightly upstream from two homeobox motifs is present in the rat DBH promoter and involved in the response of the DBH promoter to cAMP and to phorbol esters (41). There is transcriptional synergism between the homeodomain proteins Arix/Phox2a and protein kinase A pathway in regulation of DBH transcription (42). A similar CRE/AP1 motif in the human DBH gene binds to CREB (43) and its phosphorylation is implicated in the activation of DBH transcription in response to ethanol (44). However, in the rat DBH promoter, AP1 proteins and to a lesser extent CREB are involved in mediating the transcriptional response to cAMP (45). It remains to be determined if the protein kinase A and/or MEK pathways mediate the membrane initiated response of DBH to E_2 in cells expressing ERα. However, the activation of the MEK pathway may not be involved in the induction of DBH by membrane initiated E_2 signaling since activation of ERK is actually associated with cytokine suppression of DBH promoter activity, at least in neuroblastoma cells (46) and ERK1/2 is a negative regulator of the homeodomain protein Arix/Phox2a involved in DBH transcriptional regulation (47).

What might be the physiological significance of regulation of DBH transcription by oestradiol? While DBH is not generally considered the rate limiting enzyme in catecholamine biosynthesis under certain circumstances DBH can be rate limiting [rev in (48)]. A recent review (49) summarizes the evidence supporting the hypothesis that extracellular DA in the cerebral cortex originates not only from dopaminergic terminals but also from LC derived noradrenergic ones, and that DA can act not only as a precursor for NE but also as a co-transmitter. Thus an increase in DBH by oestrogen could alter the NE/DA ratio in the medial prefrontal cortex (PFC). This could be very significant as the medial PFC is involved in the regulation of cognitive and emotional processing and the LC noradrenergic projections to this region have modulatory effects on working memory and attention (50).

The regulation of DBH gene expression by oestrogen may be responsible for the greater number of DBH immunoreactive neurons in female than in male rats (11,12). This could be involved in sex specific differences in vigilance and alertness and in memory.

Overall, the findings of this study provide new mechanistic insight into the physiological and possibly patho-physiological roles of oestrogens on the regulation of DBH transcription and, on a larger scale, regulation of catecholaminergic systems.

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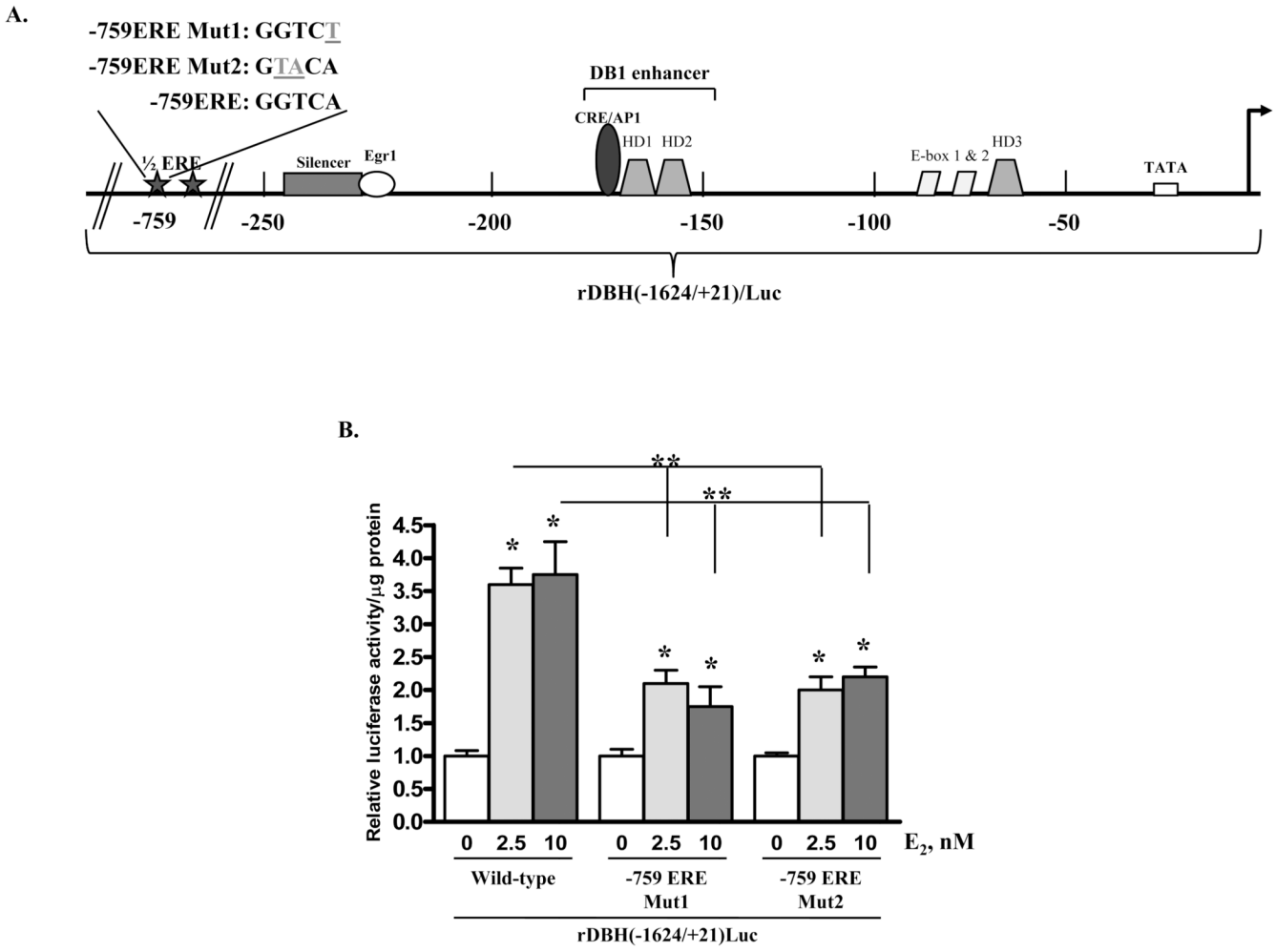

