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Differential regulation of apoptosis-associated genes by estrogen receptor alpha in human neuroblastoma cells

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Abstract.
Purpose: The neuroendocrinology of female sex hormones is of great interest for a variety of neuropsychiatric disorders. In fact, estrogens and estrogen receptors (ERs) exert neuromodulatory and neuroprotective functions. Here we investigated potential targets of the ER subtype alpha that may mediate neuroprotection and focused on direct modulators and downstream executors of apoptosis.
Methods: We employed subclones of human neuroblastoma cells (SK-N-MC) stably transfected with one of the ER subtypes, ERalpha or ERbeta. Differences between the cell lines regarding the mRNA expression levels were examined by qPCR, changes on protein levels were examined by Western Blot and immunocytochemistry. Differences concerning apoptosis induction were analysed by cell survival assays which included primary rat neurons.
Results: In this report we show a potent protection against apoptosis-stimuli in ERalpha expressing cells compared to controls lacking ERalpha. In fact, almost a complete silencing of Caspase 3 expression in SK-ERalpha cells compared to SK-01 control transfected cells was observed. In addition, prosurvival *bcl2, bag1* and *bag3* expression was highly up-regulated in the presence of ERalpha.
Conclusion: Taken together, we identified Caspase 3, BAG1 and BAG3 as key targets of ERalpha in neuronal cells that may play a role in ERalpha-mediated neuroprotection.

Keywords: Estrogen, ERalpha, neuroprotection, Caspase 3, BAG1, BAG3

1. Introduction

Estrogens and estrogen receptors are known to exert neuroprotective activities (Behl & Manthey, 2000; Behl et al., 1995, 1997; Behl, 2002; Green et al., 1997) beside their well-established role in sex specific development and physiology. It has been shown before (Dubal et al., 2006) that estrogens can protect neurons in a receptor mediated and receptor independent way. In particular, the estrogen receptor alpha (ERalpha) but not estrogen receptor beta (ERbeta), which are both expressed throughout the brain (Kuiper et al., 1998), was proposed to be involved in neuroprotection (Gamerdinger et al., 2006; Green et al., 1997; Manthey & Behl, 2006). Human SK-N-MC neuroblastoma cells stably transfected with ERalpha showed enhanced survival when exposed to the calcium ionophore, ionomycin, as well as reduced calpain enzymatic activity (Gamerdinger et al., 2006). Moreover, it has been shown that ERalpha displays neuroprotective functions against MPP^+-induced dopaminergic neuron death in

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mesencephalon primary mouse neurons (Bains et al., 2007) and against the toxicity of Alzheimer's Disease-associated amyloid beta protein (Marin et al., 2003). Furthermore, ERalpha mediated protective effects have been demonstrated in glutamate induced excitotoxicity (Sribnick et al., 2006), ischemic brain injury (Dubal et al., 2001) and in models of oxidative stress toxicity (Biewenga et al., 2005). In addition, rodents lacking estrogens showed a higher neuronal vulnerability after ischemic brain injury (Alkayed et al., 2000). Interestingly, Li and co-workers recently demonstrated that protective effects of estrogens after traumatic cerebral contusion in the pericontusional zone are mediated by ERalpha and by subsequent inactivation of Caspase 3 (Li et al., 2011).

During the last decade beneficial effects of estrogens have often been demonstrated *in vitro* and *in vivo* and a wide portfolio of possible modes of neuroprotective activity has been proposed. In the present study we demonstrate that ERalpha expressing SK-N-MC cells and estradiol-treated primary neurons are less vulnerable to peroxide induced oxidative stress or staurosporine induced apoptosis. By analysing an apoptosis-specific cDNA gene array we found that in ERalpha expressing cells *caspase* 3 expression is significantly decreased whereas the *bcl*2/*bax*-ratio and anti-apoptotic players like *bag*1 and *bag*3 are increased. Furthermore, significantly decreased caspase 3 levels and a reduced caspase 3 activity were observed in ERalpha expressing cells. These data indicate that key modulators of apoptosis are direct targets of the activity of ERalpha in neuronal cells.

2. Materials and methods

2.1. Cell culture and treatment

Human SK-N-MC cells (ATCC HTB-10) and MCF-7 cells (human breast adenocarcinoma cell line) were obtained from the American Type Culture Collection and were cultured in phenol red-free Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany) supplemented with 1 mM sodium pyruvate (Invitrogen), 10% charcoal-dextran-treated fetal calf serum, 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen). The medium of the SK-N-MC transfectants was supplemented with 2 μg/mL G418 (Calbiochem, Darmstadt, Germany). Construction of ERalpha and ERbeta containing pIRES vectors (Clontech, BD Biosciences, Mountain View, CA, USA) and subsequent stable transfection of SK-N-MC cells were described before (Manthey et al., 2001; Zschocke et al., 2002). Mock transfection was done with vector alone. The clones were analysed by RT-PCR and luciferase reporter assay in order to quantify ERalpha expression. Stock solutions of 17beta-estradiol or the ER antagonist ICI 182780 were made in 99.9% ethanol (Merck, Darmstadt, Germany), staurosporine in dimethylsulfoxide (DMSO), and hydrogen peroxide ready to use applied in final concentration from 25 nm to 1.5 mM.

Primary cortical neurons were prepared from E18 embryonic Sprague Dawley rats as described previously (Hajieva et al., 2009). Cells were treated with 10 nM 17beta-estradiol, 1 μM ICI and 100 nM staurosporine and pictures were taken with a 63× magnification.

