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ORIGINAL PAPER

Selective transcriptional regulation of aromatase gene by vitamin D, dexamethasone, and mifepristone in human glioma cells

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Abstract The human aromatase gene (*CYP19A1*) is controlled by multiple promoters that give rise to different aromatase transcripts. Its regulation has been studied in cells from multiple origins, including placenta, bone, adipose tissue, and breast cancer. However, little is known about its regulation in cells from neural origin. We assessed whether vitamin D, dexamethasone, and the glucocorticoid receptor antagonist mifepristone regulate the aromatase gene in human glioma, neuroblastoma, and breast cancer cells. The results show that these compounds enhance the activity of different aromatase promoters in glioma cells, but not in neuroblastoma and breast cancer cells. Vitamin D increased the expression of I.3, I.7, and I.4 aromatase transcripts and induced de novo expression of the I.6 transcript; dexamethasone increased the expression of I.4, PII, and I.3 transcripts and mifepristone increased the expression of PII and I.3 aromatase transcripts. The cell specific regulation of *CYP19A1* by vitamin D, dexamethasone, and mifepristone opens the possibility for cellular selective modulation of estrogen biosynthesis within the brain.

Keywords Aromatase · Glioma · Neuroblastoma · Breast cancer · Vitamin D · Dexamethasone · Mifepristone

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Introduction

The human aromatase gene, *CYP19A1*, which codifies for the enzyme that catalyzes the conversion of androgens into estrogens [1], contains a regulatory region constituted by at least 10 non-coding variants of exon I: PII, I.3, I.6, I.2, I.f, I.7, I.5, I.4, 2a, and I.1 (Fig. 1). This regulatory region is followed by a coding region, composed by 9 exons (exons II to X) [2, 3]. Each variant of exon I is under the control of a regulatory region or promoter located in the 5′ end. The selective activation of a particular promoter gives rise, by alternative splicing, to an aromatase transcript starting in the 5′ end region with the sequence of the corresponding exon I, followed by the sequence corresponding to the coding region (Fig. 1). Therefore, with independence of the identity of the non-coding variant of exon I that is transcribed into mRNA, all aromatase transcripts share the same coding region (exons II–X) and all of them, therefore, codify for the same protein.

The transcriptional regulation of *CYP19A1* has been studied in cells from multiple origins, including bone [4, 5], placenta [6], adipose tissue [7–9], or breast cancer [10, 11]. These studies have shown that there is some degree of tissue specificity in the use of each promoter. Ovary uses predominantly the promoter associated to the PII variant of exon I; adipose tissue uses predominantly variants I.3 and I.4; bone variant I.6; endothelial cells variant I.7 and fetal tissues variant I.4 [4, 12]. In addition, under pathological conditions, the activity of certain promoters is increased. For instance, promoters associated to exons PII, I.3, I.4, and I.7, are significantly up-regulated in breast cancer [13]. On the other hand, the promoters associated to exons I.2, 2a, and I.1 are active almost exclusively in the placenta [12] (Fig. 1).

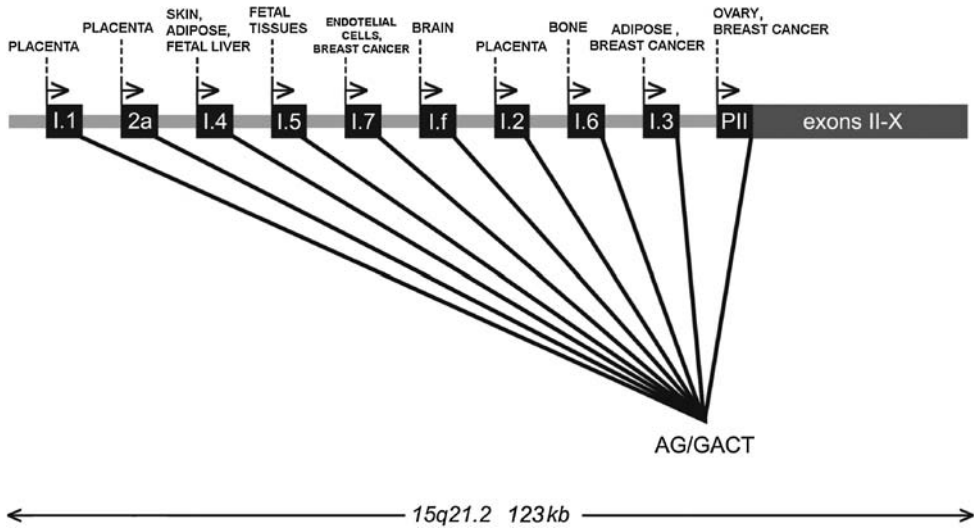

Fig. 1 Schematic representation of the human aromatase gene (*CYP19A1*) showing the different non-coding variants of exon I and the common splice site in exon II (AG/GACT). Each exon I variant is associated to a particular promoter. The selective activation of a particular promoter gives rise to an aromatase transcript with a particular exon I in the 5′end (5′ untranslated region or 5′-UTR). By means of an alternative splicing, each exon I is fused to exons II-X. Therefore, all aromatase transcripts share the same coding region (exons II-X) and all of them codify for the same protein. Based on references [3] and [14]

In the human brain, aromatase expression is under the control of the promoters associated to exons I.f, PII, I.3, and I.4 [14]. However, little is known about the transcriptional regulation of the aromatase gene in human cells of brain origin. The aromatase gene is expressed in the nervous system of most vertebrates, including fish, amphibians, birds, and mammals [15–17]. Brain aromatase regulates the development and function of neural structures involved in the control of neuroendocrine events and reproductive behavior [18–21]. Moreover, aromatase activity in the brain modulates adult neurogenesis [22], neuronal plasticity [23–26], non-reproductive behaviors [27], cognition [28–30], and the response of neural tissue to neurodegenerative stimuli [31–34]. Therefore, since brain aromatase exerts important modulatory actions under physiological and pathological conditions, the study of its regulation in human neural cells is of potential practical interest.