Figure 1.

17 β-oestradiol induces rat DBH promoter-driven luciferase activity partially through the ERE at position −759 in PC12 cells transfected with ERα. (**A**) Schematic of functional *cis*-regulatory elements of the rat DBH promoter. (**B**) PC12 cells were co-transfected with ERα expression vector and either wild-type rDBH(−1624/+21)/Luc or rDBH(−1624/+21)/Luc mutated at the −759 ERE (−759ERE/Mut1 or −759ERE/Mut2), as shown in (A). Cells were treated with 0, 2.5, or 10 nM E_2 for 24 h and assayed for luciferase activity. Levels of luciferase activity were normalized to protein levels in each sample and are expressed relative to the respective untreated control taken as 1. * p≤0.05 compared to untreated control; ** p≤0.05 compared to wild-type rDBH(−1624/+21)/Luc.

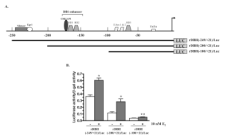

Figure 2.

Effect of truncation of the rat DBH promoter on response to E_2. (**A**) Schematic of truncations of DBH promoter. (**B**) PC12 cells were co-transfected with ERα and β-galactosidase (β-gal) expression vectors and either rDBH(−249/+21)/Luc, rDBH(−200/+21)/Luc, or rDBH(−100/+21)/Luc. Cells were treated with 0 or 10 nM E_2 for 24 h and assayed for luciferase activity. Luciferase activity is normalized to β-gal activity and expressed relative to untreated controls. * $p \leq 0.05$ compared to respective untreated control; # $p \leq 0.05$ compared to rDBH(−200/+21)/Luc treated with E_2.

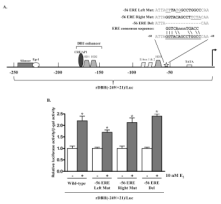

Figure 3.

The putative ERE at −56 to −43 is not a functional ERE. (**A**) Schematic of mutations of DBH promoter. (**B**) PC12 cells were co-transfected with ERα and β-gal expression vectors and either wild-type rDBH(−249/+21)/Luc or mutant rDBH(−249/+21)/Luc, mutated at the −56 ERE (−56ERE/Del, −56ERE/Right Mut or −56ERE/Left Mut). Cells were treated with 0 or 10 nM E_2 for 24 h and assayed for luciferase activity. Luciferase activity was normalized to β-gal activity and is expressed relative to the respective untreated control taken as 1. * p≤0.05 compared to untreated control.

