Gender-specific regulation of mitochondrial fusion and fission gene transcription and viability of cortical astrocytes by steroid hormones

Susanne Arnold, Gilda Wright de Araújo and Cordian Beyer
Faculty of Medicine, Institute for Neuroanatomy, RWTH Aachen University, 52074 Aachen, Germany

(Author correspondence should be addressed to S Arnold; Email: sarnold@ukaachen.de)

Abstract

Astroglia and steroid hormones such as estrogen and progesterone regulate cell growth, function, and protection in the central nervous system (CNS). It appears that astrocytes and steroids act in concert to promote cell survival under pathological conditions. With respect to the role of mitochondrial fusion and fission in energy metabolism, apoptosis, and proliferation, astrocyte mitochondria resemble a perfect intracellular target for steroids to modulate these processes, thereby promoting cell vitality after damage. We have studied the effects of estrogen and progesterone on cell viability in comparison with mitochondrial fusion and fission gene transcription in primary cortical astrocytes from female and male mouse brains. Estrogen- and progesterone-treated female astrocytes demonstrated an increase in cell number and proliferation marker accompanied by an upregulation of fusion and fission gene transcription, which were apparently balancing pro- and anti-apoptotic processes. On the other hand, male astrocytes exhibited no change in cell number after estrogen treatment, but a decrease after progesterone administration. This could be the consequence of stimulated apoptosis in male astrocytes by both steroids, which was counterbalanced by an increased proliferation in the presence of estrogen, whereas it was strengthened in the presence of progesterone. Supportively, estrogen promoted and progesterone decreased the transcription of fusion and fission genes. We suggest that estrogen and progesterone affect mitochondrial fusion and fission gene transcription in cortical astrocytes in a gender-specific way, thereby influencing mitochondrial function differently in both genders. Thus, interaction of sex steroids with mitochondria may represent one possible cause for gender differences in cellular pathology in the CNS.

Journal of Molecular Endocrinology (2008) 41, 289–300

Introduction

Estrogen (E) and progesterone (P) exert their well-known neuroprotective effects in the central nervous system (CNS) by initiating a multitude of cellular mechanisms that are often mediated by astrocytes (Azcoitia et al. 2001, Arnold 2005, Pawlak et al. 2005, Garcia-Segura & Melcangi 2006, Leonelli et al. 2007). Astrocyte mitochondria represent an interesting intracellular target organelle for steroid hormones, since they provide ample of services to neural cells including energy production, calcium buffering, and regulation of apoptosis. These processes are also known to be modulated by gonadal steroid hormones (Nilsen & Brinton 2004, Kipp et al. 2006). However, mitochondria-mediated mechanisms in steroid-dependent protection are not fully understood. In very recent studies, we and others have shown that E promotes the expression of mitochondria-encoded subunits of the respiratory chain, thereby influencing mitochondrial respiratory activity and enhancing functional efficiency (Araújo et al. 2008, Irwin et al. 2008). Mitochondrial functions are not only affected by regulation of gene expression and activity of the respiratory chain (Zheng & Ramirez 1999, Araújo et al. 2008, Irwin et al. 2008), but also by the morphology of these organelles. Two sets of proteins and their relative activities are involved in shaping the morphology of mitochondria. While mitochondrial GTPases, mitofusin 1 and 2 (MFN1 and MFN2), and optic atrophy 1 homolog (OPA1), are essential for fusion processes, fission 1 (mitochondrial outer membrane) homolog (FIS1) and dynamin-related protein 1 (DNM1L) are essential fission proteins (Santel & Fuller 2001, Rojo et al. 2002, Chen et al. 2003, Olichon et al. 2003, Cipolat et al. 2004, Gripac et al. 2004, Chen & Chan 2005). Given the highly ordered arrangement of mitochondrial networks in some cell types (Bakeeva et al. 1981), it is likely that mitochondrial shape indeed has important consequences for mitochondrial function. Under physiological conditions, mitochondria are characterized by a dynamic equilibrium of fusion and fission, whereas an imbalanced expression of fusion versus fission proteins leads to a disturbance of this equilibrium and causes dramatic changes in mitochondrial morphology and function (Yaffe 1999, Karbowski & Youle 2003). In humans, mutations in...
Mfn2 cause Charcot–Marie–Tooth neuropathy type 2A (Zuchner et al. 2004, Kijima et al. 2005). Mutations in Opa1 lead to autosomal dominant optic atrophy (Alexander et al. 2000, Delettre et al. 2000). A down-regulation of fusion proteins together with an impairment of mitochondrial fusion processes or an overexpression of fission proteins causes fragmentation of the tubular mitochondrial network (James et al. 2003, Olichon et al. 2003, Yoon et al. 2003, Lee et al. 2004, Stojanovski et al. 2004, Chen et al. 2005, Detmer & Chan 2007a,b). As a consequence, an increased susceptibility to cell death and apoptosis occurred. Knock-out mice of either Mfn1 or Mfn2 are both embryonic lethal demonstrating the essential role of mitochondrial fusion for embryonal development and cell viability (Chen et al. 2003). In contrast, overexpression of the two MFN proteins together provides some protection against different apoptotic stimuli (Sugioka et al. 2004). On the other hand, mitochondrial fission proteins (FIS1 and DNM1L) and processes are essential for cell division as well as for programmed cell death and decreased cell survivability (James et al. 2003, Yoon et al. 2003, Lee et al. 2004, Stojanovski et al. 2004). In Caenorhabditis elegans, an overexpression of fission protein DNM1L increased the number of mitochondrial divisions (Labrousse et al. 1999). FIS1 overexpression also caused mitochondrial fragmentation and aggregation (Stojanovski et al. 2004). On the contrary, a disruption of fission by overexpressing a mutant form of DNM1L or overexpression of fusion proteins provoked an elongation and formation of interconnected mitochondrial network due to enhanced mitochondrial fusion (Smirnova et al. 2001, Santel et al. 2003).