Previous studies have found that vitamin D and glucocorticoids increase aromatase expression in certain cells types, including adipose stromal cells and osteoblasts [5, 7]. Here we have analyzed the regulation of the *CYP19A1* gene in response to vitamin D, the synthetic glucocorticoid dexamethasone and the antagonist of glucocorticoids and progesterone receptors mifepristone (RU486) [35] in human glioma and neuroblastoma cells. In addition, we have compared the results with those obtained in MCF7 cells, a human breast cancer cell line in which aromatase expression is well characterized [11, 36].

Results

Glioma, neuroblastoma, and breast cancer cells express different aromatase transcripts

The three cell lines expressed aromatase mRNA under basal conditions (culture medium without serum for 36 h) (Fig. 2). However, the pattern of expression of aromatase transcripts was not identical among cell lines. Under these conditions, glioma T98G cells expressed the PII, I.3, I.f, I.7, and I.4 transcripts (Fig. 2). Moreover, in the case of the I.3 transcript, we found two variants: the full variant of the transcript (I.3) and a truncated variant (I.3T) that lacks part of the 3′ region of the I.3 exon (Fig. 2).

Under the same culture conditions, only transcripts PII and I.3 were detected in neuroblastoma BE2M17 and breast cancer MCF7 cells (Fig. 2). Interestingly, the I.3 transcript found in these cells corresponds to an unspliced variant that contains both I.3 and PII exons (Fig. 2). The I.f, I.7, and I.4 transcripts were only found very occasionally in these cells.

Vitamin D increases the expression of I.3, I.7, and I.4 aromatase transcripts and induces de novo expression of I.6 transcript in glioma cells but not in neuroblastoma and breast cancer cells

Total aromatase mRNA expression was evaluated using a pair of primers directed against the coding region of the aromatase mRNA, a region present in all aromatase

