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Characterisation of Human Oestrogen Receptor beta (ER β) Splice Variants in Neuronal Cells

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Abstract

Oestrogen receptor (ER) α and ER β are members of the ligand-activated superfamily of nuclear receptors and mediate most facets of oestrogen signalling. Several naturally occurring splice variants of each ER have been identified in the human brain, yet the biological significance of these splice variants in the brain remains unknown. In the present study, we exploit the unique structural differences of the human ER β splice variants to determine the functional significance of individual ER domains in the brain. We previously established that full-length rodent ER β (i.e. rER β 1) has constitutive transcriptional activity in neuronal cells in the absence of ligand. By contrast to the rodent splice variants, the human ER β splice variants used in the present study contain varying length truncations of exon 8, which encodes for the E/F domains. Our results reveal that, in neuronal cells, each human-specific ER β splice variant constitutively activated promoters mediated by a canonical oestrogen response element and repressed promoters mediated by activator protein-1 sites via p38 activity. From these data, we conclude that the C-terminus, encoding the AF-2 region and F domain, is not essential for the constitutive properties of human ER β . Taken together, these studies show that human-specific ER β variants are constitutively active and also provide novel insight into the contributions of the functional domains of ER β towards mediating constitutive transcription at various promoters in neuronal cells.

Keywords

oestrogen receptor β ; splice variants; ERE; AP-1; vasopressin; neurones

Oestrogen receptors (ERs) are critical regulators of many processes involved in functions of the central nervous system, including homeostasis, reproduction, memory, anxiety and synaptic plasticity (1–7). The actions of oestrogens are mediated primarily by high-affinity ER α and ER β , both of which belong to the nuclear receptor superfamily. Similar to the case in rodents, the human full-length ER β (hER β 1) is the most recently identified ER. At least three alternatively spliced variants of hER β are present in the human brain, although the exact expression patterns and precise actions of these receptor splice variants remain largely unknown (8, 9). However, the naturally occurring variations in ER β structure have the capacity to provide important clues about the functional significance of the receptor domains, ultimately giving insight into the mechanisms regulating receptor action in various tissue-specific microenvironments. The physiological importance of these variants has been recently highlighted in a study showing the elevated expression of a dominant negative rat ER β 2, which is structurally distinct from human ER β 2, diminished the effectiveness of

hormone therapy following ovariectomy in rats (10). On a molecular level, previous studies from our laboratory have demonstrated that the rat ER β splice variants constitutively activated a variety of minimal and complex promoters in neuronal cells, and this activation was not dependent on the presence of ligands [17 β -oestradiol (E₂), growth factors, etc.]; however, no such phenomenon has been reported for the human ER β splice variants. Importantly, the rodent ER β splice variants are substantively different in structure from the human-specific splice variants raising the question of relevance and translatability from the rodent studies to human health.

The structural differences in human ER β splice variants suggest the intriguing possibility that these receptors could have specific functions that are not dependent on the presence of ligands, or that they are resistant to normal ligand effects, which could have detrimental consequences for therapeutic hormone treatment strategies if these variants are highly expressed during menopause or in disease states. The human ER β variants identified to date contain variable length deletions and substitutions in exon 8 (e.g. hER β 1, hER β 2, hER β 4 and hER β 5) (Fig. 1A), resulting in serially truncated receptor proteins at the C-terminus (9). The C-terminus of ER β houses the ligand binding domain, a domain that is absolutely required for ligand-induced actions of the receptor. Notably, the hER β splice variants lack varying portions of the E and F domains, which alter their innate functional properties and ability to bind ligand. Specifically, truncations to this region render hER β 2 unable to bind E₂, whereas hER β 4 and hER β 5 have a very low affinity for E₂, such that binding would occur only in conditions with supra-physiological levels of E₂ (8).

Amino acids encoded in the E domain (ligand binding domain; LBD) form a secondary structure consisting of 12 α -helices. These helices are considered essential for ligand binding and associations with coregulatory proteins. Indeed, helices 3, 4, and 12 are arranged in a pattern that forms a 'hydrophobic pocket' called the activation function-2 (AF-2) region. Upon ligand binding, the LBD undergoes a conformational change that results in a positional shift of helix 12, which alters the opening of the pocket; however, only hER β 1 has the necessary coding region for the normal configuration of this helix. Helix 12 of hER β 2 is encoded such that it is positioned in antagonism to ligand (Fig. 1B) and hER β 3, 4 and 5 do not contain this sequence (8). Upon ligand binding, this region can interact with the common nuclear box consensus LxxLL motifs that are contained in nuclear receptor coregulatory proteins such as steroid coactivator-1 (SRC-1) (11–13). In addition to ligand binding, the E/F domains of ER α contain the interface for receptor dimerisation and possibly other regions important for the binding of coregulatory proteins (14).