2.2. Viability assays

To analyse cell viability 5000 exponentially growing cells per well were plated on 96-well plates, containing 100 μL of medium. After 24 h medium was replaced with serum-reduced (0,1% serum) medium containing 10 nM 17beta-estradiol, 1 μM ICI or ethanol as vehicle control. After additional 3 h cells were treated with increasing concentrations (0.2–1.5 mM) of hydrogen peroxide (Sigma) or staurosporine (0.25–1 μM) for 18 h. Cell viability was determined either with cell titer blue assay or by Propidium iodide (PI) exclusion assay. The cell titer blue assay (Promega) was carried out according to the following protocol: 20 μL of cell titer blue were added per well and incubated for 4 h. Subsequently viability was measured photometrically by exciting at 535 nm and determining fluorescence emission intensity at 590 nm. The PI exclusion assay was performed as supposed in Current Protocols in Neuroscience (Aras et al., 2008). At the end of the stimulation with the different substances, PI was added to the medium and incubated for 15 min in the dark. Living cells are able to exclude the dye, whereas dead cells are not, resulting in the staining of the nuclei of dead cells. Analysis was performed by microscopy and captured on film with a 20× magnification. Counting of dead and living cells was done with the program ImageJ cell counter. Calculation of the percentage of viable cells was done by comparing the dead cells with the overall number of cells. The data of the different treatments were related to the DMSO control which was set as 100% in every cell line.

2.3. Quantitative real-time reverse transcription-PCR

Total RNA from sub-confluent cell cultures was extracted using the total RNA Isolation Kit from Agilent according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany). cDNA was synthesised using Verso cDNA Kit from ABgene. Quantitative real time-reverse transcription-PCR analysis was performed in a 25 μL reaction volume containing 1 μL cDNA, 12.5 μL of 2× Absolute QPCR SYBR Green Mix (ABgene, Epsom, UK) and 0.5 μL sense and antisense primers (100 pmol) and adding 10.5 μL H_2O using the iCycler real-time thermocycler (Bio-Rad, Hercules, CA, USA). Following 15 min denaturation at 95°C 35 cycles of amplification were carried out. PCR cycle conditions were 95°C for 30 s (denaturation), 59°C for 20 s (annealing) and 72°C for 45 s (extension). The PCR cycle number that generated the first fluorescence signal above threshold (C_T) was determined. The specificity of the PCR products was confirmed by analysing the melting curve. The relative expression ratio R of target genes in SK-ERalpha, SK-ERbeta cells compared with SK-01 cells was calculated using the relative expression software tool (REST) (Pfaffl et al., 2002) with the following formula: $R = 2^{\wedge}\Delta C_T(\text{target})/2^{\wedge}\Delta C_T(\text{ref})$, with $\Delta C_T(\text{target}) = (C_T \text{ SK-01} - C_T \text{ SK-ERalpha})$ of target genes and $\Delta C_T(\text{ref}) = (C_T \text{ SK-01} - C_T \text{ SK-ERalpha})$ of GAPDH. To validate the results, calculations were repeated with a second housekeeping gene (B2M). Expression profile of apoptosis associated genes was carried out with microtiter plates (RT^2 Profiler™ PCR Array Human Apoptosis, PAHS-012A, from SABiosciences) containing 84 different specific primer samples.

2.4. Protein extraction and Western blot analysis

Western blots were performed as previously described (Gamerdinger et al., 2009). In detail: sub-confluent cells were rinsed with ice-cold phosphate buffered saline (PBS) following cell lysis in a 62.5 mM Tris, 1 mM EDTA, 2% sodium dodecyl sulfate (SDS), 10% saccharose containing buffer supplemented with phenyl-methylsulfonyl fluoride (PMSF) (5 mg/mL) and aprotinin (1 μg/mL). Samples were briefly sonicated and incubated for 5 min at 95°C. Total protein concentration was measured by using a bicinchoninic acid (BCA) based method (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard. Equal amounts (10–50 μg) were loaded on a 10–12% sodium dodecyl sulfide-polyacrylamid gel (SDS-PAGE) in 16 μL loading buffer (10% SDS, 20% glycerine, 125 mM Tris, 1 mM EDTA, 0.002% bromphenol blue, 10% beta-mercaptoethanol) and separated by electrophoresis. Subsequently, the separated samples were transferred to a nitrocellulose membrane by electroblotting. Blocking of non-specific binding sites was carried out in Tris-buffered saline, 0.05% Tween 20 containing 5% non-fat dry milk for 30 min at 20°C followed by incubation with mouse anti-caspase 3-antibody (Cell Signalling), diluted 1:1000 in TBS/T; mouse anti-Tubulin-antibody (Sigma), diluted 1:1000 in TBS/T; rabbit-anti-BAG3-antibody (Abcam), diluted 1:1000 in TBS/T or rabbit anti-cBAG-antibody (anti-BAG domain, a 151–263), diluted 1:500 in TBS/T, at 4°C over night. After washing the blots three times with TBS/T membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary anti mouse or anti rabbit antibodies (1:10000, Jackson Laboratory, West Grove, PA, USA) for 1.5 h and washed again as described above. Secondary antibodies were detected using the Super Signal procedure (Millipore) and visualised with the Fuji LAS-3000 intelligent dark box (Fujifilm, Dusseldorf, Germany). The densitometric quantification was done after normalising the results to tubulin. Analysis was performed with Aida Image Analyzer v.4.26 software (raytest GmbH, Straubenhardt, Germany). For the statistical analysis one-sample *t*-test was used.