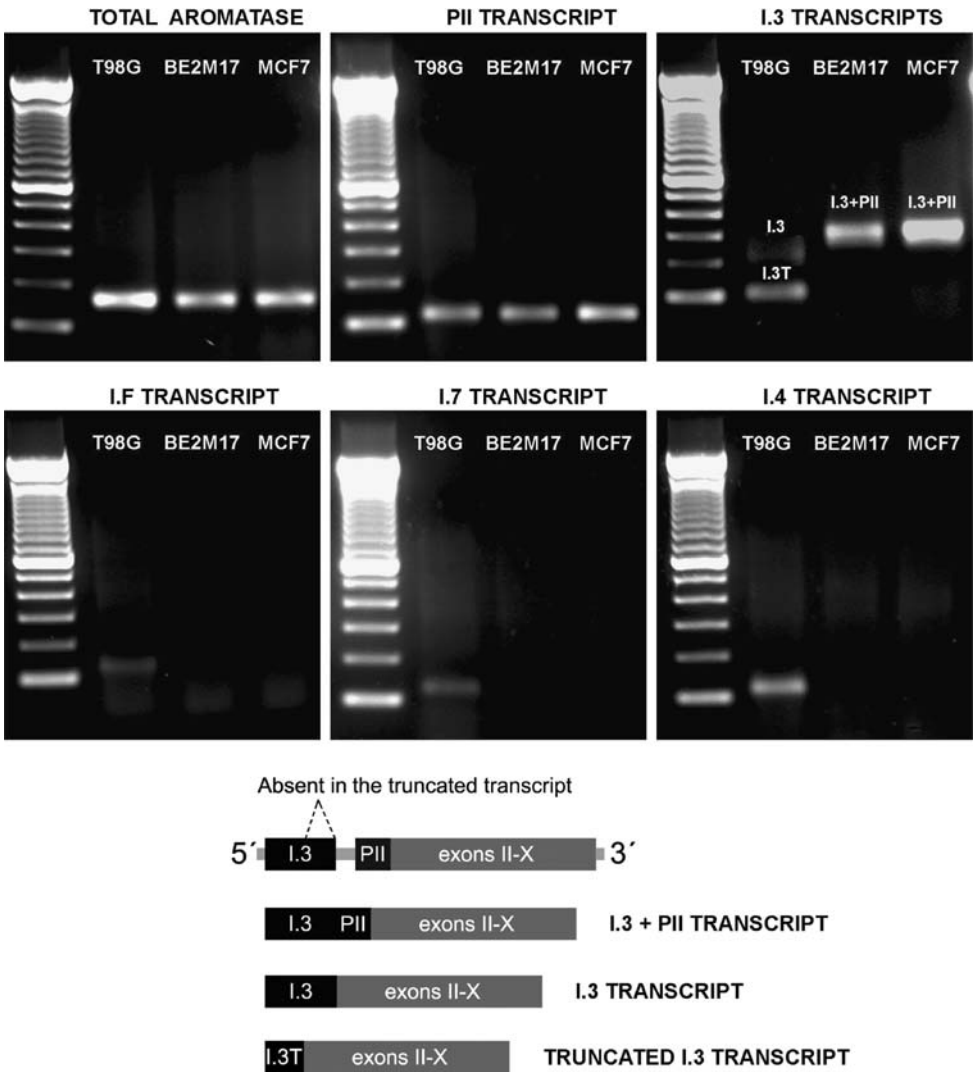

Fig. 2 PCR analysis of total aromatase mRNA and aromatase transcripts (upper panels) expressed under basal conditions in three different human cell lines: glioma T98G, neuroblastoma BE2M17, and breast cancer MCF7 cells. Left lanes, 100 bp ladder. Five different aromatase transcripts were detected in T98G cells: PII, I.3, I.f, I.7, and I.4. In the case of the I.3 transcript, two variants were found: the full variant (I.3) and a truncated variant (I.3T). In BE2M17 and MCF7 cells, two different aromatase transcripts were detected: PII and I.3. The I.3 transcript corresponds to an unspliced variant that contains both exons I.3 and PII. The bottom panel shows a schematic representation of the different I.3 transcripts found

transcripts. Vitamin D increased the expression of total aromatase mRNA to approximately 5 times the value detected in control glioma cells (Fig. 3a). This was accompanied by an increase in the expression of I.3, I.7, and I.4 transcripts to about 6, 8, and 5 times the levels in control cells, respectively (Fig. 3c, e, f). Although the levels of PII transcript were also moderately increased after vitamin D treatment (Fig. 3b), the difference did not reach statistical significance ($P = 0.08$). Moreover, vitamin D induced the de novo expression of an aromatase transcript in glioma cells, the I.6 transcript (Fig. 4).

In contrast to what it was observed in glioma cells, the expression of total aromatase mRNA in neuroblastoma and breast cancer cells remained unchanged after vitamin D treatment, compared to control conditions (Fig. 3a). On the other hand, vitamin D did not modify the expression of PII and I.3 transcripts in neuroblastoma and breast cancer cells (Fig. 3b, c) and did not induce the expression of new transcripts in these cells (Fig. 3d–f).

Dexamethasone increases the expression of I.4 aromatase transcript and, to a lesser extent, the levels of PII and I.3 transcripts in glioma cells but not in neuroblastoma and breast cancer cells

In glioma cells, dexamethasone treatment increased total aromatase mRNA expression to more than 20 times the control values (Fig. 5a). The analysis of the different aromatase transcripts in glioma cells treated with dexamethasone revealed an increase in the expression of PII, I.3, and I.4 transcripts to about 4, 2, and 60 times the control values, respectively (Fig. 5b, c, f). However, dexamethasone did not affect the expression of I.f and I.7 transcripts (Fig. 5d, e) and did not induce the expression of new transcripts in glioma cells.

In neuroblastoma and breast cancer cells, dexamethasone treatment had no effect in the levels of total aromatase mRNA (Fig. 5a) and did not change the levels of the particular transcripts expressed by these cell lines (Fig. 5b, c).