Unique from all other steroid hormone receptors, only ERs contain an F domain that, for ER β , consists of approximately 30 amino acids at the extreme end of the C-terminus. Little is known about the functional significance of the F domain on ER β , although recent studies suggest that the F domain may be important for transcriptional activation of ER α . For example, mutations to the F domain of ER β enhanced dimerisation, possibly by unmasking the dimerisation interface of the E domain (15). Importantly, one study showed that specific amino acid sequences in this region alter the transcriptional responsiveness of ER α in the presence of a typical ER agonist such as E₂ or an antagonist, such as tamoxifen or fulvestrant (ICI 182 780) (16). This change in transcriptional responsiveness may be related to the ability of the F domain to associate with coregulators. For example, alterations to the F domain of ER α also lead to increased associations with coactivators, and ultimately enhanced transcriptional activity in yeast (14).

The present study provides the first examination of transcriptional actions mediated by human ER β splice variants in neuronal cells. Specifically, we hypothesised that the E and F domains were important for conferring ligand-dependent (rather than ligand-independent or

constitutive) activity when regulating minimal promoters with an oestrogen response element (ERE) or activator protein-1 (AP-1) enhancer site, and that these effects may be a result of endogenous kinase activity. Taken together, our results demonstrate that the previously observed constitutive activity of ER β in neuronal cells is conserved between rodents and humans. Moreover, the constitutive activity is regulated independent of the C-termini truncations deleting the AF-2 region and F domain of the receptor and alternative splice variants. These data highlight the possibility that these ER β splice variants play an important functional role in the brain.

Materials and methods

Expression vectors

Plasmid expression vectors (pcDNA 3.0; Invitrogen, Carlsbad, CA, USA) containing inserts for human ER β 1, ER β 2, ER β 4 and ER β 5 were generously provided by Dr Shuk-Mei Ho (University of Cincinnati, Cincinnati, OH, USA) and have been extensively characterised (8).

Reporter constructs

The ERE-tk-luciferase reporter (generously provided by Dr P. Budworth, Case Western Reserve University, Cleveland, OH, USA) contains two copies of the vitellogenin ERE sequence coupled to the minimal tk-Firefly luciferase promoter and subcloned into pGL2-Basic plasmid (Promega, Madison, WI, USA). The AP-1-tk-Firefly luciferase promoter (generously provided by Dr C. Clay, Colorado State University, Fort Collins, CO, USA) contains three copies of the AP-1 sequence (TGACTCA) coupled to the minimal tk-Firefly luciferase promoter and subcloned into pGL2-Basic plasmid. The human arginine vasopressin (AVP) promoter-Firefly luciferase reporter construct was purchased from Switch Gear Genomics (Menlo Park, CA, USA) and contains a 929-bp insert upstream from the transcription start site of the human AVP promoter in the pSGG_prom plasmid vector. The Renilla luciferase pGL4 reporter construct (Promega) was used as an internal control for calculating plasmid transfection efficiency.

Cell culture

The cell lines used for all transient transfections were HT-22 mouse hippocampus-derived neuronal cells (generously provided by Dr D. Schubert, Salk Institute, San Diego, CA, USA) or human neuroblastoma-derived SK-N-SH cells (American Type Culture Collection, Manassas, VA, USA). HT-22 and SK-N-SH neuronal cells were maintained in phenol red-free minimal essential medium (MEM) (SK-N-SH) or MEM with Earle's salts, respectively. The medium contained 4.5% glucose and L-glutamine (Invitrogen) and was supplemented with $1 \times$ non-essential amino acids, and 10% foetal bovine serum (FBS) or dextran charcoal-stripped FBS (Hyclone Laboratories, Logan, UT, USA). Cells were grown to 70% confluency and all transient transfection experiments were performed within ten passages.

