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Transcriptional activity of human brain estrogen receptor-α splice variants: Evidence for cell type-specific regulation

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ABSTRACT

Estrogen receptor α (ERα) isoforms with complex types of alternative splicing are naturally present in the human brain and may affect canonical receptor signaling. In the present study we investigated transcriptional activity of common ERα splice variants from this group with different molecular defects: MB1 (intron retention), TADDI (small deletion between exons 3 and 4 with an insert), the Δ (deletion) 3*–7*/819 (complete skipping of exons 4, 5 and 6 and partial deletion of exons 3 and 7) and the Δ3–6 (lacking exons 3, 4, 5 and 6) in HeLa and M17 cells upon stimulation with (17β)estradiol or insulin-like growth factor 1 (IGF-1). In HeLa cells, all these splice variants showed the dominant negative function that was more pronounced for the TADDI. In M17 cells the dominant negative variants appeared to be the MB1 and the Δ3–6, whereas TADDI turned out to be a clearly dominant positive variant. In M17 cells mRNA levels of Δ3–6 and Δ3*–7*/819 variants increased following (17β)estradiol administration. In Hela cells (17β)estradiol up-regulated the IGF-1 receptor mRNA levels in cultures transfected with MB1, TADDI and Δ3*–7*/819. Our data demonstrate that ERα splice variants show differential levels of the transcriptional activity in a cell type-specific way and that IGF-1 signaling pathways are differentially employed in a cell-type specific manner depending on the level of the discrete ERα splice variants expressed. Functional properties of various ERα splice variants and their cell type-specificity should, thus, be considered as potential confounders of estrogen therapy effects on the brain.

1. Introduction

Estrogen effects on cognitive functions in climacteric period remain a controversial issue (Henderson and Diaz Brinton, 2010; Hogervorst and Bandelow, 2010; Brinton, 2009; Gleason et al., 2005). Estrogens mediate their actions largely through the cognate receptors – estrogen receptor (ER) – α and β (O'Lone et al., 2004; Roepke et al., 2009; Micevych and Dominguez, 2009). In Alzheimer's disease (AD) ERα expression is changed in the brain areas that are involved in the regulation of memory (Ishunina and Swaab, 2008a). Moreover, in addition to the wild type (wt) ERα the human brain

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expresses a large variety of ERα splice variants that are present concomitant with the wt ERα or as predominant/sole forms of the ERα and their ratio changes at menopause and in AD (Ishunina and Swaab, 2008b, 2009, 2012). For that reason, functional roles of various ERα splice variants and their effect on the full length receptor signaling are crucial for our understanding of estrogen therapy effects on the brain. While functional properties of the ERα single exon skipping variants are well known (Bollig and Miksicek, 2000; García Pedrero et al., 2003; Wong and Weickert, 2009), no such data were available for the complex types of alternative splicing. Therefore, in the present study we aimed at determining the functional activity of ERα isoforms that are representative examples from our recent classification groups of ERα splice variants in the human brain (Ishunina and Swaab, 2012). This study focuses on MB1 (mamillary body, exon1), TADDI, Δ (deletion) 3*–7*/819 and Δ3–6 ERα splice forms. MB1 was first identified in the mamillary body of an AD patient. It is lacking 168 nucleotides in the exon 1 that encode a large portion of the transactivation function 1 of the ERα (Ishunina et al., 2005; Ishunina and Swaab, 2008b). TADDI isoform was first found in the human hippocampus. It is lacking 31 base pairs at the junction between exons 3 and 4 with 13 nucleotides from the exon 2 being inserted into this splice site (Ishunina et al., 2007; Ishunina and Swaab, 2009). Δ3*–7*/819 was first identified in the human cingulate gyrus and in the hypothalamus. Exons 3 and 7 are deleted partially and exons 4, 5 and 6 are skipped completely in this variant. The net deletion comprises 819 nucleotides and is due to the alternative usage of 5′ and 3′ splice sites (Ishunina and Swaab, 2012). Δ3–6 was found in the hypothalamus, pituitary gland and amygdala. It is devoid of exons 3, 4, 5 and 6 and represents an example of quadruplet exon skipping variants (Ishunina and Swaab, 2012). Δ3*–7*/819 and Δ3–6 are lacking a large portion of the ligand binding domain that is encoded by exons 4, 5, 6, 7 and 8 (Fig. 1).

Estrogen signaling is not limited to estrogen receptors and was shown to interact with that of the insulin-like growth factor 1 (IGF-1). It was reported that estradiol treatment leads to the association of the ERα and IGF-1 receptor (IGF-1 R) and activation of the IGF-1 R. This interaction can also be enhanced by the IGF-1 alone. Moreover, the IGF-1 may activate the ERα in the absence of estradiol and regulate the ERα-mediated transcription on its own (Garcia-Segura et al., 2010; Varea et al., 2010). The interaction of estradiol with the IGF-1 is particularly relevant for brain neurogenesis and neuroprotection (Garcia-Segura et al., 2010). In the present study we investigated whether the IGF-1 can act only through the canonical ERα or whether its effects can also be mediated via alternatively spliced ERα forms.