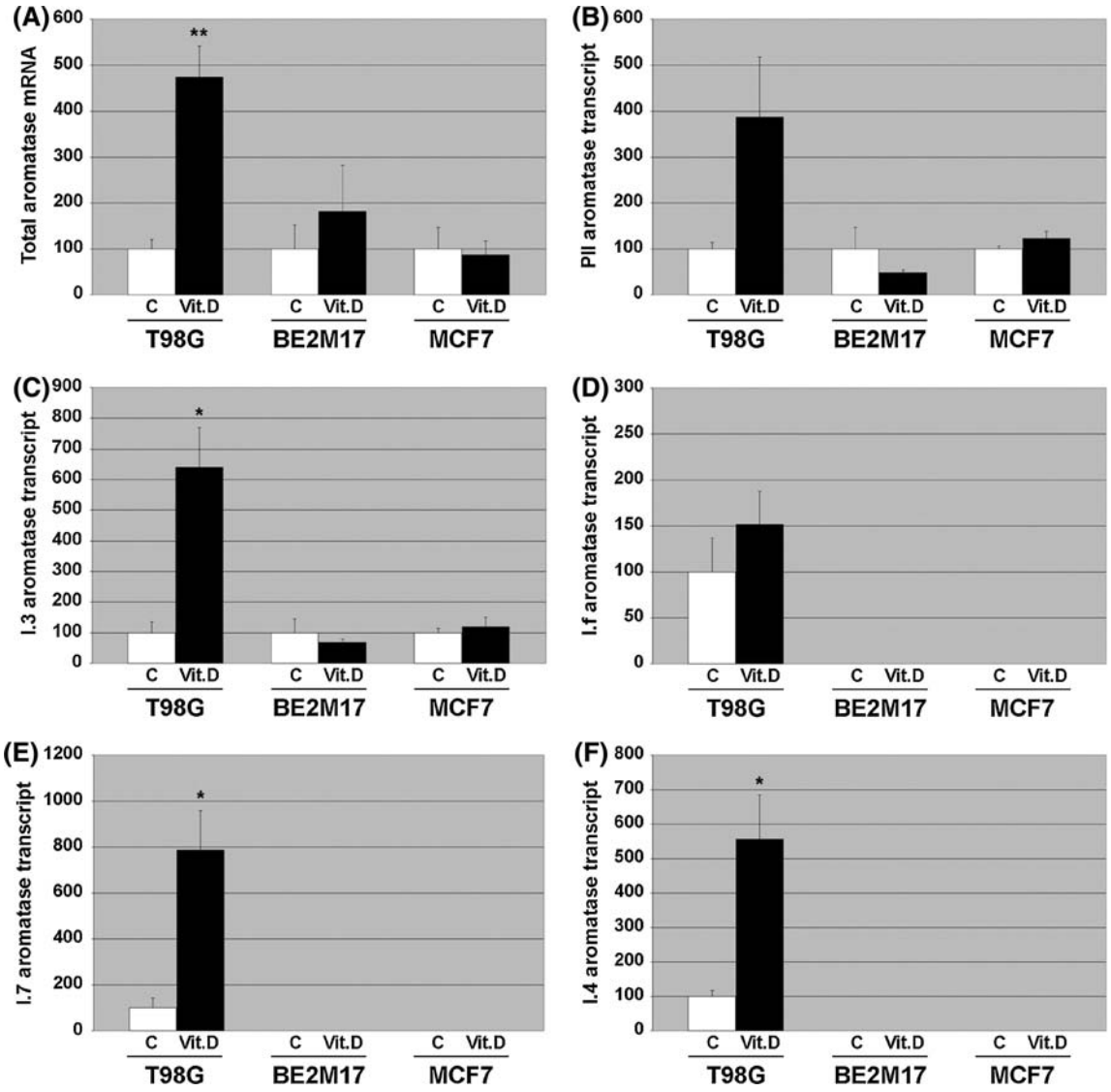

Fig. 3 Quantitative PCR analysis of total aromatase mRNA expression and aromatase transcript levels in glioma (T98G), neuroblastoma (BE2M17), and breast cancer (MCF7) cells treated with vitamin D (100 nM) or vehicle for 24 h. A, Total aromatase mRNA. B, PII transcript. C, I.3 transcript. D, I.f transcript. E, I.7 transcript. F, I.4 transcript. mRNA values are expressed in arbitrary units. Data represent the mean ± standard error of the mean (SEM) from 3 to 5 independent experiments. Asterisks, statistical difference with respect to control values ($^*P < 0.05$, $^{**}P < 0.01$, Student's *t*-test). C, control. Vit.D, vitamin D

Furthermore, dexamethasone did not induce the expression of new transcripts in neuroblastoma and breast cancer cells (Fig. 5d–f).

Mifepristone increases the expression of PII and I.3 aromatase transcripts in glioma cells but not in neuroblastoma and breast cancer cells

In glioma cells, mifepristone significantly increased the expression of total aromatase mRNA (Fig. 6a) and the expression of PII and I.3 transcripts (Fig. 6b, c) to about 10, 7, and 20 times the levels observed under control conditions, respectively. However, in neuroblastoma and breast cancer cells, the expression of total aromatase mRNA (Fig. 6a) and the expression of aromatase transcripts PII and I.3 (Fig. 6b, c) were not affected by mifepristone treatment. Since mifepristone is an antagonist of progesterone receptors, we tested the effect of progesterone on the expression of aromatase by glioma cells. However, the expression of total aromatase mRNA and the expression of PII and I.3 transcripts were unaffected in glioma cells by the treatment with the hormone (Fig. 7).

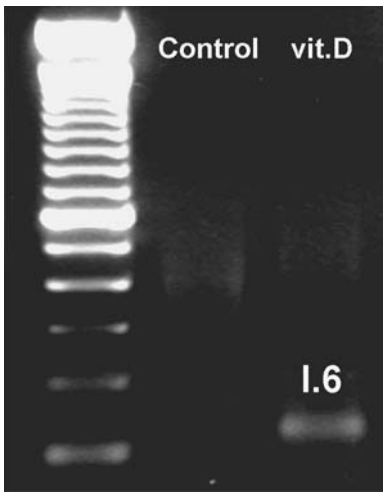

Fig. 4 PCR analysis showing the de novo expression of I.6 transcript in glioma T98G cells in response to vitamin D (Vit.D) treatment. Left lane, 100 bp ladder

Discussion

Aromatase expression in glioma, neuroblastoma, and breast cancer cells is under the control of different promoters

In the present study we have identified the promoters of the aromatase gene that control the expression of the enzyme in human glioma, neuroblastoma, and breast cancer cell lines under basal conditions and in response to vitamin D, dexamethasone, and mifepristone. Our results show that in glioma T98G cells the aromatase gene is under the control of the promoters associated to exon I variants PII, I.3, I.f, I.7, and I.4, as demonstrated by the presence in these cells of the corresponding transcripts. With the exception of the I.7 transcript, this pattern of aromatase transcripts is identical to that found in the human cerebral cortex [14]. The presence of the I.7 transcript has been described mainly in endothelial cells and it is up-regulated in breast cancer tissue [37]. Under the same culture conditions, in the neuroblastoma BE2M17 and breast cancer MCF7 cells the aromatase gene appears to be controlled exclusively by the promoters associated to exons PII and I.3, since only PII and I.3 transcripts were detected in these cells.

In the case of I.3 transcript, we found that glioma cells express two variants: the full variant of the transcript (I.3) and a truncated variant (I.3T). The I.3T variant has been previously described in breast tissue [38] and, more recently, in the human cerebral cortex [14]. In contrast, in neuroblastoma and breast cancer cells the I.3 transcript detected form corresponds to an unspliced variant that contains both exons I.3 and PII. Since both the activation of the promoters associated to exon PII and exon I.3 will give rise to transcripts containing exon PII in these cells, we were unable to determine the relative proportion of pure PII transcripts and those corresponding to the unspliced I.3 transcript variant containing exon PII. The presence of all these multiple I.3 transcript variants may be the result of the use of multiple splicing donor sequences located in the boundaries of each exon I [39]. The physiological or physiopathological meaning of these variants remains unknown.