Considering the severe consequences of imbalanced mitochondrial fusion and fission for neurodegeneration, we studied the effect of E and P on the transcription level of mitochondrial fusion and fission genes in cortical astrocytes in vitro. Astrocytes were chosen since they are the outstanding cell type in the CNS supporting neuronal viability and function. It is apparent that neurodegenerative processes and their steroid responsiveness often occur in a gender-specific way (Miller et al. 1998, Roof & Hall 2000, van den Eeden et al. 2003, Dluzen & McDermott 2004, Kenchappa et al. 2004, Baba et al. 2005, Marchetti et al. 2005). Therefore, we have analyzed female and male astroglia separately.

Materials and methods

Materials

All chemicals were obtained from Roth (Karlsruhe, Germany), whereas reagents for molecular biological techniques and cell culture were purchased from Invitrogen, unless indicated otherwise.

Animals

BALB/c mice were purchased from Harlan Winkelmann GmbH (Borchen, Germany). All procedures were performed in strict accordance with the published welfare rules for the care and use of laboratory animals at the University Clinic Aachen and the government of the State of Nordrhein-Westfalen, Germany.

Preparation of primary astrocytes

Astrocytes were prepared from postnatal day 1 BALB/c mice (Pawlak et al. 2005, Horvat et al. 2006). The gender of the donor was determined by visual inspection of the anogenital distance that is larger in males (Beyer et al. 1991). Brains from decapitated mice were removed and transferred in preparation buffer consisting of 10 mM HEPES, 154 mM NaCl, 10 mM glucose, 2 mM KCl, and 15 μM BSA. The brain cortices were separated and meninges removed. Afterwards, the brain tissue was incubated in PBS containing 0-1% (w/v) trypsin and 0-02% (w/v) EDTA for 15 min and minced with a Pasteur pipette and filtered through a 50 μm nylon mesh. The cell suspension was centrifuged at 300 g (Eppendorf, Hamburg, Germany) for 4 min. The cell pellet was re-suspended and the cell suspension plated onto poly-L-ornithine-coated (Sigma–Aldrich) culture dishes and cultured in Dulbecco’s modified Eagle’s medium (PAA, Coelhe, Germany) supplemented with 20% (v/v) fetal calf serum (PAA), 50 U/ml penicillin, 50 μg/ml streptomycin, 0-25 μg/ml amphotericin B (Fungizone), and 2 mM glutamine (Glutamax). Cultures were maintained at 37°C in a humidified atmosphere of 95% air/5% CO2. Before reaching confluence, astrocytes were trypsinized and plated at lower density. Sub-confluent cells were incubated in neurobasal medium (NBM) supplemented with 0-2% (v/v) B27, penicillin, streptomycin, Fungizone, and t-glutamine for 48 h. Astrocytes were subsequently used for experiments.