2. Results

2.1. *Natural profile of cell lines*

HeLa cells appear to express the wt ERα mRNA as shown by amplification of transcripts from exons 2 to 8, from exons 2 to 4, from exons 3 to 5 (Fig. 2). Small fragments (~400 to 500 bp) of wt ERα mRNA were consistently detected in the samples of untransfected Hela cells (Fig. 2). Using Q-PCR, the presence of the short ERα mRNA amplicons (Table 2) was revealed in all HeLa cultures (normalized mRNA levels ~0.0014). Moreover, HeLa cells were found to produce a small amount of ERα mRNA splice variants: the Δ3–6 and TADDI (normalized mRNA levels $\sim 3.69 \times 10^{-6}$ and 3.1×10^{-4}, respectively). When HeLa cells were transfected with the plasmid bearing the full length ERα, resulting in the higher level of the canonical ERα mRNA (normalized mRNA level 1.07), more splice variants (MB1, Δ3*–7*/819, Δ3–6) and at a higher quantity (normalized mRNA levels 0.114, 0.110, 0.0004 respectively) were uncovered. This observation shows that the amount of ERα splice variants may depend on the level of the canonical ERα mRNA. HeLa cells showed low level of the IGF-1 mRNA expression (normalized mRNA levels $\sim 2.76 \times 10^{-5}$) and high level of the IGF-1R mRNA (normalized mRNA levels ~0.013).

Similar to HeLa cells, M17 cells expressed both canonical and alternatively spliced ERα mRNAs at baseline level. Transcripts from exons 3 to 5 and from exons 2 to 8 of the canonical ERα mRNA were detected using the RT-PCR (Fig. 2F) and short fragments were detected with Q-PCR at the cycle threshold of ~21 to 23. In M17 cell line Δ3–6 variant was detected at the cycle threshold ~26. Like in the HeLa cells mRNA levels of the IGF-1R were higher than those of the IGF-1.

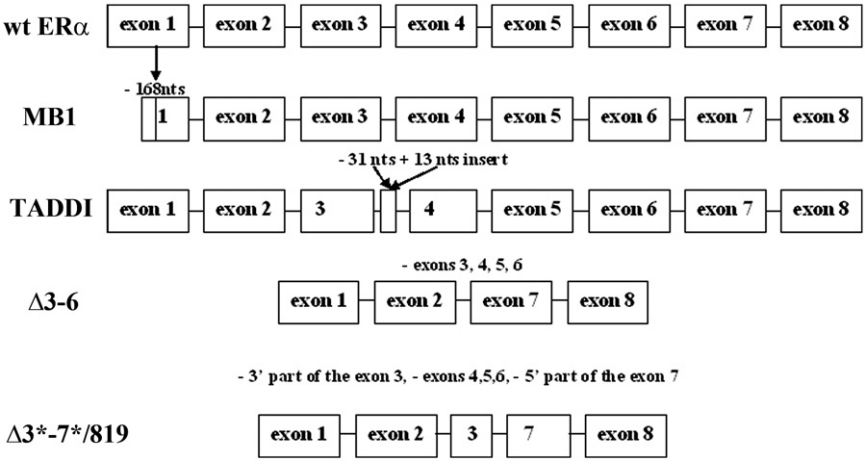

Fig. 1 – Schematic representation of molecular defects in the DNA constructs of ERα splice variants.