Vitamin D, dexamethasone, and mifepristone stimulate aromatase expression and the activation of different aromatase promoters in glioma cells but not in neuroblastoma and breast cancer cells

Our findings indicate that vitamin D, dexamethasone, and mifepristone enhance the expression of total aromatase mRNA in glioma cells. However, the mechanism used by these molecules for the induction of aromatase expression seems to be different. On the basis of the aromatase transcripts detected and its expression levels, vitamin D appears to act mainly activating promoters associated to exon I variants I.3, I.7, and I.4 as well as by the de novo activation of the promoter associated to variant I.6. Dexamethasone also stimulates the activation of the promoters associated to variants I.3 and I.4 in glioma cells, particularly to variant I.4. In addition, dexamethasone activates the promoter associated to variant PII. On the contrary, dexamethasone does not seem to activate the promoters associated to exon I variants I.7 and I.6. Finally, mifepristone stimulates aromatase gene expression in glioma cells through an increase in the activation of the promoters associated to exon I variants PII and I.3. Therefore, vitamin D, dexamethasone, and mifepristone regulate aromatase expression in glioma cells by the selective regulation of a different set of promoters of the *CYP19A1* gene.

The increased I.3 transcript expression in glioma cells in response to vitamin D is in agreement with previous findings in osteoblasts [4, 5]. Since the promoter associated to exon variant I.3 does not seem to contain any vitamin D response element (VDRE), the regulation of I.3 transcript expression by vitamin D may depend on a VDRE-independent mechanism. The mechanism may involve an interaction of vitamin D, through the activation of the Jun kinase pathway, with an AP1 motif present in the promoter associated to exon variant I.3 [40]. Our findings show that vitamin D may also increase aromatase expression in glioma cells by the activation of the promoters associated to exon I variants I.4, I.6, and I.7. The promoter associated to exon I variant I.4 has several putative VDRE motifs [41] and an AP1 motif [7]. The promoter associated to exon variant I.6 contains also several AP1 motifs [39]. All these motifs would be potential targets of vitamin D. However,