Cell treatment

Cortical astrocytes were placed on culture dishes in a humidified atmosphere at 37°C in the presence of NBM and were treated with 10−7 M 17β-estradiol (E) or P for 24 h. To inhibit nuclear estrogen receptors (ER) and progesterone receptors (PR), cells were simultaneously treated with 10−8 M ICI 182 780 (ICI; Tocris, Bristol, UK) and 10−6 M Rti 3021-022 (Rti; Sigma–Aldrich) respectively. Cells of the same preparation maintained under identical conditions except for hormone and antagonist treatments served as controls.

Cell viability

Primary astrocytes from brain cortices were treated as described above and mentioned in the figure legends.
In brief, cells were plated on cover slips, treated, and stained with 1 μg/ml Hoechst 33342 Trihydrochlorid (Hoechst, Invitrogen) for 5 min under culturing conditions. Subsequently, cells were washed twice with PBS, fixed with 4% (w/v) paraformaldehyde (Merck) for 15 min at room temperature, washed twice with PBS, and mounted in Mowiol (Merck). Hoechst-stained normal, apoptotic, and necrotic cell nuclei were detected with a Hitachi HV-C20A Camera connected to a fluorescence microscope (Leica Microsystems, Wetzlar, Germany) at excitation wavelength of 365 nm. Cell nuclei were scored accordingly to their specific morphological characteristics (Horvat et al. 2006) from the images taken by using the Adobe Photoshop CS2 software (Adobe Systems Inc). Viable and apoptotic cells were counted from five distinct areas of the same size per cover slip from three to five independent experiments. Cell numbers were calculated as percentage of the ratio of sample cell number to control cell number referred to as 100%. Additionally, cells were stained with propidium iodide to visualize necrotic astrocytes. Before the cells were stained with 500 nM propidium iodide for 10 min, they were fixed in 100% (v/v) ice-cold methanol at −20°C for 10 min, washed, and incubated in 2×SSC (0.3 M NaCl, 0.03 M sodium citrate (pH 7.0)) for 20 min. Fluorescent necrotic cells were visualized at excitation wavelength of 535 nm and using an emission filter of a wavelength >605 nm and quantified the same way as Hoechst-stained samples.

Reverse transcription

Total RNA was isolated from astrocyte cultures using PeqGold RNA pure (PeqLab, Erlangen, Germany). The procedure was performed according to the instruction of the manufacturer. RNA concentration was measured photometrically using BioPhotometer (Eppendorf). RNA integrity was tested randomly by 1% (w/v) agarose denaturing gel electrophoresis and ethidium bromide staining and visualized under UV-illumination. First-strand complementary DNA (cDNA) was synthesized from 0.5-μg total RNA. In brief, total RNA dissolved in 7 μl diethyl pyrocarbonate water was pre-incubated at 85°C for 5 min and placed immediately on ice. Subsequently, the reaction buffer consisting of 8 U/μl M-MLV reverse transcriptase, 8 mM dithiotreitol, 40 mM Tris–HCl, 60 mM KCl, and 2.4 mM MgCl2, and 0.4 mM each dNTP (Roti-mix PCR3, Roth) was added to RNA giving a final volume of 12.5 μl. After 1 h incubation at 37°C, reverse transcription was stopped by heat-inactivating the enzyme at 70°C for 15 min. Addition of water instead of RNA served as negative control. Transcripts of 18S ribosomal RNA (18S rRNA) and hypoxanthine guanine phosphoribosyl transferase (Hprt), spanning over intron–exon borders, served as control for RNA purity and normalization of sample quantity.