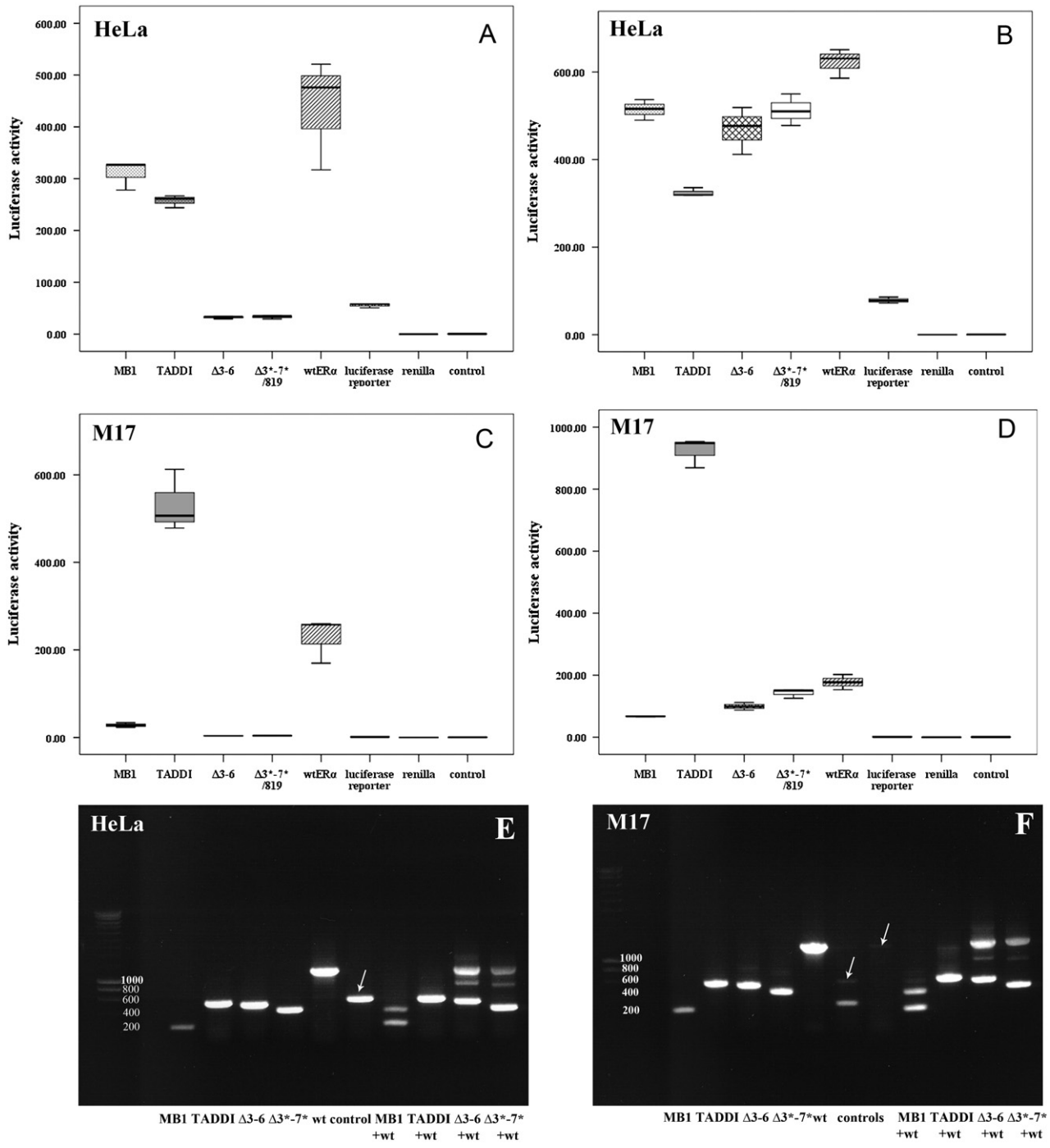

Fig. 2 – Graphs showing differences in the transcriptional activity in HeLa (A, B) and M17 (C, D) cells transfected with alternatively spliced (MB1, TADDI, Δ3–6, Δ3*–7*/819) and/or wild type estrogen receptor-α constructs following estradiol treatment. Controls represent cell cultures that were transfected with luciferase reporter gene and pRL-TK vector (Renilla), pRL-TK vector (Renilla) only and blank controls without any constructs and vehicle. Wt indicates cultures that were transfected with the full length ERα and luciferase reporter construct. Data are presented normalized to pRL-TK vector (Renilla) expression as mean ± S.E.M. of three independent cultures and were reproducible in separate experiments ($p < 0.001$). A – HeLa cells were transfected with mutant or wtERα and luciferase reporter constructs. B – HeLa cells were transfected with mutant+full length ERα+luciferase reporter constructs except for the wt and control cultures. C – M17 cells were transfected with mutant or wt ERα and luciferase reporter constructs. D – M17 cells were transfected with mutant+full length ERα+luciferase reporter constructs except for the wt and control cultures. E, F – RT-PCR confirmation of the target amplicon expression in corresponding HeLa (E) and M17 (F) cell cultures. Lane 1 is a marker, lanes 3–6 represent amplicons that were transfected with mutant receptors, lane 7 – transcript containing exons 2, 3, 4, 5, 6, 7 and 8 of the canonical ERα mRNA amplified from cells transfected with the full length ERα construct, lane 8 shows a transcript containing exons 3, 4, and 5 amplified from control untransfected cultures, lanes 9–12 (E) and 10–13 (F) represent amplicons that were transfected with both mutant and full length ERα constructs. Lower bands represent splice variants, upper bands – fragments of the wt ERα mRNA. Extra bands in between the wt and splice variants in last two lanes turned out to be the corresponding splice forms. Lane 9 (F) – amplicon containing exons 2, 3, 4, 5, 6, 7 and 8 amplified from untransfected cultures.

Table 1 – Primer pairs used to amplify fragments of the canonical and alternatively spliced ERα mRNAs.