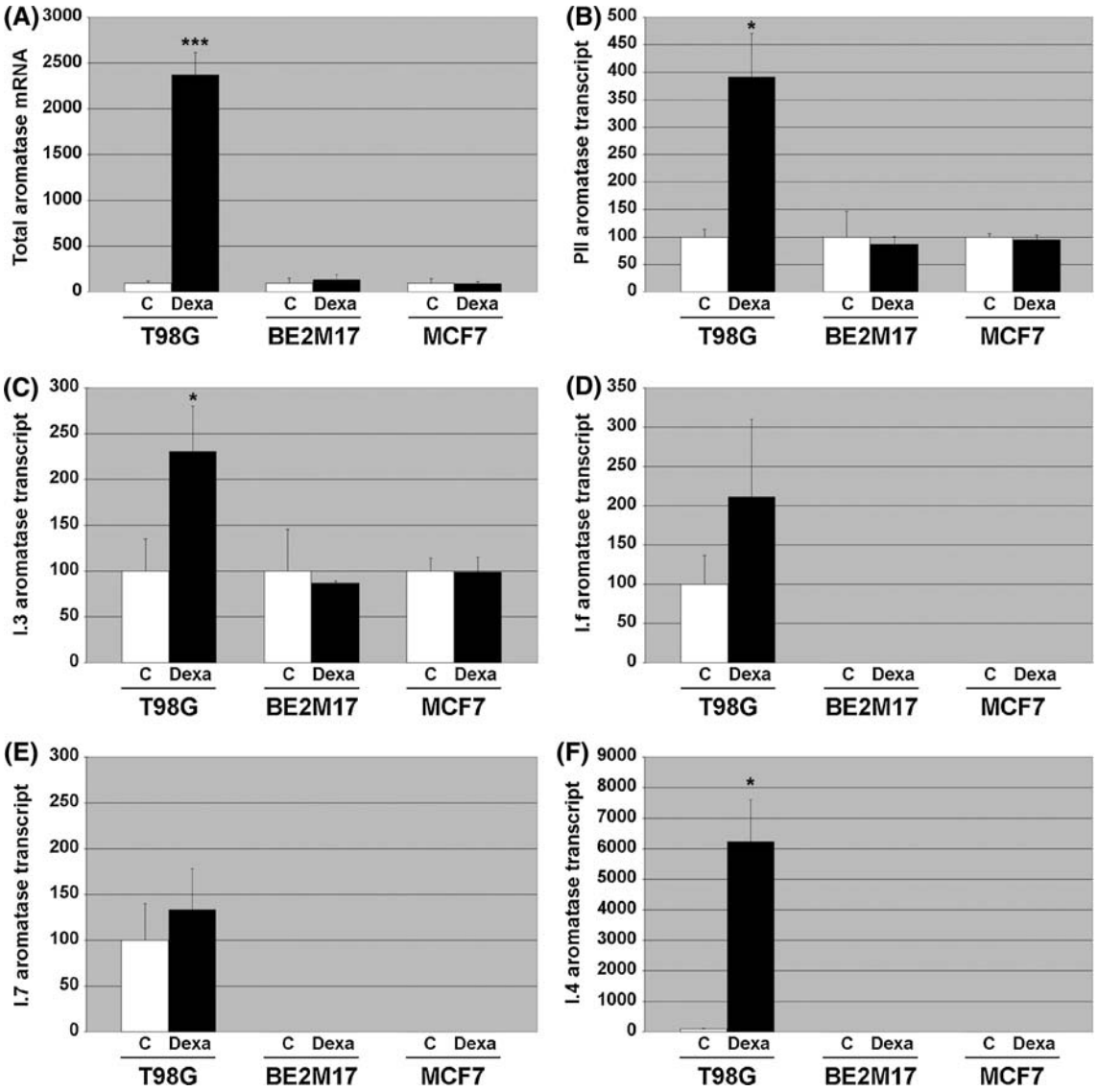

Fig. 5 Quantitative PCR analysis of total aromatase mRNA expression and aromatase transcripts levels in glioma T98G, neuroblastoma BE2M17, and breast cancer MCF7 cells treated with dexamethasone (100 nM) or vehicle for 24 h. **a** Total aromatase mRNA. **b** PII transcript. **c** I.3 transcript. **d** I.f transcript. **e** I.7 transcript. **f** I.4 transcript. Left lanes, 100 bp ladder. mRNA values are expressed in arbitrary units. Data represent the mean ± SEM from 3 to 5 independent experiments. Asterisks, statistical difference with respect to control values (*$P < 0.05$, ***$P < 0.001$, Student's *t*-test). C: control. Dexa, dexamethasone

the de novo induction of I.6 transcript in glioma cells by vitamin D was unexpected, since vitamin D does not modify the expression of this transcript in osteoblasts, where the I.6 transcript is constitutively expressed [4, 5]. The mechanism of activation of the promoter associated to exon I variant I.7 in response to vitamin D remains also to be elucidated.

The potent induction of the promoter associated to exon I variant I.4 in response to dexamethasone in glioma cells is in agreement with previous data in adipose stromal cells [7], osteoblasts [5, 42], and fibroblasts [43]. The induction would depend on the presence in this promoter of a glucocorticoid response element (GRE) motif [7]. In fact, the deletion of this motif completely abolishes the activation of the promoter by glucocorticoids, at least in fibroblasts [43]. The activation of the promoter associated to exon I variant I.3 by dexamethasone in glioma cells is also in agreement with previous findings in osteoblasts [5]. This promoter contains a putative GRE motif [44]. Interestingly, a putative response element for glucocorticoids and androgens has been also identified in the promoter associated to exon I variant I.f [45]. However, according to our data, the promoter associated to this exon variant in glioma cells seems to be insensitive to dexamethasone. In contrast, GRE motifs have not been identified in the promoter associated to exon I variant PII, which was regulated by