Transient transfections

HT-22 cells were plated at a density of 0.2×10^5 cells/well in 96-well plates and allowed to grow to 70–80% confluency until 24 h before transfection. SK-N-SH cells were plated at a similar density and allowed to grow to 70–80% confluency until 48 h before transfection. Immediately before transfection, the media was removed, and cells were washed once with $1 \times$ PBS. Regular media was replaced with media containing 10% charcoal dextran-stripped FBS to eliminate the presence of exogenous steroids or growth factors. Transfections were carried out using a lipid-mediated transfection reagent in accordance with the manufacturer's instructions (Fugene6; Roche Molecular Biomedical, Indianapolis, IN,

USA). Cells were then incubated with the transfection media complex in stripped media containing the empty vector, hER β 1, 2, 4 or 5 expression vectors for 12 h, which was then replaced with phenol red-free Dulbecco's modified Eagle's medium containing dextran charcoal-stripped fetal bovine serum. After a total of 24 h in stripped media, vehicle, hormone and/or kinase inhibitor treatments were given for an additional 12 h. The hormone compounds were diluted in 100% EtOH and used at a final concentration of 100 nM in 0.001% EtOH: E₂ (Sigma-Aldrich Co. St Louis, MO, USA), 5 α -androstane-3 β , 17 β -diol (3 β -diol) and ICI 182 780 (Steraloids, Newport, RI). The p38 inhibitor SB 202190 was diluted in nuclease-free water and phosphoinositide 3-kinase (PI3K) inhibitor LY 294002 was diluted in 100% EtOH; both were used at a final concentration of 10 μ M. Control reporter (Renilla luciferase) and reporter (Firefly luciferase) activity was measured a total of 36 h post-transfection using the Dual Luciferase Reporter Assay system (Promega) in accordance with the manufacturer's instructions. Relative light units for each construct were measured using the Synergy HT multimode plate reader (BioTek Instruments Corp., Winooski, VT, USA) and represented as a ratio of Firefly : Renilla. Luciferase substrates (100 μ l/well) were added to cells using an automatic injector system. All constructs were transfected in replicates of six wells within each assay, and each transfection assay was repeated in a minimum of three independent experiments. Independent experiments were compared by calculating the percentage change from empty vector controls. Furthermore, each experiment was performed using a minimum of three different preparations for each plasmid reporter construct and expression vector.

Electromobility shift assay (EMSA)

Synthesis of hormone receptor proteins—Human ER β expression vector plasmids (1 μ g each) were used to synthesise receptor proteins *in vitro* using the TnT-coupled rabbit reticulocyte lysate system (Promega) with T7-RNA polymerase according to manufacturer's directions.

Oligonucleotides—Double-stranded oligonucleotides containing the vitellogenin consensus ERE sequence were ³²-P end-labelled with T4 polynucleotide kinase. The percentage of ³²-P incorporation was determined and labelled probes with greater than 50% ³²-P incorporation were used for EMSAs.

Gel electrophoresis—Receptor protein lysates were incubated with 100 nM E₂ or 0.001% ethanol (vehicle control) for 18 h before gel electrophoresis. Following ligand-binding, receptor lysates were incubated with 1 $\times$ gel shift binding buffer [20% glycerol, 5 mM MgCl₂, 2.5 mM ethylenediaminetetraacetic acid, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCL, 0.25 mg/ml poly(dI-dC)-poly(dI-dC)] for 10 min. Specific binding reactions were also incubated with 500–1000 fold excess of unlabelled ERE oligonucleotide. Nonspecific binding was tested using the ³²P-SP1 oligonucleotide (data not shown). After an initial 10-min incubation, ³²P-ERE was added and incubated for an additional 20 min. DNA–protein complexes were resolved on a 6% Novex DNA retardation bis-acrylamide gel (Invitrogen) for 20 min at 250 V. Gels were dried on a vacuum gel dryer at 80 °C for 2 h before autoradiography.

Autoradiography and analysis—Dried gels were exposed to X-ray film (Biomax MS; Eastman Kodak Company, New Haven, CT, USA) for 12 h at –70 °C. Gel bands were scanned and optical density was quantified using IMAGE J (NIH, Bethesda, MD, USA). Relative densitometry of replicate gels were averaged and data are reported as the mean $\pm$ SD density of pixels.

Site-directed mutagenesis

Site-directed mutagenesis was used to delete the putative AP-1 regulatory site from the human AVP promoter. The imperfect AP-1 site, TGACTCC, located –611 bp upstream of the transcription start site, was deleted by directing primers to the region (forward: 5'-CCTCTCATTCTGTGTCCCTACGACGGCGG-3'; reverse: 5'-CCGCCGTCGTAGGGACACAGAATGAGAGG-3') using the Quik Change XL system (Stratagene, La Jolla, CA, USA) in accordance with the manufacturer's instructions.

Statistical analysis

Differences among hormone treatment groups for individual receptors were analysed by one-way ANOVA followed by Tukey's honestly significant difference test. Post-hoc comparisons between control groups (empty-vector + vehicle; receptor + vehicle) were analysed using Student's *t*-test. $P < 0.05$ was considered statistically significant. All transfection data are represented as the percentage change compared to vehicle-treated, promoter + empty vector controls.