Real time-PCR analysis

Quantitative real-time PCR (RT-PCR) analysis of fusion and fission genes, pro-/anti-apoptotic, as well as proliferation markers was performed using SYBR Green technology and carried out by the iQ5 detection system (Bio-Rad). Forward and reverse primers (Table 1) for specific amplification of Mfn1, Mfn2, Fis1, Dnm1l, a proliferation marker, proliferating cell nuclear antigen (Pcna), an anti-apoptotic marker, B-cell lymphoma protein 2 (Bcl2), and a pro-apoptotic marker, BCL2-associated X protein (Bax), were designed eliminating the possibility of amplifying genomic DNA. For each set of primers, a basic local alignment search tool (BLAST, NCBI) search revealed that sequence homology was obtained specifically for the target gene. The cDNA from untreated control and treated cell samples obtained after reverse transcription was diluted 1:10 and added to a solution containing 5 μM primers and IQ SYBR Green Supermix (Bio-Rad) consisting of 25 U/ml iTaq polymerase, 50 mM KCl, 20 mM Tris–HCl, 0.2 mM each dNTP, 3 mM MgCl2, SYBR Green I, and stabilizers. The RT-PCR protocol was composed of an initial denaturation step for 3 min at 95°C followed by 40 cycles consisting of 10 s at 95°C, 30 s at the appropriate for the target gene annealing temperature (53°C – Mfn1, Mfn2; 58°C – Fis1, Dnm1l; 59°C – Bcl2, Bax, Pcna; 60°C – 18S rRNA; 61°C – Hprt), 30 s at 72°C, and 10 s at 78°C. To obtain melting curves for the resulting PCR products, a final step was added to the RT-PCR consisting of 81 cycles of increasing temperature from 55°C to 81°C by 0.5°C for 10 s each step. The PCR products were quantified using the relative ΔCt method. Relative quantification relates the PCR signal of the target transcript to that of 18S rRNA in treated cells with respect to untreated cells. A test for an approximately equal efficiency of target amplification was performed by looking at ΔCt value variations with template dilutions. 18S rRNA and Hprt served as endogenous control in the validation experiments. The absolute value of the slope of log input amounts versus ΔCt should be ~−3.3 and the efficiency ~100%. The validation experiments passed this test. The results are expressed as an average of triplicate samples of at least three independent experiments for control and treated cells.

Statistical analysis

Data are presented as means ± S.E.M. of at least three to five independent experiments. For statistical analysis, data were analyzed by ANOVA followed by a post hoc Tukey’s test and regarded as statistically significant at P < 0.05.
Results

Gender-specific effects of steroid hormones on cell viability and apoptosis

Cortical astroglial cell cultures prepared from postnatal mouse brains revealed a high yield (>95%) of glial fibrillary acidic protein (GFAP)-positive cells, as determined by immunocytochemistry against the GFAP, an astrocyte-specific protein marker. Neurons, oligodendrocytes, and microglia were virtually absent from astrocyte cell cultures (Pawlak et al. 2005, Horvat et al. 2006). Astrocytes from both genders express ER and PR as studied by RT-PCR for ERα, ERβ, and PR (data not shown).