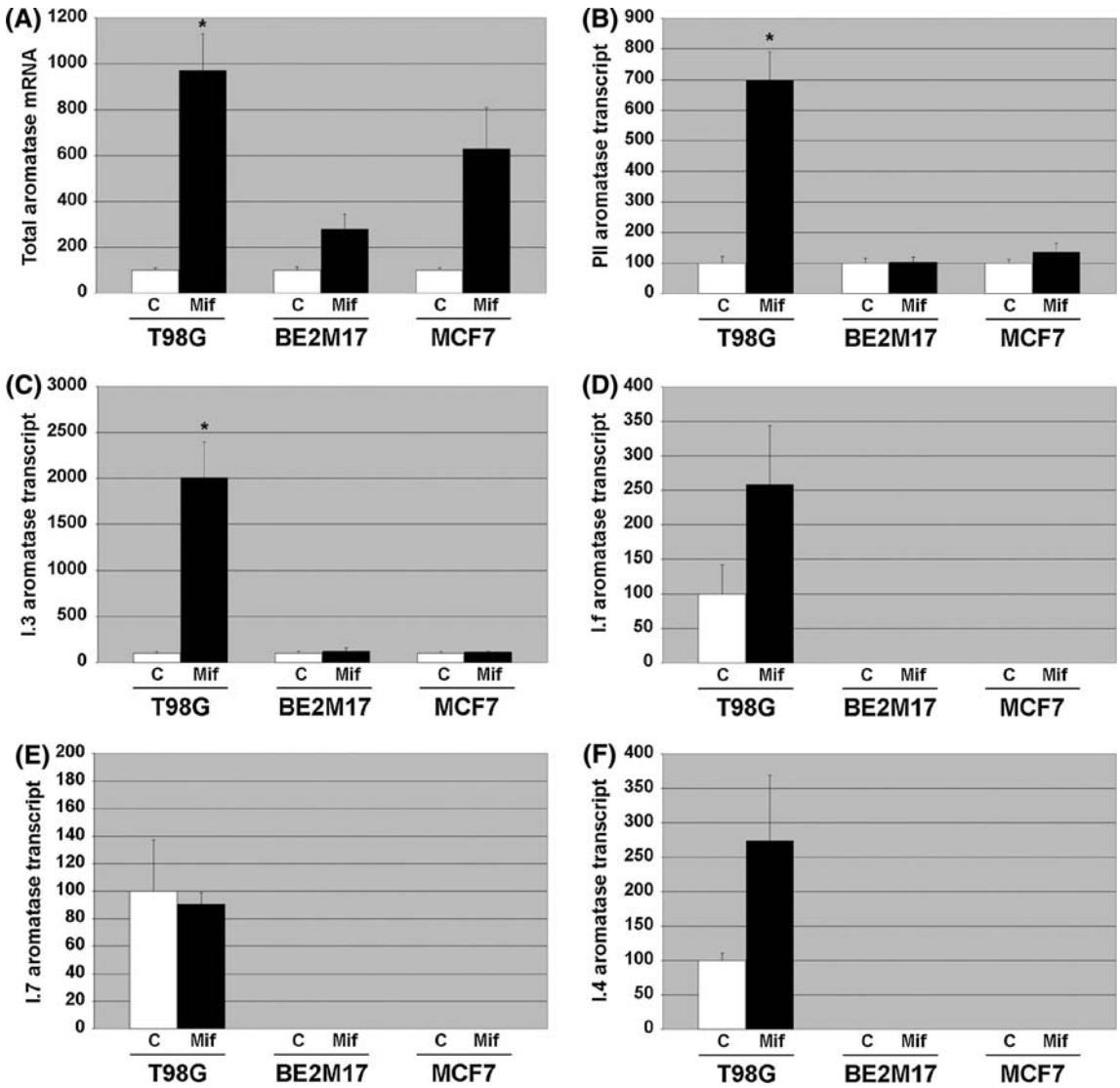

Fig. 6 Quantitative PCR analysis of total aromatase mRNA expression and aromatase transcripts levels in glioma T98G, neuroblastoma BE2M17, and breast cancer MCF7 cells treated with mifepristone (20 μM) or vehicle for 24 h. **a** Total aromatase mRNA. **b** PII transcript. **c** I.3 transcript. **d** I.f transcript. **e** I.7 transcript. **f** I.4 transcript. mRNA values are expressed in arbitrary units. Data represent the mean ± standard error of the mean (SEM) from 3 to 5 independent experiments. Asterisks, statistical difference with respect to control values (*$P < 0.05$, Student's *t*-test). C: control. Mif, mifepristone

dexamethasone in glioma cells. Therefore, its induction by dexamethasone in glioma cells is probably mediated by a GRE-independent mechanism.

Mifepristone treatment elicited an increase to about 10 times in total aromatase mRNA expression in glioma cells and the evaluation of the contribution of each aromatase transcript to the effect of mifepristone revealed a notable induction of the promoters associated to exon I variants PII and I.3. The similarity in the effects of dexamethasone and mifepristone on aromatase mRNA expression in glioma cells was unexpected, since mifepristone is an antagonist of glucocorticoid receptors [35]. Indeed, several studies have shown that mifepristone is able to block the stimulating effect of glucocorticoids on the transcription of the enzyme in other cell types [46, 47]. Moreover, mifepristone exerts additional effects, including the antagonism of progesterone receptors [35]. Progesterone receptors have been identified in human gliomas [48] and these receptors are involved in the down-regulation of aromatase expression in breast cancer cells [49]. Therefore, mifepristone may potentially up-regulate aromatase expression via the antagonism of progesterone receptors. However, our findings showing that progesterone did not significantly affect aromatase expression in glioma cells suggest, although do not prove, that mifepristone may up-regulate aromatase expression in these cells by a progesterone receptor

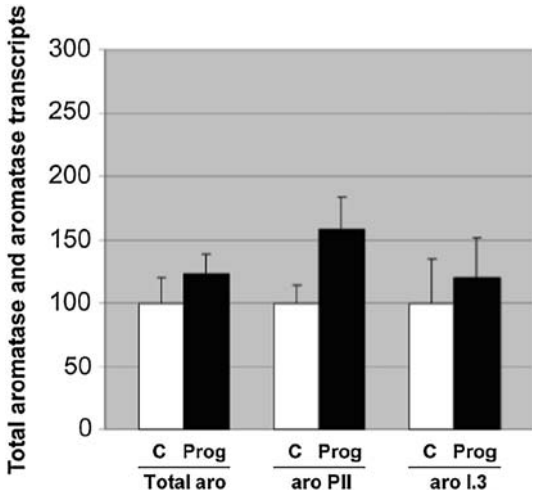

Fig. 7 Quantitative PCR analysis of total aromatase mRNA expression and aromatase transcripts II and I.3 levels in glioma T98G cells treated with progesterone (1 μM) or vehicle for 24 h. mRNA values are expressed in arbitrary units. Data represent the mean ± standard error of the mean (SEM) from 3 to 5 independent experiments. C: control. Prog, progesterone

independent mechanism. Interestingly, in vivo and in vitro studies have identified neuroprotective actions of mifepristone that do not seem to be mediated by its action on glucocorticoid or progesterone receptors [50, 51]. The mechanisms involved in the activation of aromatase promoters in glioma cells in response to mifepristone remain however unknown.

In summary, our results indicate that aromatase expression in glioma, neuroblastoma and breast cancer cells is under the control of different promoters under basal conditions. Moreover, the treatments with vitamin D, dexamethasone, and mifepristone are able to selectively enhance the activity of a different set of aromatase promoters in glioma cells. The identification of factors that increase aromatase expression in neuronal and/or glial cells but not in peripheral tissues may allow the development of pharmacological treatments to selectively increase the levels of the neuroprotective steroid estradiol in the nervous system. In this regard, it is of interest that vitamin D, dexamethasone, and mifepristone affect the expression of aromatase in glioma cells, but not in neuroblastoma and breast cancer cells. The cell specific regulation of the aromatase gene by vitamin D, dexamethasone, and mifepristone opens the possibility for the selective modulation of aromatase expression and estrogen biosynthesis in the central nervous system. Our findings also suggest that vitamin D may promote local estradiol synthesis within the brain by increasing aromatase expression in glial cells. Since local estradiol synthesis plays an important role in the maintenance of neural function [18–34, 52], our findings open the question on the possible contribution of modifications in brain aromatase expression to the mood and cognitive alterations reported in older people with vitamin D deficiency [53]. Finally, it is tempting to speculate with the possible use of vitamin D supplementation to improve neural cell function and neuroprotection by enhancing brain aromatase expression.