2.5. Immunocytochemistry

Cells were plated on cover slips in 24-well plates and fixed with 4% paraformaldehyde. Subsequently, cells were permeabilized and blocked in PBS/0,5 % Triton-X100 and PBS/3 % bovine serum albumin, respectively. After incubation with caspase 3 antibody (1:500) overnight at 4°C cells were incubated with anti-rabbit-Cy3 (Sigma Aldrich C2306; 1:200) for 2 h and counterstained with 4,6-diamidino-2-phenylindole (DAPI; 1 μg/ml). Cells were analysed by microscopy using an inverted Zeiss Axiovert 200 microscope equipped with a SPOT RT CCD-camera from Diagnostic Instruments (Visitron) with a magnification of 63×.

2.6. Data analysis

Data processing, calculation and statistical analysis were done by using Sigma Stat software featuring

one-sample Student's *t*-test or pair wise comparison with one way ANOVA. Values are shown in means ± S.D. and considered to be significant at $p < 0.05$; = * $p < 0.01$; = ** $p < 0.001$ = ***.

3. Results

3.1. Increased survival of cells expressing ERalpha

To investigate possible beneficial effects of ER signalling on cell survival in paradigms of apoptosis, we employed human SK-N-MC neuroblastoma cells ectopically expressing ERalpha and beta (Manthey & Behl, 2006; Zschocke et al., 2002). In a first step we analysed the resistance of ERalpha expressing cells against hydrogen peroxide (H_2O_2)-induced acute oxidative cell death. The ERalpha expressing neuroblastoma cells showed a 17beta-estradiol-independent significant increased survival compared to control-transfected SK-01 cells (Fig. 1A). Following preincubation with 17beta-estradiol (E2) 3 h before toxin application a further increase in oxidative stress resistance was only observed in ERalpha expressing cells (Fig. 1C) as compared to control-transfected cells (SK-01; Fig. 1B). The protective effect of E2 in SK-ERalpha cells could be blocked by applying the complete ER antagonist ICI 182 780 (Fig. 1C). Equal findings were observed in MCF-7 cells employed as control cell line endogenously expressing ERalpha (Lappano et al., 2012; Wu et al., 2011). Consistently, an enhanced survival of ERalpha expressing SK-N-MC cells after H_2O_2 treatment could also be detected by PI-staining as an additional measure of cell viability (Fig. 1G). In SK-N-MC cell clones expressing exclusively ERbeta no significant neuroprotective effects against H_2O_2 or staurosporine, neither in the absence nor in the presence of 17beta-estradiol, could be observed with the cell titer blue assay as well as with the PI staining (data not shown and Fig. 1G).

Interestingly, SK-ERalpha cells also showed an increased survival in the presence of the kinase inhibitor staurosporine (Fig. 1D) which is known to induce apoptosis via the downstream activation of caspase 3 (Chae et al., 2000) or caspase-independent cell death via decrease of the mitochondrial membrane potential (Déas et al., 1998). In ERalpha expressing cells, cell death was induced only at rather high staurosporine concentrations. Control transfected SK-N-MC cells lacking ERalpha expression (SK-01) are highly sensitive to staurosporine even at a low concentration of 25 nM (Fig. 1E). A significant resistance could again be detected after prestimulation with 17beta-estradiol in SK-ERalpha cells (Fig. 1F).

In addition, a protective effect of 17beta-estradiol could also be detected in rat primary neurons challenged with staurosporine (Fig. 1H). Neurons pretreated with 17beta-estradiol before staurosporine administration, showed more intact neurons with neuronal processes. The prosurvial effect of 17beta-estradiol could be blocked by cotreatment with ICI 182 780, indicating protective activities of ER also in primary neurons.

3.2. Regulation of apoptosis genes by ERalpha

Next we analysed a gene array carrying apoptosis-specific genes (PAHS-012A); a total of 84 targets were spotted on the array. To do so, we performed qPCR employing RT-PCR of mRNA of ERalpha expressing cells compared to mock transfected control cells (SK-01). Significant alterations of prominent mediators of apoptosis were found in SK-ERalpha cells compared to SK-01 cells (Table 1). Interestingly, ERalpha expressing cells showed an up-regulation of the mRNA for *bcl2* (2.81 fold), a well-known anti-apoptotic factor, while the mRNA of the pro-apoptotic *bax* remained unaltered. Furthermore, *nol*3 which acts as an apoptosis repressor was up-regulated (5.24 fold). But most importantly, we found a significant down-regulation of the key mediators of apoptosis, the *caspases*, including *caspase* 7 (−2.08 fold) and *caspase* 3 (−5.31 fold), the

Table 1
Expression profile of apoptosis-associated genes in SK-ERalpha cells. Apoptosis gene array by qPCR (here a selection of genes is shown) indicates a differentiated expression profile of apoptosis genes in SK-ERalpha cells compared to control transfected SK-01 cells ($n = 2$)

Gene name	Accsession no.	Fold change in SK-ERalpha
bag1	NM_004323	2.13
bag4	NM_004874	−5.31
bax	NM_004324	1.19
bcl2	NM_000633	2.81
bcl2l11	NM_006538	7.16
card6	NM_032587	−22.78
caspase3	NM_004346	−5.31
caspase7	NM_001227	−2.08
dapk1	NM_004938	−266.9
ltbr	NM_002342	263.2
nol3	NM_003946	5.24

central downstream executioner caspase. In addition, *card*6 which contains a Caspase recruitment domain was massively down-regulated (−22.78 fold), as well as *dapk*1 (−266.9 fold) which is known as a positive mediator of programmed cell death triggered by interferon γ (Feinstein et al., 1995). In contrast, we found the mRNA of the *lymphotoxin beta receptor* (*ltbr*), which can trigger apoptosis in certain non neuronal cell types, highly up-regulated (263.3 fold). Nevertheless, we observed a reduced expression for the majority of apoptosis inducers and executors. Interestingly, the same results were observed in the endogenous ERalpha expressing cell line MCF-7 (data not shown).