In a first attempt, we have analyzed gender-specific effects of E- and P-treatment on proliferation and apoptotic death of cortical astrocytes. Female and male astrocyte cultures were treated with 10⁻⁷ M E or P in the absence or presence of the appropriate receptor inhibitor, i.e. ICI and Rti as inhibitors for ER and PR respectively. Viable, apoptotic, and necrotic astrocytes were distinguished by fluorescent labeling of cell nuclei with Hoechst 33342. Before and after treatments, no significant changes in the number of necrotic cells were detected (0–2 cells per total number of ~100 cells; data not shown). This finding was supported by experimental data obtained from propidium iodide staining that labels necrotic cells (data not shown). Due to the fact of a constant number of necrotic astrocytes, we focused on the determination of the number of viable and apoptotic astrocytes before and after hormone application. Treatment of cortical astrocytes from female brains with E or P for 24 h induced a significant increase in the number of Hoechst-stained viable cells by ~50% (Fig. 1A). Co-application of P and PR inhibitor suppressed this effect, whereas the ER inhibitor did not affect the E-mediated effect (Fig. 1A). Independent of the steroid hormone applied, the number of apoptotic cells was not significantly changed in female astrocyte cultures (Fig. 1B). In contrast, the application of E did not exert any effect on the number of viable male cells, whereas P caused a decrease (Fig. 1C). The latter effect was counteracted by Rti. Compared with female astrocytes, opposite effects were observed concerning apoptosis of male astrocytes. Both hormones provoked a significant three- to fourfold induction that was partially suppressed by ICI and completely antagonized by Rti (Fig. 1D).

In the next step, we measured transcription levels of pro-, anti-apoptotic, and proliferation markers (Bax, Bcl2, and Pcn respectively) to figure out, whether changes in the viable and apoptotic cell numbers resemble changes in the marker gene expression. Primers used for RT-PCR amplification are given in Table 1. They were designed as intron over-spanning sequences except for 18S rRNA. Only single bands at correct molecular weight according to Table 1 were observed for the PCR products of the housekeeping gene Hp6t and the genes of interest in an

Table 1 Information about primers used in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession no.</th>
<th>Primer sequences 5’–3’</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mfn1a</td>
<td>NM_024200</td>
<td>CAG AGA AGA GGG TTT ATT CA ACT CAT CAA CCA AAA CAG AT</td>
<td>180</td>
</tr>
<tr>
<td>Mfn2b</td>
<td>NM_133201</td>
<td>TGA ATG TTG TTG TTG TTG AAAG TGC TGT TGT CTA AAT GT TTT GTG GTG TGT AGG ACT GG TTT GTG AGG TTC TGT CCT GA</td>
<td>175, 241</td>
</tr>
<tr>
<td>Dnm1f</td>
<td>NM_152816</td>
<td>CTA CAG GGG TGC AGG AGA AA AGA TGG ACT GGT AGG CAT GG CAT CCC AGC TTC ACA TAA CC GCA ATG CGA CTC ACC AAC CAT TT AGG GGA CCT GAG GTT TAT TG TCA GGA GCA ATC TTC AAA GG TTT TTC ACA AAA GCC ACT CC</td>
<td>228</td>
</tr>
<tr>
<td>FisIa</td>
<td>NM_025562</td>
<td>AGA TGG ACT GGT AGG CAT GG AGA TGG ACT GGT AGG CAT GG CTA CAG GGG TGC AGG AGA AA AGA TGG ACT GGT AGG CAT GG</td>
<td>175</td>
</tr>
<tr>
<td>Bcl2b</td>
<td>BC089016</td>
<td>GCA ATC CGA CTC ACC AAC CAT TT AGG GGA CCT GAG GTT TAT TG TCA GGA GCA ATC TTC AAA GG TTT TTC ACA AAA GCC ACT CC</td>
<td>228</td>
</tr>
<tr>
<td>Baxc</td>
<td>NM_007527</td>
<td>GGCG CTA CCA CAT CCA AGG AA AGG GGA CCT GAG GTT TAT TG TCA GGA GCA ATC TTC AAA GG TTT TTC ACA AAA GCC ACT CC</td>
<td>194</td>
</tr>
<tr>
<td>Pcnld</td>
<td>X53068</td>
<td>CTA CAG GGG TGC AGG AGA AA AGA TGG ACT GGT AGG CAT GG CTA CAG GGG TGC AGG AGA AA AGA TGG ACT GGT AGG CAT GG</td>
<td>175</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>X00686</td>
<td>CTA CAG GGG TGC AGG AGA AA AGA TGG ACT GGT AGG CAT GG CTA CAG GGG TGC AGG AGA AA AGA TGG ACT GGT AGG CAT GG</td>
<td>241</td>
</tr>
<tr>
<td>Hprt</td>
<td>NC_000086.6</td>
<td>CTA CAG GGG TGC AGG AGA AA AGA TGG ACT GGT AGG CAT GG CTA CAG GGG TGC AGG AGA AA AGA TGG ACT GGT AGG CAT GG</td>
<td>248</td>
</tr>
</tbody>
</table>