Material and methods

Cell cultures and treatments

Three human cell lines were studied: the glioma T98G, the neuroblastoma BE2M17 and the breast cancer MCF7 cell lines. All of them were obtained from American Type Culture Collection (ATCC; Middlesex, England). Cell lines were expanded in Dulbecco′s Modified Eagle Medium (DMEM) with nutrient mixture F–12 (DMEM/F12; 1:1; Gibco, 11039; California, USA) containing 10% fetal bovine serum.

For the study of the transcriptional regulation of the aromatase gene, cell lines were cultured for 12 h in DMEM/F12 without serum, in a density near confluence. After this period, cultures received a treatment with 100 nM dexamethasone (Sigma, D4902; Missouri, USA), 100 nM vitamin D (Sigma, D1530), 20 μM mifepristone (Sigma, M8046), or 1 μM progesterone (Sigma, P6149). About 24 h later, cells were lysated and RNA was extracted. For each treatment, 3–5 independent experiments were performed.

Data were analyzed with the Student's *t*-test (control versus treatment). Level of significance was set to $P < 0.05$. Analysis was done with GraphPad prism 4 software.

RNA extraction, retrotranscription, and real time PCR analysis

To efficiently remove genomic contamination during RNA purification, RNA extraction was carried out using the RNeasy Mini Kit (Qiagen GmbH; Hilden, Germany) with a DNase treatment (RNase-Free DNase Set, Qiagen GmbH), following the manufacturer's instructions. For retrotranscription (RT), RNA was incubated for 1 h at 37°C, with M-MLV reverse transcriptase (Promega, Madison, WI, USA) and random primers (Gibco Life Technologies, Barcelona, Spain) followed by inactivation for 15 min at 70°C. Real time PCRs were done in an ABI Prism 7000 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Assays were done in 96-well optical plates. For each reaction, 5 μl of a 1:4 dilution of the RT products were amplified in a total volume of 15 μl. Final concentrations were 0.3 mM for each primer (Table 1) and 1X SYBR

Table 1 List of primers used to amplify total aromatase mRNA (Total aro), aromatase transcripts (PII, I.3, I.6, I.2, I.f, I.7, I.5, I.4, 2a, and I.1) and 18S. Tm: melting temperature. *GenBank sequence. The sequences for designing the primers PII, I.3, and I.6 were based on references [38] and [39], respectively

Primers for total aromatase, aromatase transcripts, and 18S			
Transcript	Forward primer	Reverse primer	Tm
Total aro *NM_000103.2, NM_031226.1	5′-GGTATATGGAGAATTCATGCGAGTC–3′	5′-ATATGATGCCTTTCTCATGCATACC-3′	60°
PII (38)	5′-ACCCTCTGAAGCAACAGGAGCTAT-3′	5′-ACCATCTTGTGTTCCTTGACCTCAG-3′	60°
I.3 (38)	5′-ACTCAAGGGCAAGATGATAAGGTTC-3′	5′-ACCATCTTGTGTTCCTTGACCTCAG-3′	60°
I.6 (39)	5′-AGGGATTACAAAACCTGGCTGAA-3′	5′-ACCATCTTGTGTTCCTTGACCTCAG-3′	60°
I.2 S96437	5′-CTCCTCAGCCACTTGTAAGCATAAC-3′	5′-ACCATCTTGTGTTCCTTGACCTCAG-3′	60°
I.f *D29757	5′-GAAAAGCCACCTGGTTCTTAAACA-3′	5′-ACCATCTTGTGTTCCTTGACCTCAG-3′	60°
I.7 *AF419337	5′-TCCCAACAAGAAGATGAAGTGTTGT-3′	5′-ACCATCTTGTGTTCCTTGACCTCAG-3′	60°
I.5 *S71536	5′-AGGCAAAGGGTAAGTGGTTTCATTC-3′	5′-ACCATCTTGTGTTCCTTGACCTCAG-3′	60°
I.4 *L21982	5′-TCCTGGCTCCAAGTAGAACGTGAC-3′	5′-ACCATCTTGTGTTCCTTGACCTCAG-3′	60°
2a	5′-TGGTGAAATTCAGCCTGTGGATTC-3′	5′-ACCATCTTGTGTTCCTTGACCTCAG-3′	60°
I.1	5′-GGAAGGTGAAGAAGAACTTATCCTATCAG-3′	5′-ACCATCTTGTGTTCCTTGACCTCAG-3′	60°
18S *NR_003286	5′-CGCCGCTAGAGGTGAAATTCT-3′	5′-CATTCTTGGCAAATGCTTTCG-3′	60°

GREEN PCR Master Mix (Applied Biosystems). Each sample was amplified in triplicate. The amplifications consisted of 40 cycles at 95°C for 15 s (denaturation) and 60°C for 1 min (annealing and extension) (Table 1).

Standard curves for each gene were performed at a series of dilutions with a pool of cDNA. The baseline and threshold values were adjusted with the ABI Prism software to obtain the best standard curve and these conditions were fixed to compare different experiments. The mean of every triplicate was considered to be the quantity of mRNA per sample. Data for every transcript were normalized to 18S values [54]. In every run, wells without template were included in order to detect possible contaminations.

For visualizing and sequencing the PCR products, each mixture was electrophoresed in 2% (w/v) ethidium bromide-stained agarose gels. Then, bands were excised and cDNA was purified using the QIAquick PCR purification Kit (Qiagen GmbH). One hundred nanograms of each sample were sequenced (Automatic Sequencing Center, CSIC, Madrid, Spain) with the corresponding forward or reverse primer. The obtained sequence was aligned with the expected sequence of each transcript obtained from the GenBank.

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