Since caspase 3 is the key downstream executioner caspase of apoptotic cell death, we further investigated *caspase* 3 at the protein and activity level. In fact, in Western blots caspase 3 was not detectable at all in SK-ERalpha cells compared to SK-01 control cells (Fig. 2A) and a densitometric analysis revealed only an extremely weak signal (Fig. 2B). In control transfected SK-01 cells as well as in SK-subclones expressing ERbeta caspase 3 protein was found. Interestingly, in human breast cancer MCF-7 cells as non-neuronal controls known to endogenously express ERalpha at high levels, also no caspase 3 signal could be detected supporting our findings. These results could be strengthened by immunocytochemistry (Fig. 2C): compared to SK-01 cells, ERalpha expressing cells, SK-ERalpha and MCF-7, showed just weak signals in the caspase 3 staining, consistent with the Western blotting data.

3.3. Increased expression of BAG1 and BAG3 in ERalpha-expressing cells

In addition to the aforementioned *caspase* genes also the expression of other apoptosis-associated genes has been found to be differentially regulated in ERalpha cells. Interestingly, the expression of *bcl*2 and *bcl2l*11, another member of the *bcl*2 gene family, was increased in the presence of ERalpha (an increase of approx. 2.8 fold for *bcl*2 and of approx. 7 fold for *bcl2l*11; Table 1). While Bcl2l11 functions as a pro-apoptotic molecule, Bcl2 is a known inhibitor of apoptosis. BAG1(Bcl2-associated athanogene 1) was identified as an enhancer of the Bcl2 anti-apoptotic activity (Takayama et al., 1995), and BAG3 (Bcl2-associated athanogene 3) is known to protect non-small cell lung cancer cells from apoptosis, by regulating Bcl2 and Bcl-XL expression (Zhang et al., 2011). The expression of the anti-apoptotic *bag*1 is enhanced 2.13 fold in SK-ERalpha cells. A more differentiated expression profile was observed in ERalpha expressing cells since BAG4, another anti-apoptotic protein interacting with Bcl2 (Antoku et al., 2001), was down-regulated (−5.31 fold). Immunoblot analysis of BAG1 revealed a strong up-regulation of all BAG1 isoforms as a result of an alternative translation initiation from one mRNA (Fig. 3A), validating our results of the apoptosis-gene array analysis. BAG3, another member of the BAG-protein family with anti-apoptotic function, has also been shown to be up-regulated in ERalpha expressing cells (Fig. 3). In addition of the role of BAG1 as enhancer of Bcl2 activity, BAG1 has been recently shown to be involved in protein degradation via the ubiquitin-proteasome system, whereas BAG3 was linked to protein clearance via macroautophagy in aged and acutely stressed cells (Gamerdinger et al., 2009). Both genes are up-regulated in ERalpha expressing cells as observed in array independent qPCR analysis (data not shown; *bag*3 primer were not spotted on the employed cDNA gene array). As shown in Fig. 3A the expression of BAG3 and the different isoforms of BAG1 (BAG1L 50 kDa, BAG1M 46 kDa, BAG1 36 kDa and BAG1S 29 kDa) are increased in SK-ERalpha cells (approx. up to 240% for BAG3 (Fig. 3B) and up to 346% for BAG1 (Fig. 3C)). Interestingly, the same effect could again be shown in MCF-7 cells (up to 296% for BAG3 and up to 475% for BAG1 (Fig. 3B, C)).

Taken together, our results indicate a differentiated expression profile of apoptosis-, and anti-apoptosis-related genes in ERalpha expressing cells. Also, an increased resistance of ERalpha expressing cells against H_2O_2 and staurosporine could be shown. These results lead to the suggestion that the modulation of apoptosis genes caused by ERalpha affects cell survival in favor of anti-apoptotic signaling pathways. Most importantly, the almost complete down-regulation of the expression of caspase 3, a key executor of apoptosis, is highly significant.

3.4. Reduction of caspase 3-specific BAG3-cleavage products in the presence of ERalpha

To investigate the functional relevance of the expression findings in the context of apoptosis, we have performed immunoblot analysis of BAG3 cleavage products (Fig. 4). BAG3 is known to be cleaved by