Transcript	Forward primer	Reverse primer	The size of the product (bp)
(1) wt ERα mRNA	AACCGTCCGCAGCTCAAGATC	TCGATTATCTGAATTTGGCCTGTAGAA	393
(1) MB1 isoform	AACCGTCCGCAGCTCAAGATC	TCGATTATCTGAATTTGGCCTGTAGAA	225
(2) wt ERα mRNA	CACCAACCAGTGCACCATTGA	AGCAAGTTAGGAGCAAACAGTA	558
(2) TADDI isoform	CACCAACCAGTGCACCATTGA	AGCAAGTTAGGAGCAAACAGTA	540
(3) wt ERα mRNA	GCAGAGAAAGATTGGCCAGTA	CTGGCGCTTGTGTTTCAACAT	329
(4) wt ERα mRNA	AGTATGGCTATGGAATCTGC	ACTTTTGCAAGGAATGCGATG	1228
(4) Δ3*–7*/819 isoform	AGTATGGCTATGGAATCTGC	ACTTTTGCAAGGAATGCGATG	409
(4) Δ3–6 isoform	AGTATGGCTATGGAATCTGC	ACTTTTGCAAGGAATGCGATG	502

Table 2 – Primer pairs used in Q-PCR.

Transcript	Forward primer	Reverse primer	Description
wt ERα	CCTCCTCATCCTCTCCCACAT	GGCACCACGTTCTTGCACT	Join exons 7 and 8
wt ERα	CGATGATGGGCTTACTGACCA	AAATCCACAAAGCCTGGCAC	Link exons 4 and 5
MB1	AGGGCGCCGCCTACGAGTTCA	TAGTAGGGCACCTGCGCGTTG	Reverse primer is over the specific splice site of MB1
TADDI	AAG TGGGAATGAT GAAAG ATTGG	ATCATCTCTCTGGCGCTTGT	Forward primer is over the specific splice site of TADDI
Δ3*–7*/819 Isoform	GGACATAACGACTATATGTGTCCAGCC	AGCCGCTGGTGCTGCTGGTT	Reverse primer is over the specific splice site
Δ3–6 Isoform	TGTGAGGGCTGCAAGGCCTT	GCTGGACAGAAATGTGTACACTCCTTG	Reverse primer is over the specific splice site
IGF-1 mRNA	AGGGGCTTTTATTTCAACAAGCCCA	CCCTCCGACTGCTGGAGCCAT	Forward primer is over the splice site between exons 3 and 4 that encode the active protein
IGF-1 receptor mRNA	GCACCAATGCTTCAGTTCCTTCCA	TTCGATGCTGAAAGAACGTCCAAGG	Forward primer is over the splice site between exons 7 and 8

2.2. *Transcriptional activity of ERα splice variants and of the ERα full length receptor*

2.2.1. *HeLa cells*

Compared to the full-length ERα, (17β)estradiol-stimulated transcriptional activity of the MB1 ($p=0.033$), TADDI ($p=0.001$), Δ3–6 and Δ3*–7*/819 ($p<0.001$) ERα splice variants was significantly decreased (Fig. 2A). Transcriptional activity in the cultures transfected with the splice variants Δ3–6 and Δ3*–7*/819 was lower but still comparable to the natural ERα signaling ($p=1.000$). MB1 and TADDI showed higher level of signaling than the Δ3*–7*/819 and the Δ3–6 variants ($p<0.001$). No significant differences in the transcriptional activity between MB1 and TADDI or between Δ3*–7*/819 and Δ3–6 splice forms were present ($p=1.000$) (Fig. 2A).

When the splice variants were co-expressed with the full-length ERα (Fig. 2B), the transcriptional activity initiated by (17)estradiol, was lower ($p=0.005$ for MB1+wt ERα, $p<0.001$ for TADDI+wtERα and Δ3–6+wtERα, $p=0.004$ for Δ3*–7*/819+wt ERα) than that of the wtERα alone but was higher than in the control cultures ($p<0.001$).

2.2.2. *M17 cells*

In the neuroblastoma M17 cells (17β)estradiol-stimulated transcriptional activity of the MB1, Δ3–6 and Δ3*–7*/819 ERα splice variants was significantly lower ($p<0.001$) than that of the wtERα and did not substantially differ from control cultures (Fig. 2C). There were no significant differences between the MB1, Δ3–6 and Δ3*–7*/819 variants. However, transcriptional activity of TADDI significantly exceeded that of the wtERα ($p<0.001$) (Fig. 2C). The same differences were observed in the cultures that did not receive (17β)estradiol, although transcriptional activity of the wtERα and its splice variants was 2–5 times lower compared to corresponding cultures to which (17β)estradiol was given.

Following co-expression with the full-length ERα, transcriptional activity of the MB1 and the Δ3–6 initiated by (17β)estradiol enhanced, but was lower ($p<0.001$ and $p=0.006$ respectively) than of the wtERα alone. Most robust reduction in signaling was observed in the MB1 cultures (Fig. 2D). Transcriptional activity of the TADDI was much higher not only compared to the other splice variants but also compared to the wtERα ($p<0.001$) and was ~30% higher than in the cultures transfected with the TADDI without the wtERα. Signaling detected in cultures transfected with the Δ3*–7*/819+wtERα did not differ from that of the wtERα alone ($p=1.000$). In each series of cultures transcriptional activity was significantly higher than in all three controls. The results appeared to be reproducible in independent experiments. Highly significant correlation coefficients were determined between two sets of data obtained in repeated experiments (rho values range from 0.926 to 0.994, $p<0.001$).