Results

hER β splice variants bind a consensus ERE in the absence of ligand

The classical model of ER action requires that the receptor first bind to a ligand or undergo an activational event before it is capable of binding to DNA at an ERE. This model contrasts with previous data from our laboratory and others showing that rodent ER β 1 and ER β 2, and human ER β 1, bind an ERE consensus sequence in the absence of ligand (17).

Notwithstanding these previous binding studies, it was unknown whether human-specific ER β 2, ER β 4 or ER β 5 had the ability to bind a consensus ERE in the absence of ligand. Therefore, EMSAs were performed to determine whether hER β 1, hER β 2, hER β 4 and hER β 5 could bind a consensus ERE sequence in the absence of E₂ and also to determine whether the presence of E₂ altered DNA binding. The results obtained showed that all human ER β splice variants caused a strong shift of ERE-P³² oligos, demonstrating the ability of all human-specific splice variants to bind an ERE in both the presence and absence of E₂ (Fig. 2A). Similar to the rodent splice variants reported previously, there were no significant differences between vehicle and E₂ treated lysates that contained hER β 1, hER β 2, hER β 4 or hER β 5 (Fig. 2B). These results suggest that the presence of E₂ does not enhance, nor is it required, for hER β 1, hER β 2, hER β 4 or hER β 5 to bind an ERE, which is consistent with their reported inability to bind E₂ with high affinity. A scrambled sequence ERE oligo and competition with 1000-fold excess unlabelled ERE effectively demonstrated specific DNA binding to an ERE for hER β 1 (Fig. 2, lanes 1,2) and each of the splice variants (data not shown). In control experiments, none of the splice variants caused a shift in EMSAs using a SP-1- P³² oligo, indicating the selectivity of these splice variants for the ERE oligo (data not shown).

Apo-hER β splice variants activate ERE -and AP-1-mediated promoter activity

Liganded ER-mediated activation or repression of promoter activity is accomplished through direct DNA binding at an ERE site when the receptor is in the cis-acting conformation, or in the trans-acting conformation through protein : protein tethering at alternative regulatory transcription sites, such as an AP-1 site. To test the transcriptional activation of ERE- or AP-1- mediated promoters by human-specific ER β splice variants, we co-transfected hippocampal- derived HT-22 neuronal cells with a 2xERE-tk-luciferase reporter construct or an AP-1-tk-luciferase reporter construct, and varying concentrations of expression vectors containing full-length hER β 1, hER β 2, hER β 4 or hER β 5 (Figs 3 and 4). All hER β splice variant expression vectors significantly increased basal ERE-tk-luc activity

in the absence of ligand (Fig. 3). Furthermore, we found that there were no significant differences between the increasing concentrations of expression vectors, with the exception of hER β 2, because the lowest dose did not cause a statistically significant increase in promoter activity at an ERE. Notably, with the exception of hER β 2, all of the other hER β splice variants increased ERE-tk-luciferase activity with concentrations as low as 0.0375 μ g/well and maintained consistent activation at all doses, demonstrating the ability of these ER β splice variants to constitutively activate ERE-mediated promoter activity.

ER-mediated regulation of promoters through an AP-1 site requires protein associations with members of the Jun and Fos family of proteins. Therefore, AP-1-mediated promoter regulation can be highly complex and variable depending upon the cellular context. For example, rodent ER β has been shown to have both activational and repressive actions on AP-1-mediated promoter activity (18, 19). In these experiments, we examined transcriptional activity of the human-specific ER β splice variants on AP-1-mediated promoter activity in hippocampal HT-22 neuronal cells. Our results showed that hER β 1, hER β 2, hER β 4 and hER β 5 significantly repressed basal AP-1 mediated promoter activity (Fig. 4) in a constitutive manner. Unlike our observations using the ERE-tk-luc reporter construct, there were significant differences between the highest and two lowest concentrations of ER β 1 plasmid and the highest and lowest plasmid concentrations of ER β 5 (Fig. 4).