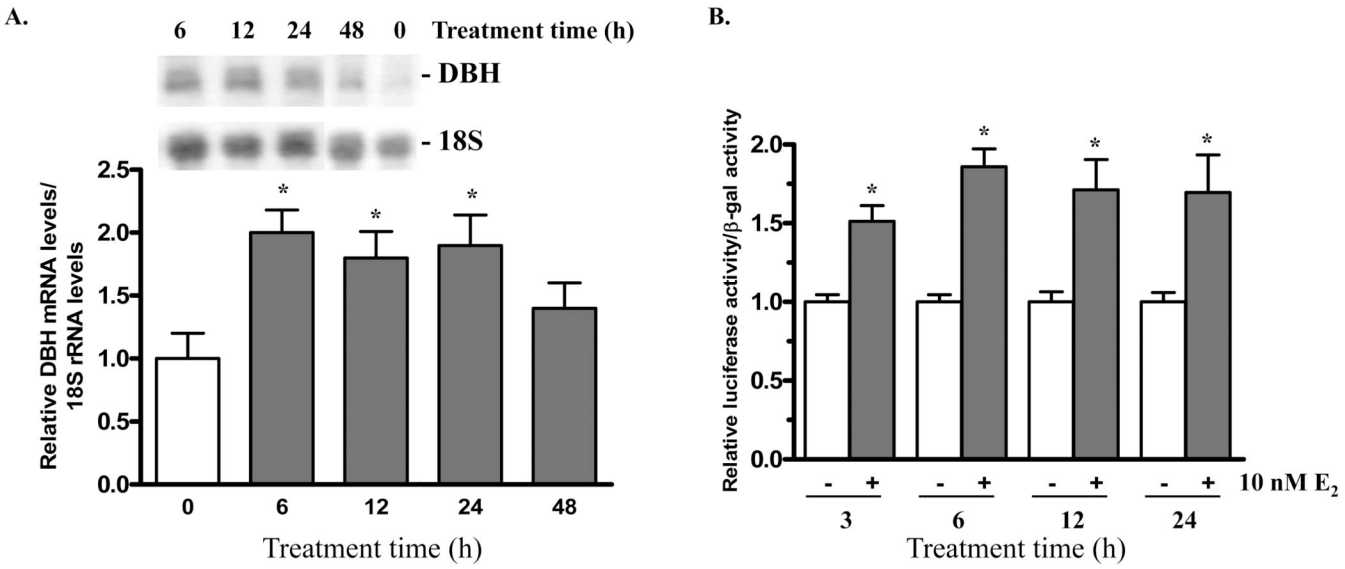

Figure 4.

17 β-oestradiol induces increased rat DBH promoter activity and elevated mRNA levels within hours. (**A**) PC12 cells were transfected with ERα expression vector and treated with 10 nM E_2 for 0, 6, 12, 24, or 48 h. Total RNA was isolated and analyzed by northern blot. DBH mRNA levels are normalized to 18S rRNA levels and expressed relative to the 0 h group and taken as 1. n = 4–5 replicates per group * p≤0.05 compared to 0 h control. (**B**) PC12 cells were co-transfected with ERα and β-gal expression vectors and rDBH(−200/+21)/Luc. Cells were treated with 0 or 10 nM E_2 for 3, 6, 12 or 24 h. Luciferase activity was normalized to β-gal activity and is expressed relative to the respective untreated control taken as 1. * p≤0.05 compared to untreated control.

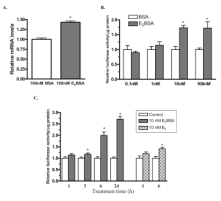

Figure 5.

A membrane impermeable form of estradiol, E_2BSA, induces rat DBH promoter activity and increases rat DBH mRNA levels. (**A**) PC12 cells were transfected with ERα expression vector and treated with 100 nM E_2BSA or BSA for 24 h. Total RNA was isolated and analyzed by quantitative RT-PCR. DBH mRNA levels are normalized to total RNA and expressed relative to the BSA-treated control taken as 1. n=6 *p≤0.05 compared to BSA-treated control.

(**B**) PC12 cells were co-transfected with ERα expression vector and rDBH(−249/+21)/Luc, treated with 0.1, 1, 10, or 100 nM E_2BSA or BSA for 24 h and assayed for luciferase activity. Luciferase activity is normalized to total protein and expressed relative to the respective BSA-treated control taken as 1. *p≤0.05 compared to BSA-treated control. (**C**) PC12 cells were co-transfected with ERα expression vector and rDBH(−249/+21)/Luc, treated with 10 nM E_2BSA or BSA (control), or 10 nM E_2 or vehicle (control), for 1, 3, 6, or 24 h and assayed for luciferase activity. Data are normalized to total protein and expressed relative to the respective control and taken as 1. *p≤0.05 compared to respective control.

Table 1

Primers for deletion, mutation, and truncation of rDBH/Luc reporter constructs

rDBH/Luc Construct	Forward (top) and reverse (bottom) primers (listed 5’ to 3’)
−759 ERE/Mut1	GCTGAGCAATAGGAAGGTCTCAACTTCGGACTGGTGG CCACCAGTCCGAAGTTGAGACCTTCCTATTGCTCAGC
−759 ERE/Mut2	GCTGAGCAATAGGAAGTACAAAACTTCGGACTGGTGG CCACCAGTCCGAAGTTTTGTACTTCCTATTGCTCAGC
−56 ERE/Del	CCAGACAAATGTGATTACAACCCCACCGAAC GTTCGGTGGGGTTGTAATCACATTTGTCTGG
−56 ERE/LeftMut	CCACCAGACAAATGTGATTACTTATGGCCTGGCCCAACCCCACC GGTGGGGTTGGGCCAGGCCATAAGTAATCACATTTGTCTGGTGG
−56 ERE/RightMut	GACAAATGTGATTAGGTACAGCCTTCTACAACCCCACCGAACAGACATAA TTATGTCTGTTCGGTGGGGTTGTAGAAGGCTGTACCTAATCACATTTGTC
rDBH(−200/+21)/Luc	CGAGGTCGACGGTATCATGGCCATCTGCTTC GAAGCAGAATGGCCATGATCACGTCGACCTCG
rDBH(−100/+21)/Luc	GAGGTCGACGGTATTGGGAGAGCCAC GTGGCTCTCCCAATACCGTCGACCTC