IGF-1 on its own (50 ng/ml) failed to trigger transcriptional activity since there were no significant differences in the values obtained from cultures to which IGF-1 was administered and cultures with only vehicle added ($p=0.582$; correlation coefficient rho=0.976, $p<0.001$).

RT-PCR amplification of the ERα mRNA fragments enclosing the splice sites of the MB1, TADDI, Δ3*–7*/819, Δ3–6 and subsequent sequencing of the PCR products confirmed the presence of each ERα splice variant or the wt ERα mRNA in the corresponding transfected cultures (Fig. 2EF, Table 1). Furthermore, Q-PCR with the primers overlapping the specific splice sites of the targeted ERα mRNA isoforms (Table 2) showed that these transcripts were present at high levels (cycle threshold ~15 to 19).

3. mRNA levels of the alternatively spliced and canonical ERα mRNAs

In the separate set of cultures transfected with either the mutant or the full length receptor, possible changes in mRNA levels of various ERα mRNA isoforms (MB1, TADDI, Δ3*–7*/819, Δ3–6, wt), IGF-1 mRNA and IGF-1R mRNA upon (17β)estradiol treatment were examined (Fig. 3). (17β)Estradiol appeared not to alter the level of any of the studied splice variants in either transfected or non-transfected HeLa cells (Fig. 3A). However, mRNA levels of Δ3–6 and Δ3*–7*/819 variants were two-fold up-regulated ($p=0.026$ ($F=5.96$, $df=4$) and $p=0.055$ ($F=3.11$, $df=4$) respectively) in the corresponding M17 cultures (Fig. 3C). The expression of the IGF-1 mRNA did not show significant changes following (17β)estradiol treatment in HeLa cells, but was enhanced in M17 cultures transfected with Δ3*–7*/819 ($p=0.008$, $F=0.046$, $df=4$). The level of the IGF-1 receptor mRNA was up-regulated by (17β)estradiol in the HeLa cultures transfected with the MB1 ($p=0.034$, $F=2.46$, $df=4$), TADDI ($p=0.007$, $F=2.87$, $df=4$) and Δ3*–7*/819 ($p=0.049$, $F=2.52$, $df=4$) (Fig. 3B). No changes in the expression of reference genes upon (17β)estradiol administration were detected in any of the cultures (p-values range from 0.193 to 0.910).

4. Discussion

In the present study we found that ERα splice variants MB1, TADDI, Δ3–6 and Δ3*–7*/819 show differential transcriptional activity in HeLa and M17 cells. In HeLa cells all these variants appeared as dominant negative, whereas only two of them (MB1 and Δ3–6) were dominant negative in M17 cells. Moreover, the splice variant TADDI demonstrated opposite effects in these cell lines. It was dominant negative in HeLa and dominant positive in M17 cells. Such cell type-specific functioning of mutant receptors suggests differential employment of co-activators and co-repressors. It should be mentioned that the cell specificity in transcriptional activity has been also described for the Δ5 variant of ERα in COS-1 and HeLa cells (Desai et al., 1997). The Δ3–6 and the Δ3*–7*/819 variants lack significant portions of the ligand binding domain. Such deletions cause inability of the truncated receptors to bind (17β)estradiol and result in the absence of signaling on their own as shown by the lowest level of the transcriptional activity comparable to that of the control cultures in the present study. Although both of them suppressed wtERα signaling to a substantial extent in HeLa cells, Δ3*–7*/819 was not acting as a dominant negative variant in M17 cells as concluded from the experiments where it was co-expressed with the full-length receptor. The fact that the Δ3*–7*/819 does not significantly diminish the wtERα function whereas the Δ3–6 does can be explained by the differences in their deletions. While the exon 3, a part of which is encoding the DNA binding domain, is deleted partially in the Δ3*–7*/819 isoform, it is completely absent in the Δ3–6 isoform. Therefore, the alteration in the DNA-binding domain in the Δ3–6 is the cause of a more pronounced effect on the wt ERα signaling. MB1 appeared as the dominant negative variant in both HeLa and M17 cells. Despite its ability to bind estrogens and estrogen responsive elements as evidenced by its basal level of transcriptional activity (Fig. 2A,C), this variant is significantly repressing the signaling of the full length receptor (Fig. 2B,D). Although, the molecular fault in TADDI seems to be small compared to the other studied variants (only 31 nucleotides deleted with the 13 nucleotides insert), its location in the position of the nuclear localization signal seems to explain significant reduction in the transcriptional activity (Fig. 2A,B). However, its dominant positive activity in M17 cells may rather be related to the cell type-specific co-activators/co-repressors involved in ERα signaling.

Both canonical and mutant ERα showed transcriptional activity in the absence of exogenous estrogen, but the level of their signaling in this condition was 2–5 times lower than in the presence of (17β)estradiol.