ER β agonists and antagonists do not alter hER β splice variant constitutive regulation of ERE- and AP-1-mediated promoters

Human-specific ER β 2, ER β 4 and ER β 5 have very limited binding affinity for E₂, despite the fact that ERs are classified as ligand-activated nuclear receptors. Therefore, to determine whether the inherent structural differences in the C-terminus of the human-specific ER β splice variants alter the ability of agonists or antagonists to activate ERE- or AP-1-mediated promoter activity, we assessed ERE- and AP-1-tk-luciferase activity in the presence of E₂ and 3 β -diol (an ER β -selective agonist), or the ER antagonist ICI 182 780. Consistent with experiments shown in Figs 3 and 4, the presence of hER β splice variants alone caused a statistically significant constitutive increase in ERE-mediated promoter activity (Fig. 5A, black bars). Conversely, a significant constitutive hER β -mediated repression was observed for all of the splice variants on AP-1-mediated promoter activity (Fig. 5B, black bars). As expected, E₂ further potentiated the constitutive hER β 1-mediated effects at an ERE, but not AP-1 site, whereas the ER antagonist, ICI 182 780 abolished the constitutive hER β 1-mediated response at both an ERE and AP-1 site (Fig. 5). The presence of ligand, either agonist or antagonist, did not alter the constitutive effects of hER β 2, hER β 4 or hER β 5 on ERE- or AP-1-mediated promoter activity (Fig. 5). Moreover, the selective ER β agonist 3 β -diol did not further potentiate the constitutive effects of hER β 1 on ERE- and AP-1 mediated promoter activity (Fig. 5). Taken together, these results suggest that the presence of ligand does not alter the basic constitutive transcriptional activity of the human ER β splice variants at these promoter elements in neurones and that ligand-mediated activity (agonists and antagonists) is restricted to the full-length hER β 1.

hER β -mediated repression of human AVP (hAVP)-luciferase promoter is dependent upon AP-1 and p38 activity

AVP regulates a number of neurological processes, including stress and anxiety, and it is well established that E₂ and ERs alter vasopressinergic systems (20–23). To assess the transcriptional efficacy of hER β 1, hER β 2, hER β 4 and hER β 5 in the context of a complex human promoter, we used an hAVP reporter construct containing the proximal 929 bp of the AVP promoter. We co-transfected the hAVP-luciferase reporter construct with the individual hER β splice variants into human neuroblastoma-derived SK-N-SH cells and

measured luciferase activity. Our results showed that all human-specific ER β splice variants significantly repressed hAVP promoter activity in a constitutive manner, which was blocked by ICI 182 780 for hER β 1 (Fig. 6A). Consistent with our results using the ERE and AP-1 constructs, all other splice variant/ligand combinations did not alter the constitutive repression of hAVP promoter activity.

The results from the complex hAVP promoter were strikingly similar to the results obtained using the AP-1 minimal promoter construct shown in Fig. 5(B). Therefore, we hypothesised that the primary element regulating this portion of the hAVP promoter activity would be an AP-1 site. Indeed, examination of the hAVP promoter sequence showed the presence of an imperfect AP-1 site located 611 bp upstream from the transcription start site. To examine whether the constitutive repression in hAVP promoter activity was a result of this imperfect AP-1 site, we used site-directed mutagenesis to create a mutant promoter construct lacking the imperfect AP-1 site (hAVP Δ AP-1) and then subjected the hAVP Δ AP-1 to the same reporter gene analysis described in Fig. 6(A). Site-directed mutagenesis of the imperfect AP-1 site eliminated the constitutive repression of transcriptional activity that was detected for the full-length hAVP promoter in the presence of hER β 1, hER β 2, hER β 4 or hER β 5, and also allowed for significant activation of the AVP promoter by hER β 1, hER β 4 and hER β 5 (Fig. 6B). The presence of agonists, E₂ or 3 β -diol had no effect hAVP Δ AP-1 promoter activity mediated by any of the hER β splice variants compared to vehicle-treated controls. However, unlike our previous results obtained using the minimal promoters, the antagonist ICI 182 780 was unable to reduce the constitutive activation of hER β 1-mediated hAVP Δ AP-1 activity back to baseline levels.