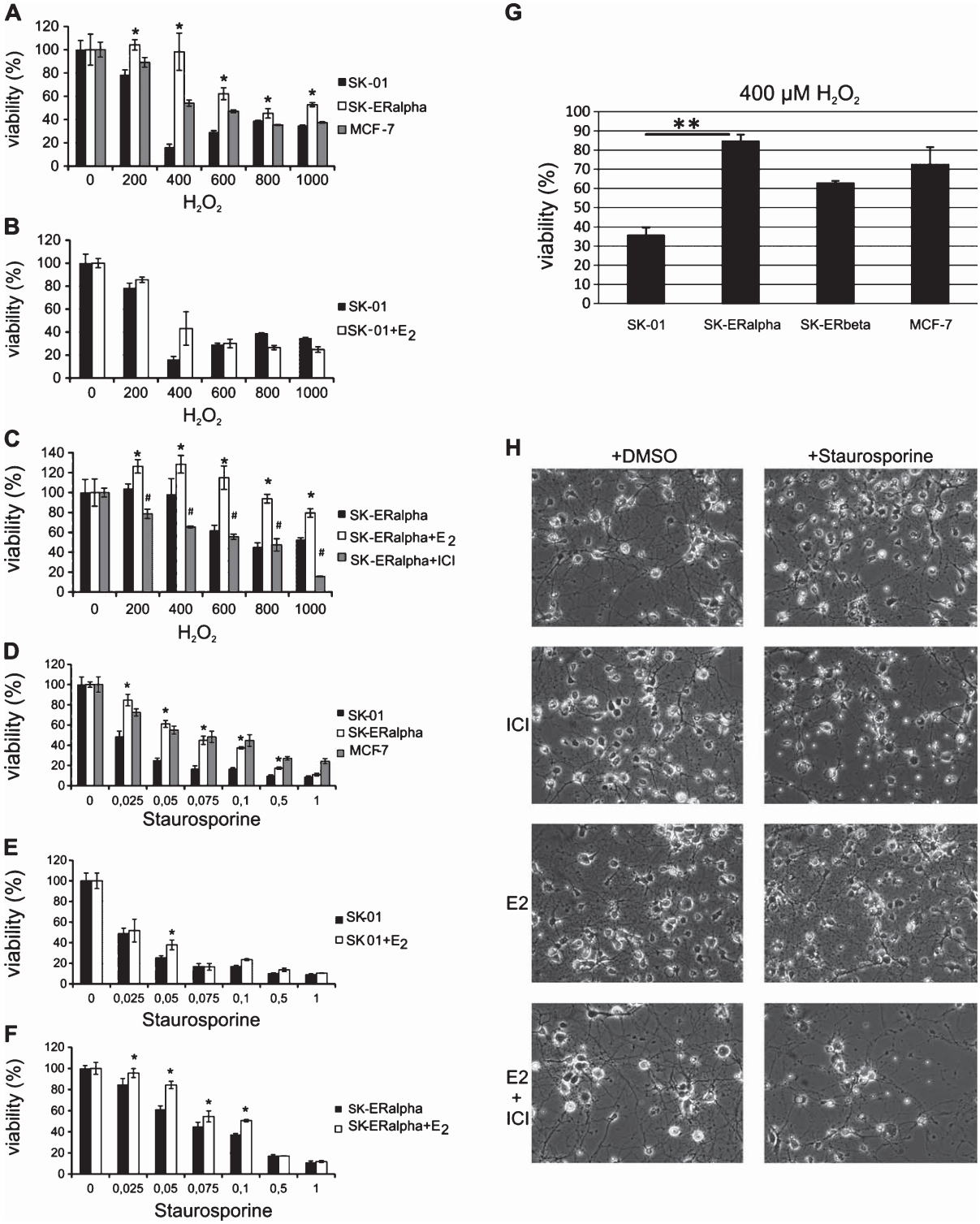
A
viability (%)
SK-01
SK-ERalpha
MCF-7
H2O2
B
SK-01
SK-01+E2
C
SK-ERalpha
SK-ERalpha+E2
SK-ERalpha+ICI
D
Staurosporine
E
F
G
400 µM H2O2
SK-ERbeta
H
+DMSO
+Staurosporine
ICI
E2
E2 + ICI

Fig. 1.

caspase 3 (Wang et al., 2010) and, therefore, is a useful indicator for caspase 3-activity. Hence, we have incubated cells with staurosporine to induce caspase 3 activity. Indeed, the amount of BAG3 cleavage products following staurosporine treatment was reduced (relative to full length BAG3) in SK-ERalpha cells, while as the amount of BAG3-cleavage products in SK-01 cells was highly increased upon staurosporine treatment (Fig. 4A, right panel). Again, MCF-7 cells showed a result similar to the SK-ERalpha expressing cells, while ERbeta expressing cells displayed a BAG3-cleavage pattern comparable to control transfected SK-01 cells. As revealed by densitometric analysis the BAG3-cleavage products were reduced by approx. 80–90% in both ERalpha ectopically and endogenously expressing cells (Fig. 4B). Taken together, these results suggest that the observed enhanced survival of ERalpha expressing cells under apoptosis inducing conditions may be due (at least in part) to the different regulation of the expression profile of apoptosis and apoptosis-associated genes.

4. Discussion

Our results present a link between the expression of ERalpha in neuroblastoma cells and the down-regulation of main players modulating apoptosis, most importantly, the key executioner caspase, *caspase* 3, leading to an increased resistance against hydrogen peroxide-induced oxidative stress and staurosporine-induced apoptosis. In addition to the observed down-regulation of *caspase* 3, the increased *bcl*2/*bax*-ratio furthermore supports the suppression of apoptosis caused by ERalpha expression. Interestingly, the ERalpha-mediated protection against neuronal apoptosis is occurring already in the absence of 17beta-estradiol. In fact, we have previously observed effects of ERalpha without further addition of 17beta-estradiol in the context of silencing *caveolin 1* and *caveolin 2* gene expression in SK-ERalpha cells (Zschocke et al., 2002). Here, we show that the expression of *caspase 3* appears almost completely silenced in SK-ERalpha cells independent of ligand binding.

Caspase 3 is the main enzymatic executioner during apoptosis and is known to degrade distinct cellular structural and regulatory proteins (Fischer et al., 2003; Pop & Salvesen, 2009). Initial induction of the intrinsic caspase cascades ultimately leading to apoptosis mainly depends on the Bcl2/Bax ratio which is responsible for Cytochrome c release, apoptosome formation and the following activation of effector caspases (Jiang & Wang, 2004). Our current data from human neuroblastoma cells are consistent with earlier results concerning ER regulated expression of *bcl*2 family members (Yao et al., 2007). We suggest that the underlying mechanism of neuroprotection may in part depend on the ERalpha driven transcriptional regulation of apoptosis genes, including *caspase* 3 and *caspase* 7 as well as *bcl*2, *bag*1 and *bag*3. Interestingly, the anti-apoptotic protein BAG3 is a target of caspase cleavage (Virador et al., 2009; Wang et al., 2010) and our results show a strong stabilisation of the full length protein, further supporting the almost complete silencing of *caspase* 3 expression and its activity. Residual BAG3 cleavage products might be caused via processing through caspase 1 or 8 which are also capable of producing BAG3 fragments (but to a lower extent) (Wang et al., 2010) and which are not regulated in our experimental setup (data not shown). The multifunctional protein BAG1 also has been shown to be involved in various cell processes linked to cell survival (Doong et al., 2002; Townsend et al., 2003, 2005) and is up-regulated in our neuroblastoma cell system expressing ERalpha. Furthermore, it has been shown that BAG1 can bind and regulate nuclear hormone receptors (Froesch et al., 1998). Interestingly, the BAG1L isoform which is known to interact and stimulate the activity of estrogen receptors (Cutress et al., 2003) is also up-regulated in our