Treatment with 1 nM (17β)estradiol did not change the levels of the wild type and alternatively spliced ERα mRNAs in HeLa cells in the cultures over expressing mutant or the full length receptors as well as in non-treated cultures, although this dosage was sufficient to trigger the overall transcriptional activity. Indeed, the mechanism of how estrogen can regulate the ERα and other genes is not completely understood. This issue is much complicated by a large variety of target DNA sequences that can be used by activated estrogen receptors for direct genomic interactions. The ERα gene possesses at least three estrogen responsive elements (EREs), which, however, are considered as half-EREs or half-palindromic (O'Lone et al., 2004). Half-EREs are associated with weaker estrogen responsiveness (Gruber et al., 2004). Furthermore, compelling evidence suggests that ERα may indirectly associate with promoters or enhancers via binding (tethering) to other transcriptional factors like Sp1, activating transcription factor (ATF)-2/c-jun, ATF-2/cAMP response element binding protein (CREB), ATF-1/CREB, activator protein 1 (AP-1) (jun/fos), nuclear transcription factor-Y and the long splice isoform of the estrogen receptor related β (O'Lone et al., 2004; Gruber et al., 2004; Welboren et al., 2007; Bombail et al., 2010). For example, the IGF-1 gene contains the AP-1 regulatory sequence through which ERα can indirectly associate (O'Lone et al., 2004). The mentioned caveats may partially explain the lack of significant changes in the wtERα and IGF-1 mRNA after 20 h of estradiol treatment in our study by cell type specificity of additional factors involved in ERα-mediated transcription. Although, ~2 fold decrease in the ERα mRNA