The mechanisms by which hER β acts as a constitutive transcription factor remain unclear; however, phosphorylation of the receptor has been implicated as a potential signal to activate nuclear receptors in the absence of cognate ligands. Specifically, p38 kinases, members of the mitogen-activated protein kinase family, have been identified as potential regulators of N-terminal phosphorylation of hER β (24). We tested the effects of endogenous kinase activity on the constitutive actions of hER β 1 using specific kinase inhibitors. A blockade of p38 activity using the kinase inhibitor, SB 202190, restored hER β 1-mediated AP-1 repression to baseline levels, which was similar to treatment with ICI 182 780 (Fig. 7B). Notably, concomitant administration of the p38 kinase inhibitor and E₂ not only restored baseline promoter activity, but also significantly enhanced AP-1-mediated promoter activity (Fig. 7B). Next, we tested whether inhibition of p38 kinase activity would block the constitutive repression observed by all of the hER β splice variants on hAVP promoter activity. Our results showed that inhibition of p38 kinase blocked the repression of hAVP activity mediated by both hER β 1 and hER β 2, but not by hER β 4 or hER β 5 (Fig. 8B). Similar to the results observed with p38 kinase inhibition at the AP-1 site, when the kinase inhibitor was administered concomitant with E₂, the promoter activity was significantly increased above baseline (Fig. 8B). We also tested whether inhibition of another signalling pathway, AKT/PI3K, would abolish hER β -mediated constitutive activity. Notably, PI3K has not been previously implicated in mediating hER β transcriptional activation. Using the PI3K inhibitor LY294002, we found no effect of PI3K inhibition on hER β -mediated activity of either promoter (Figs 7A and 8A), suggesting that this pathway is not involved in the constitutive activation of hER β .

Discussion

The key novel findings obtained in the present study demonstrate that human-specific ER β splice variants exhibit marked constitutive activity in neuronal cells at both minimal and complex promoters, which can be blocked by inhibition of endogenous p38 kinase activity, raising the possibility that these splice variant receptors are important mediators of centrally-

regulated processes in the presence and absence of cognate ligands. Our studies demonstrate that the human-specific ER β splice variants are largely unresponsive to ligand and induce modest, yet significant constitutive increases in ERE-mediated promoter activity, and robust decreases in AP-1-mediated promoter activity. Although the changes in ERE-mediated promoter activity were modest, these fine-tuned changes could have important biological consequences. Taken together, these data contribute to our overall understanding of ER splice variants in neuronal cells.

We have presented strong evidence that the human-specific ER β splice variants bind DNA at a canonical ERE sequence, and modulate transcription in a ligand-independent manner at both ERE- and AP-1-mediated minimal promoters. Treatment with E₂ and 3 β -diol did not significantly enhance the constitutive effects of hER β 1 on ERE- or AP-1-mediated promoter activity; however, the antagonist ICI 182 780 abolished all ER β 1-mediated constitutive activity. Previous ligand binding analyses have shown that both rodent and human ER β splice variants have a decreased ability to bind ligands compared to full-length hER β 1 (8, 25). Indeed, ligands, whether agonist or antagonist, had no effect on ERE- or AP-1-mediated promoter activity via the splice variants hER β 2, hER β 4 and hER β 5, which is consistent with their reported inability to bind ligand (8). Interestingly, the results shown in Figures 3 and 4 demonstrate a trend toward a dose-responsive effect of receptor concentration on promoter activity, which may be significant in brain regions where varying proportions of particular splice variants are expressed. The results from the present study suggest that further investigation of these splice variants is warranted, particularly concerning co-expression of the splice variants. These data indicate that hER β splice variants are transcriptionally active, and therefore could be physiologically relevant in neuronal cells despite fluctuations in steroid hormone levels.

The human ER β splice variants in the present study are truncated at the C-terminus, which is useful for studying one of the two functional activation regions (AF-1 compared to AF-2) because there is a natural disruption in the AF-2 region of the splice variants. We and others have shown that both human and rodent ER β display constitutive activation (in the complete absence of growth factors or other nontraditional ligands) in many different promoter contexts and cell systems (17–19, 26–31). Previous studies describe ligand-independent activation events (e.g. phosphorylation and coactivator recruitment to murine and human ER β) occurring primarily at the N-terminal AF-1 domain, whereas ligand-dependent activation occurs at the C-terminal AF-2 domain (18, 19, 26). We also propose that the N-termini of hER β s may function in concert with other activational events originating from domains A–D that depend upon the cellular context to facilitate a basal level of transcription. In congruence with published reports of other ERs (24, 32, 33), we hypothesised that p38-mediated phosphorylation of N-terminal serines (S87 and recently S105) could be an activating signal for the constitutive action of hER β s. With this in mind, we inhibited p38 and PI3K activity and measured the effects on hER β -mediated repression of AP-1 and hAVP promoter activity. Consistent with the literature p38, and not PI3K, inhibition blocked the activity of hER β 1 and, representing a novel finding, p38 inhibition blocked the dominant negative actions of hER β 2 on a complex promoter. However, p38 inhibition did not block the repressive actions of hER β 4 and 5. It is possible that p38 may phosphorylate a portion of the missing C-termini of hER β 4 and 5; however, there are no p38 consensus sequences present on this portion of the receptor to support this prediction. A more likely explanation for the inability of p38 kinase inhibition to restore promoter activity would be that a misfolding of hER β 4 and 5 occurs as a result of these variants lacking of a large region encoding the E and F domains. It is possible that this region is important for allowing a favourable conformation that results in the ability for the receptor to be constitutively active at certain promoters. Also, the presence of E₂ enhanced the hER β 1-mediated activation of both AP-1 and hAVP promoters when administered with SB 202190.