Fig. 1. Increased survival of ERalpha expressing clonal cells and primary neurons. Survival after exposure to oxidative stress induced by hydrogen peroxide (A–C, G) or staurosporine (D–F) for 18 h (A–C) is strongly enhanced in SK-ERalpha expressing cells as shown by micro titer blue assay (A–F) as well as with the Propidium iodid exclusion assay (G).17beta-estradiol (E2) pretreatment (10 nM) significantly increased the viability of ERalpha expressing cells after incubation with hydrogen peroxide (C); the 17beta-estradiol- mediated protection is reversible by the estrogen receptor antagonist ICI 182780. An increased resistance is also detected in SK-ERalpha cells after incubation with staurosporine (E, F). (*P*-values: $^{*}p < 0.05$ vs. SK-01 cells in (A), (D), (E) and $^{**}p < 0.01$ vs. Sk-01 cells in (G); *P*-values $^{*}p < 0.05$ vs SK-ERalpha without E2 pretreatment in (C), (F) and $^{\#}p < 0.05$ vs. E2-treated SK-ERalpha cells; $n = 3$). The human cell line MCF-7 is used as control expressing endogenous ERalpha (A, D, G). The protective effect of E2 against staurosporine was also found in cortical primary neurons from rat (H). Neurons pretreated with 10 nM E2 before staurosporine stimulation display more vital neurons with neuronal processes as compared to controls or following treatment with ICI 182 780 before toxin administration.

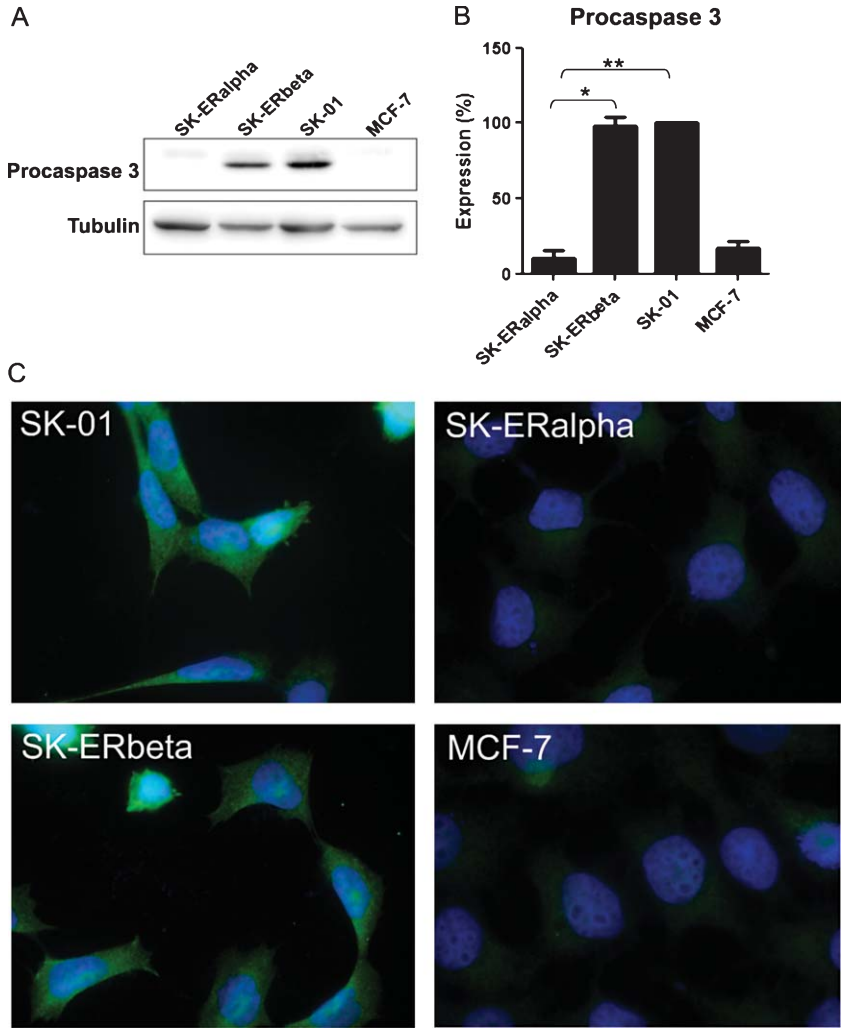

Fig. 2. Decreased caspase 3 levels in ERalpha expressing cells. (A) Western blot analysis of caspase 3 levels indicates that caspase 3 is almost not detectable in SK-ERalpha and MCF-7 cells, whereas SK-01 cells, or SK-cells expressing exclusively ERbeta clearly show caspase 3 expression. (B) Densitometric analysis of Western blots represented in (A); for densitometric quantification, results were normalised to tubulin. ($^{*}p < 0.05$; $^{**}p < 0.01$, $n = 3$). Furthermore, cells were analysed by immunocytochemistry for caspase 3 (green channel) and nuclei were counterstained with DAPI (blue channel) (C). Compared to SK-01 cells, ERalpha expressing cells, SK-ERalpha and MCF-7, showed only very weak caspase 3 staining, supporting the Western blotting data.