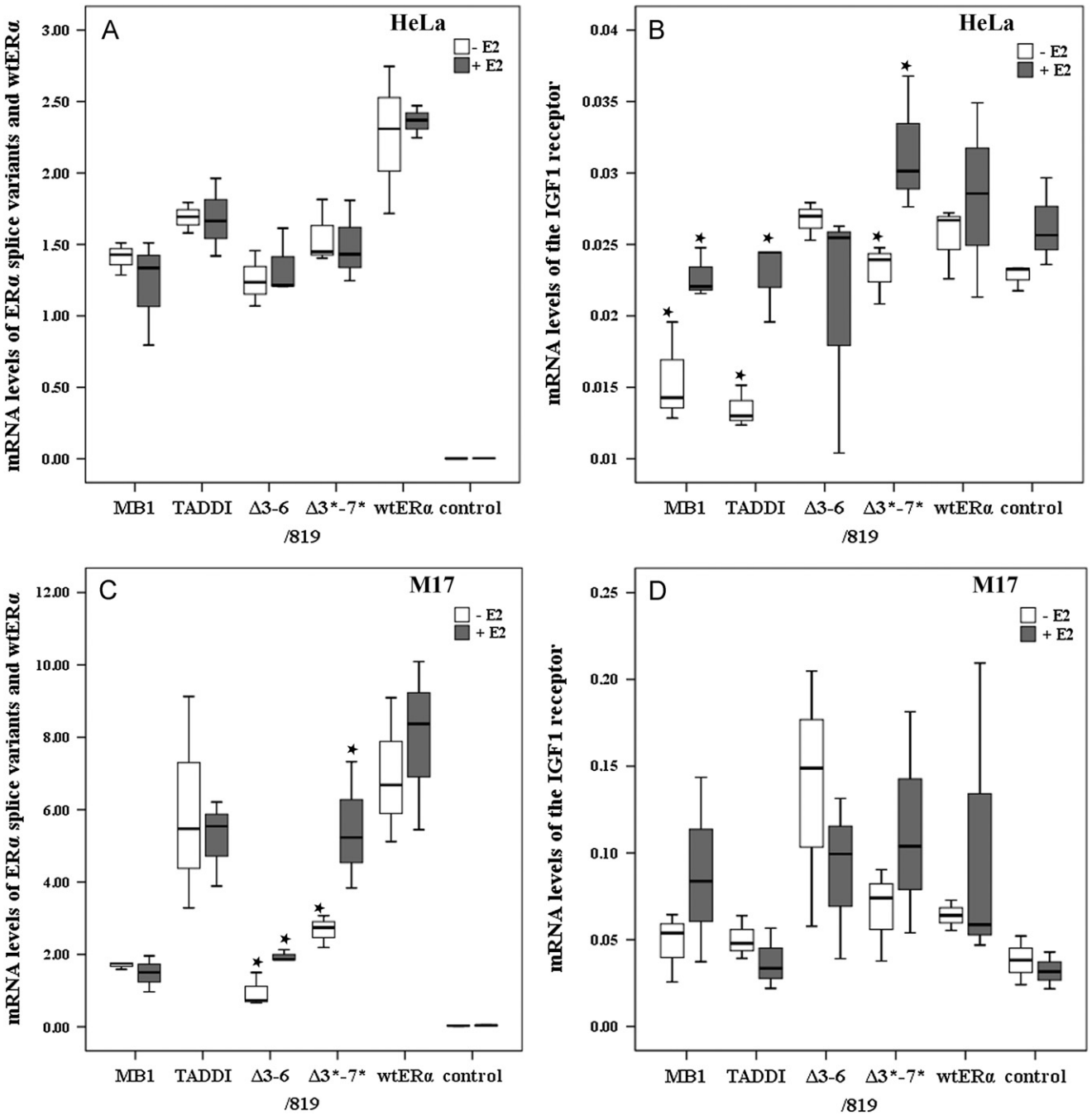

Fig. 3 – mRNA levels of alternatively spliced and canonical ERα (A, C) and of the insulin-like growth factor 1 receptor (B, D) in estradiol-treated (dark columns) and non-treated (white columns) HeLa (A, B) and M17 (C, D) cells normalized to the geometric average of two reference genes (elongation factor-1α and ribosomal protein S27a). Data are presented as mean ± SEM of three independent cultures. Statistically significant differences are indicated by stars.

levels after 10 nM estradiol treatment was reported in tet-inducible ERαHA cells (Fowler et al., 2006), this comparison was made relative to cultures that received 0.1% ethanol. It was well reported that ethanol at concentrations of 0.01% and 0.1% is able to increase the ERα protein content in cancer cells (Wang et al., 2010; Etique et al., 2004). At a concentration of 0.1% ethanol increased 1.7 fold the ERα mRNA and triggered ERα ligand independent activation (Etique et al., 2007). On the other hand, ethanol was shown to attenuate the effects of estradiol (Chen et al., 2009; Wang et al., 2010). Even 0.01% ethanol concentration was sufficient to abolish the 10 nM estradiol impact on cell growth in GH4C1 pituitary tumor cells (Wang et al., 2010). Therefore, it is not clear whether a decrease in the ERα mRNA levels upon 10 nM

estradiol treatment reported by Fowler et al. (2006) was just an attenuation of the ethanol effect. In the present study the concentration of ethanol added was kept at 0.001% which can be regarded as non-ethanol treated (Etique et al., 2007).

Interestingly, in M17 cells estradiol administration increased mRNA levels of Δ3–6 and Δ3*–7*/819 isoforms and did not change the expression of MB1 and TADDI in the cultures where they were over expressed. Together, these data show that (17β)estradiol may provide cell type-specific effects on transcriptional levels of some ERα splice variants.

Another interesting finding was the (17β)estradiol-induced up-regulation of the IGF-1 receptor mRNA in HeLa cultures expressing MB1, TADDI and Δ3*–7*/819 variants. This observation demonstrates that in the presence of the high amount of the ERα splice variants with conserved DNA-binding domain (17β)estradiol may potentially increase its signaling through the IGF-1R-coupled pathways. However, because the MB1, TADDI and the Δ3*–7*/819 isoforms show decreased transcriptional activity, this effect on the IGF-1R gene expression is indirect. In accordance with our data it has been previously reported that in prostate cancer cells the IGF-1R is up-regulated by estrogens via a nongenotropic pathway including the cyclic AMP response element-binding protein (Genua et al., 2009). Naturally high levels of the IGF-1R mRNA in all examined cell lines (originate from cervical cancer or neuroblastoma) in the present study agree well with the overexpression of the IGF-1R in prostate cancer cells (Genua et al., 2009).

In conclusion, our data demonstrate that ERα splice variants show differential levels of transcriptional activity in a cell type-specific manner and that estrogen treatment alone is insufficient to regulate the expression of wtERα mRNA and its alternative splicing in transfected HeLa cells. However, the fact that in M17 cells estradiol increased expression of the variants with compromised ligand binding domain and did not change the level of MB1 and TADDI clearly indicates that ERα mRNA alternative splicing is regulated in a cell-type specific way. This means that the effects of estrogen therapy on different tissues and even on different regions within the brain will not be similar. Functional properties of various ERα splice variants and their cell type-specificity should, thus, be considered as potential confounders of estrogen therapy effects on the brain.

5. Experimental procedures

5.1. *Plasmids*

DNA sequences encoding the MB1 (Ishunina et al., 2005), TADDI (Ishunina et al., 2007), Δ3*–7*/819 and Δ3–6 (Ishunina and Swaab, 2012) estrogen receptor α splice variants were cloned into the pDream2.1/MCS vector by GenScript (Piscataway, NJ, USA, http://www.genscript.com). Plasmids bearing the full length ERα – pSG5 HEGO (Tora et al., 1989; Koehorst et al., 1994) and the reporter construct for the luciferase assay – 17M ERE-G-luc (Metzger et al., 1995) were generous gifts of Prof. P. Chambon and Prof. P. Burbach. Fifty nanograms of each plasmid DNA were subcloned in DH5α competent *Escherichia coli* cells according to the standard procedure (Life Technologies, Bleiswijk, The Netherlands). The next day the plasmid DNA was purified according to the NucleoBond/Xtra Midi protocol (Macherey-Nagel, Düren, Germany). Resultant DNA concentrations were checked using the Agilent Nano drop (Agilent, Amstelveen, The Netherlands).