This may reflect an alternate mechanism for transcriptional activation of hER β 1 when oestrogens are present. Although we report that p38 inhibition blocks the repressive effects of hER β 1 and 2, we cannot determine from these studies whether this effect is the result of the direct phosphorylation of the receptors. Overall, the conclusions drawn from the present study exclude only the possibility that the C-terminus (part of the E and all of the F) domains are required for constitutive activity of the receptor in neuronal cells, and that p38 kinase inhibition blocks the repressive actions of hER β 1 and 2. It stands to reason that there are multiple mechanisms working in concert to support the constitutive activity of ER β , including post-translational receptor modifications, cell-type and response element specific associations with coregulators, and even allosteric modulation through DNA : receptor interactions.

The complexity of AVP gene regulation by ERs is most apparent in physiological studies where the actions of ERs have been shown to be both anxiogenic and anxiolytic *in vivo* (1, 42). The findings of the present studies revealed that, unlike the rodent ER β splice variants, which increased AVP promoter activity in the absence of ligand, the human-specific ER β splice variants uniformly decreased human AVP promoter activity. The human and rodent AVP promoters are highly homologous in the 1000-bp region proximal to the transcription start site, with the exception of an imperfect AP-1 site located 611 bp upstream of the hAVP transcriptional start site, which is absent in the rodent promoter. Deletion of this site resulted in significant activation of promoter activity mediated by hER β 1, hER β 4 and hER β 5, mimicking our previous findings in the rodent system. Conversely, hER β 2 displayed a trend towards activation of this promoter, although the differences were not significant from control. Antagonism with ICI 182 780 did not block hER β 1-mediated ligand-independent activation of hAVP when the AP-1 site was removed. This lack of antagonism suggests that ligand-independent activation of the hAVP Δ AP-1 promoter may be quite different from traditional ERE-mediated promoter activation, where ICI 182 780 blocks activation by hER β 1. These data imply that unique regulatory elements may exist in the proximal rodent and human AVP promoters that could conserve some aspects of hER β -mediated AVP promoter activity in the absence of AP-1-mediated activity. Indeed, the region responsible for ligand-independent activation by ER β in both the rodent and human promoters did not contain classical ERE or AP-1 sequences (29).

Overall, the results of the present study provide insight into the structural requirements for ER β -mediated constitutive activity, which appear to be independent of the C-terminal domains. We also confirmed the lack of splice variant-mediated constitutive effects on various promoters. Taken together, these data contribute significantly to the growing body of information detailing the molecular mechanisms of oestrogens receptor signalling. Importantly, these studies demonstrate the novel potential for human ER β to mediate transcriptional activation of a variety of genes in the absence of ligand in the brain.

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Fig. 1.

Schematic representation of specific human oestrogen receptor (hER β) splice variants (A) Representation of human ER β splice variants. Domains A–D are identical, however the E/F domains contain significant truncations (hER β 4, hER β 5) or alterations in sequence (hER β 2, Black box) (B) Amino acid sequence of C-termini of hER β splice variants. Bold letters represent amino acids comprising helix 11. Underlined amino acids are representative of helix 12 in ER β 1 and its corresponding sequence alignment with hER β 2.

(B)

Treatment	Vehicle				100 nmol/l Oestradiol			
Receptor	hERβ1	hERβ2	hERβ4	hERβ5	hERβ1	hERβ2	hERβ4	hERβ5
Relative OD (± Std)	216260 (± 7316)	211077 (± 26)	211083 (± 5)	211079 (± 24)	211093 (± 3)	211067 (± 18)	204405 (± 9414)	211080 (± 30)

Fig. 2.

Ligand-independent DNA binding activity of human oestrogen receptor (hER)β1, hERβ2, hERβ4 and hERβ5. (A) *In-vitro* translated hERβ splice variant proteins were incubated with 0.01% EtOH (Vehicle, lanes 3–6) or 100 nM 17β-oestradiol (E₂) (lanes 7–10) for 18 h at 4 °C before incubation with 0.2 pM ³²P-ATP labelled vitellogenin consensus oestrogen response element (ERE) oligonucleotide. The binding product was resolved on a 6% DNA retardation gel for 35 min at 200 V. Gels were subsequently dried and exposed to autoradiography. To determine DNA binding specificity, unlabelled oligonucleotide was added in 1000-fold excess of ³²P-ERE (lane 1). A scrambled ERE sequence was used as a negative control (lane 2). (B) Densitometric quantification of gel bands. Data are shown as the mean ± SD.