cell model. Co-silencing of BAG1 and BAG3 caused an enhanced cell predisposition to death in cell lines and primary neurons (Aveic et al., 2011), underlining our findings that on the other hand ERalpha expressing cells with increased levels of BAG1 and BAG3 show an enhanced survival under oxidative stress conditions. BAG1 and its family member BAG3 maintain the expression of the anti-apoptotic proteins Bcl2, BCL-XL and MCL1 (Aveic et al., 2011; Zhang et al., 2011). Beside the apoptosis-related impact of these molecules, they represent key players of cellular protein control. BAG1 controls proteasome-dependent protein degradation, whereas BAG3 is involved in the clearance of aggregated proteins in post-mitotic aged neurons (Gamerdinger et al., 2011). Therefore, neuroblastoma cells expressing ERalpha might extend proliferation and cell survival via a variety of pathways. Up-regulation of *bcl*2 can directly be linked to transcriptional ERalpha functions because estrogen response elements (EREs) are present in the promoter of the *bcl*2 gene (Bourdeau et al., 2004; Perillo et al., 2000) whereas down-regulation of *caspases*

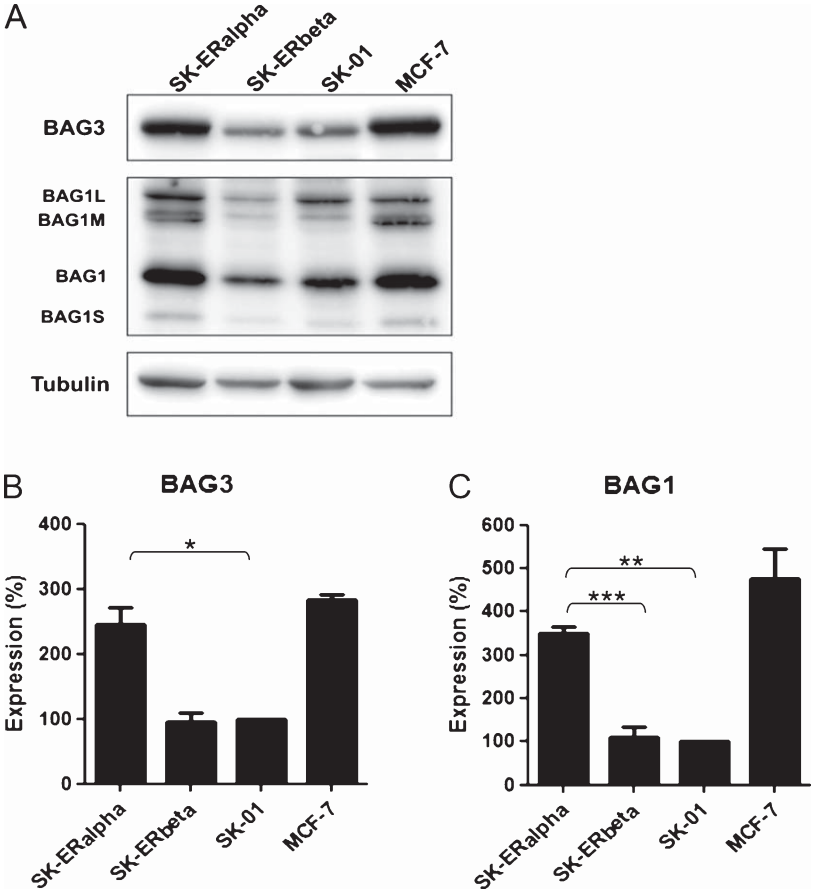

Fig. 3. Increased BAG1 and BAG3 protein levels in ERalpha expressing cells. (A) Immunoblot analysis, performed on the same blot as caspase detection, revealed a strong up-regulation of BAG1 and BAG3 in SK-ERalpha cells compared to mock transfected SK-01 cells. MCF-7 cells, which endogenously express ERalpha also showed stronger BAG1 and BAG3 immunoreactivity. (B, C) Densitometric analysis of Western blots represented in (A). For densitometric quantification, results were normalised to tubulin ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ $n = 3$).

could probably be explained with non-classical cytosolic effects of ER or the upstream cross-talk of ER signalling. ERs present in the cytoplasm can crosstalk with various signalling pathways, including the PI3-kinase pathway (Behl, 2002; Simoncini et al., 2000).

Nevertheless, our gene expression analysis in ERalpha cells is not clear-cut in demonstrating an exclusive down-regulation of apoptosis mediating genes and up-regulation of apoptosis preventing genes. In fact, a more differentiated picture on the expression profile needs to be developed. The presence of ERalpha did not result in an expression profile solely indicating anti-apoptotic features, since, for instance, increased mRNA levels of the apoptosis inducer *bcl2l*11 were also found. Also, mRNA levels of the *ltbr*, which can in fact trigger apoptosis, was found to be highly up-regulated (263,3 fold) in ERalpha expressing cells, which could be explained by a compensatory counter regulation of the cells following the blockade of certain apoptosis signalling pathways. Our findings suggest (1) that there is a fine-tuned balance of an apoptosis/anti-apoptosis network that can be influenced by ERalpha and (2) a massive down-regulation of *caspase* 3 might be sufficient to prevent apoptosis induction. Since the cells employed for the expression analysis are tumour cells (human neuroblastoma SK-N-MC, human breast cancer MCF-7 as control expressing endogenous ERalpha), our findings may also indicate that an ERalpha-driven constant inhibition of apoptosis as induced, for instance, even by general apoptosis inducers such as staurosporine may lead to changes in tumour cell physiology. Indeed, many breast cancer tumour lines are depending on ERalpha and estrogen and an increased risk for breast