5.2. *Cell line transfection.*

Experiments were performed in two cell lines: non-neuronal HeLa cells derived from cervical cancer material and neuroblastoma M17 cells from dopaminergic neurones (Bowen et al., 2004). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamax, 100 μg of streptomycin, 100 units of penicillin and 10% fetal bovine serum (FBS) (Invitrogen, Bleiswijk, The Netherlands). M17 cells were maintained in Opti-MEM with 10% FBS (Invitrogen, Bleiswijk, The Netherlands). Prior to transfection M17 cells were seeded in Opti-MEM with 0.5% FBS. One day before transfection the cells were split from plastic flasks using 0.25% trypsin and distributed equally in 24 well plates at a concentration of 5×10^4 cells per well. The next day they were transfected with 0.4 mg of each DNA construct using the calcium phosphate precipitation technique for the HeLa cells (Naldini et al., 1996) and lipofectamine 2000 (Invitrogen, Bleiswijk, The Netherlands) for the M17 cells. In final experiments four replicate cultures of cells were transfected with each of the mentioned mutants and/or the wt ERα and with the 17M ERE-G-luc to monitor transcriptional activity of canonical and alternatively spliced ERα isoforms (three for luciferase assay and one to examine the type of the transcript expressed). All cultures except for the blank controls (see below) were co-transfected with 2 ng of pRL-TK vector (Renilla) (Promega, Leiden, The Netherlands). Transcriptional activity was stimulated by 1 nM (17β)estradiol (Sigma Aldrich, St. Louis, USA) or 50 ng/ml of the insulin-like growth factor 1 (IGF-1) (Biaffin GmbH & Co KG, Kassel, Germany) 20 h before the cells were harvested. The dosages of (17β)estradiol and of the IGF-1 were verified in appropriate experiments (Wong and Weickert, 2009; Rajski et al., 2010). The concentration of ethanol added as a solvent of (17β)estradiol was 0.001%. To determine changes in the expression level of various splice variants following estrogen treatment six replicate cultures were transfected with each mutant or the full length ERα. Three of such cultures received 1 nM estradiol 20 h before RNA was collected and three cultures were maintained in the absence of exogenous (17β)estradiol.

5.3. *Luciferase assay*

Dual luciferase reporter assay was performed according to the manufacturer's instructions (Promega, Leiden, The Netherlands). Following removal of the medium and rinsing in phosphate buffered saline (PBS) the cells were collected in the lysis buffer solution and centrifuged at 12 000 rpm. Ten microlitres of the supernatant containing the cell lysate were then mixed with 100 μl of luciferase assay reagents and measured in a Luminometer (Berthold Technologies, Vilvoorde, Belgium). From each cell lysate two samples were taken for the measurement of luciferase activity expressed as light produced. In every experiment three controls were used: (1) control

cultures transfected with luciferase reporter gene, pRL-TK vector receiving estradiol, (2) control cultures transfected with pRL-TK vector receiving estradiol and (3) control cultures that were not transfected and were not given estradiol. First control with luciferase reporter gene accounts for the natural ERα signaling. The last control indicates the level of the background that was always very low (normalized values <0.7).

5.4. *PCR amplification*

Amplification of the cDNA from cell cultures was performed for two reasons: (i) to control the expression of the target sequences in experiments assessing the transcriptional activity of the ERα splice variants and (ii) in separate Q-PCR experiments aimed to determine changes in the transcriptional levels of the ERα splice variants following (17β)estradiol treatment. RNA was extracted with a Trizol reagent (Invitrogen, Bleiswijk, The Netherlands) according to the manufacturer's instructions. Reverse transcription (RT) reactions and PCR amplification of the ERα mRNA fragments that included the splice sites of the target ERα mRNA isoforms were performed as described in Ishunina and Swaab (2012) using the primer pairs listed in Table 1.

Q-PCR reactions were carried out in the 7300 Real Time PCR System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) as described in Ishunina and Swaab (2012) with the primer pairs shown in Table 2. All samples were analyzed in duplicate. The specificity of the Q-PCR reactions was examined by determining the melting point that showed the presence of a single product per each Q-PCR reaction. If no template (sterile water) was added, Q-PCR was negative for each of the primer sets. The mRNA levels were assessed using the $2^{-}\Delta\Delta^{ct}$ method (Applied Biosystems, Perkin Elmer)). For normalization of the target cDNAs, the geometric mean of two reference genes was employed: elongation factor 1α (EF1α) and ribosomal protein S27a (rS27a) as described earlier (Hope et al., 2003; Ishunina et al., 2005; Ishunina and Swaab, 2012).

5.5. *Statistical analysis*

Differences in the transcriptional activity of ERα splice variants, the full length ERα and of the basal amount of ERα in Hela cells were assessed using the ANOVA with Bonferroni Post Hoc test. Changes in the expression levels of the canonical and alternatively spliced ERα mRNAs as well as those of the IGF-1 and IGF-1R mRNA following (17β)estradiol treatment were examined with the independent-samples t-test. Spearman's test was used to test for correlations between similar data sets derived from independent experiments. $p<0.05$ was considered to be significant.

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