Fig. 3. Apo-human oestrogen receptor (hER) β splice variants on oestrogen response element (ERE)-mediated promoter activity. HT-22 cells were transiently transfected with 0.15 μ g ERE-luciferase reporter construct and increasing amounts of plasmid expression vectors containing hER β 1 (A), hER β 2 (B), hER β 4 (C) or hER β 5 (D). Data represent the percentage change in relative light units compared to empty vector controls. *P < 0.05: statistically significant differences from empty vector control.

Fig. 4.

Apo-human oestrogen receptor (hER) β splice variants on activator protein-1 (AP-1)-mediated promoter activity. HT-22 cells were transiently transfected with 0.15 μ g of AP-1-luciferase reporter construct and increasing amounts of expression vectors containing hER β 1 (A), hER β 2 (B), hER β 4 (C) and hER β 5 (D). Data represent the percentage change in relative light units compared to empty vector controls. *Statistically significant differences from empty vector control ($P < 0.05$). # $P < 0.05$ and ! $P < 0.05$: statistical significance between groups.

Fig. 5.

Effects of 17β -oestradiol (E_2), 17β -diol (3β -diol) and ICI 182 780 on human oestrogen receptor (hER) β splice variant-mediated oestrogen response element (ERE) and activator protein-1 (AP-1) promoter activity. HT-22 cells were transiently transfected with $0.15\ \mu\text{g}$ of (A) 2x-ERE- or (B) AP-1-luciferase reporter constructs and $0.15\ \mu\text{g}$ of expression vectors containing hER β 1, hER β 2, hER β 4 or hER β 5. Twenty-four hours post transfection, cells were treated with vehicle (0.001% EtOH), 100 nM of E_2 , 3β -diol or ICI 182 780 for 12 h. Data represent the percentage change in relative light units compared to empty vector controls. * $P < 0.05$: statistically significant differences from empty vector, vehicle-treated control.

Fig 6.

Effects of 17β -oestradiol (E_2), 17β -diol (3β -diol) and ICI 182 780 on human oestrogen receptor (hER) β splice variant-mediated arginine vasopressin (AVP) promoter activity before and after deletion of an activator protein-1 (AP-1) site. SK-N-SH cells were transiently transfected with 0.15 μ g of (A) human AVP (hAVP)-luciferase or (B) hAVP $\Delta 611 - 604$ -luciferase reporter constructs and 0.15 μ g of expression vectors containing hER β 1, hER β 2, hER β 4 or hER β 5. Twenty-four hours post transfection, cells were treated with vehicle (0.001% EtOH), 100 nM E_2 , 3β -diol or ICI 182 780 for 12 h. Data represent the percentage change in relative light units compared to empty vector, vehicle-treated controls. * $P < 0.05$; statistically significant differences from control.

Fig. 7.

Role of phosphoinositide 3-kinase (PI3K) and p38 kinase inhibition on human oestrogen receptor (hER)β1-mediated repression of activator protein-1 (AP-1) promoter activity. HT-22 cells were transiently transfected with 0.15 μg of AP-1-luciferase reporter construct and 0.15 μg of an expression vector containing hERβ1. Twenty-four hours post transfection, cells were treated with vehicle (0.001% EtOH), 100 nM 17β-oestradiol (E₂), or (A) 10 μM LY294002, or (B) 10 μM SB202190 and 100 nM ICI 182 780 for 12 h. Data represent the percentage change in relative light units compared to empty vector, vehicle-treated controls. *P < 0.05: statistically significant differences from control.

Fig. 8.

Role of phosphoinositide 3-kinase (PI3K) and p38 kinase inhibition on human oestrogen receptor (hER) β splice variant-mediated repression of human AVP (hAVP) promoter activity. SK-N-SH cells were transiently transfected with 0.15 μ g of hAVP-luciferase reporter construct and 0.15 μ g of an expression vector containing hER β 1, hER β 2, hER β 4 or hER β 5. Twenty-four hours post transfection, cells were treated with vehicle (0.001% EtOH) or 100 nM 17 β -oestradiol (E₂), (A) 10 μ M LY 294002 or (B) 10 μ M SB202190 and 100 nM ICI 182 780 for 12 h. Data represent the percentage change in relative light units compared to empty vector, vehicle-treated controls. *P < 0.05: statistically significant differences from control.