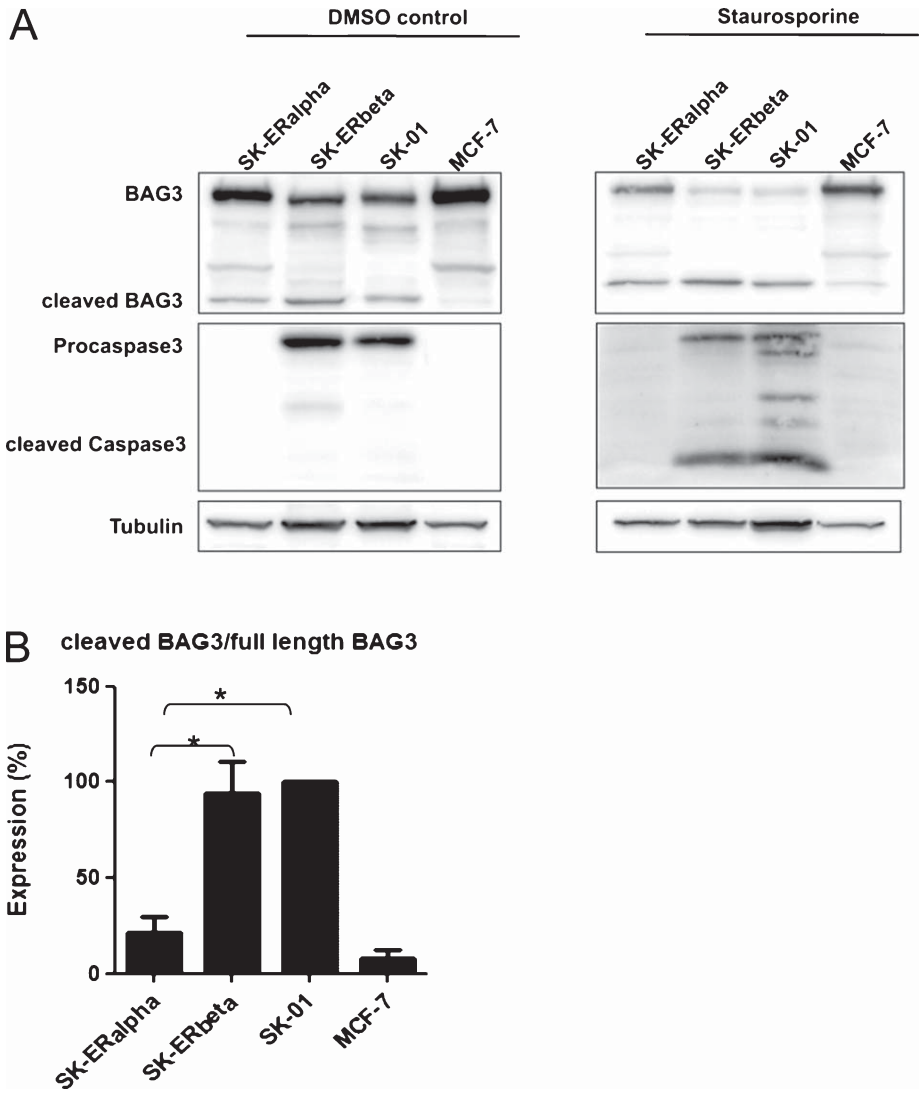

Fig. 4. Decreased caspase 3-specific BAG3-cleavage products in ERalpha expressing cells. (A) Western blot analysis of caspase 3-specific BAG3-cleavage products revealed decreased BAG3-cleavage products in ERalpha expressing cells compared to SK-01 (and SK-cells expressing ERbeta). Full length BAG3 is clearly increased in ERalpha expressing cells despite the treatment with the apoptosis inducer staurosporine. In contrast, cells lacking ERalpha expression showed cleaved caspase 3 bands indicating a staurosporine induced acvtivation of caspase 3 and consequently also an enhanced BAG3 cleavage. (B) Densitometric analysis of full length BAG3 and caspase-specific BAG3-cleavage products within the staurosporine treatment group (right panel of Western blot in A). The graph shows the ratio of caspase-specific BAG3-cleavage products to the corresponding full length BAG3 band. As control for equal protein loading, blots were re-probed with an antibody directed against tubulin ($^{*}p < 0.05$; $n = 3$).

cancer following estrogen replacement therapy was demonstrated by the women's health initiative (Clark, 2007). An increased general cancer risk was described after hormone replacement in the women's health study (Wise et al., 2005). Considering these problems a few approaches might nevertheless be worthwhile to proceed (Brann et al., 2007; Van Horn & Manson, 2008). For instance, receptor independent antioxidative effects of estrogens (Moosmann & Behl, 2002) and also epigenetic gene silencing effects mediated by ERalpha (Zschocke et al., 2002) have been described. A further molecular understanding of ER dependent downstream pathways seems to be necessary, with a special focus on ER subtype-specific activities

(ERalpha vs. ERbeta). With respect to estrogen and ER-driven protection of neurons, it needs to be clarified whether the transcriptional regulation of the apoptosis network via ERalpha described here in neuroblastoma cells, is potentially also occurring in non-transformed neurons displaying a lower ER expression level. If so, one could consider to directly modulate the apoptosis network in neurons with selective ERalpha agonists and selective ER modulators, ultimately, leading to neuroprotection. The data summarised here present potential molecular targets of ERalpha in neuronal cells that merit further investigation in order to better understand neuroprotective activities of ERs.

Declaration of interest

All authors declare that there is no conflict of interest.

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