aMitofusin 1.  
bMitofusin 2.  
cDynamin-related protein 1.  
dFission 1 (mitochondrial outer membrane) homolog.  
eB-cell lymphoma protein 2.  
fBcl2-associated X protein.  
gProliferating cell nuclear antigen.  
hHypoxanthine guanine phosphoribosyl transferase.
agarose gel. This indicates that the isolated RNA was free of genomic DNA impurities. In female cortical astrocytes, E exerted no significant effect on the transcription of anti- and pro-apoptotic marker genes, whereas P induced an approximately sixfold upregulation of Bcl2 and Bax transcription (Fig. 2A), which was partially downregulated by Rti. Unlike females, male astrocytes responded to E application with an increased transcription of the apoptotic marker Bax and a decreased transcription of anti-apoptotic Bcl2 (Fig. 2C). Treatment of male astrocytes with P caused a decreased transcription of both marker genes. The P effect was completely inhibited by Rti, whereas ICI reversed only the E effect on Bcl2 transcription.

In female astrocytes, Pcna transcription was five- to eightfold upregulated, irrespective of the hormone (Fig. 2B). Again, ICI did not prevent the E effect, but Rti partially abolished the P effect. Pcna transcription levels in male astrocytes were stimulated by E, but significantly downregulated by P (Fig. 2D). ICI showed no effect. Here again, Rti antagonized the effect.

**Gender-specific effects of steroid hormones on fusion and fission gene transcription**

After treatment of female and male cortical astrocytes with E or P, the transcription levels of Mfn1, Mfn2, Fis1, and Dnm1l were determined by RT-PCR (Table 1, Fig. 3). In female astrocytes, E stimulated Mfn2 but not Mfn1 transcription, whereas P exposure caused an increase in both fusion transcripts (Fig. 3A). In male astrocytes, E decreased Mfn1 but increased Mfn2 transcription, whereas P downregulated both fusion gene transcript levels (Fig. 3C). Regarding fission gene transcription, E and P stimulated both, Dnm1l and Fis1, in female astrocytes (Fig. 3B). In male astrocytes, E also increased Dnm1l and Fis1 transcription, but P decreased the transcription of both fission genes (Fig. 3D). Here again, ICI did not antagonize the E effects in female astrocytes, whereas Rti blocked partially the P effect (Fig. 3A and B). In male astrocytes, ICI blocked only the E effect on Mfn1 transcription, and Rti reversed the P effect on the transcription of all fusion and fission genes (Fig. 3C and D).
Correlation analyses of Mfn1 with Bcl2 transcripts and Dnm1l with Pcn1 for female and male astrocytes are given in Fig. 4A–D. It becomes evident that Mfn1 correlates well with Bcl2 and Dnm1l with Pcn1 for both steroid hormones and both genders (Fig. 4A and C as well as B and D respectively) by showing similar transcription levels. Several observations concerning gender-specific differences of the hormonal regulation deserve attention: i) In females, P increased Mfn1, Bcl2, and Dnm1l, Pcn1 transcript levels (Fig. 4A and B); in males, gene transcription was decreased (Fig. 4C and D). ii) Treatment of astrocytes with E caused higher transcript levels of Mfn1, Bcl2 and Dnm1l, Pcn1 in females than in males (Fig. 4A and B versus C and D). In females, Mfn1 and Bcl2 are not changed by E, but decreased in males. Dnm1l and Pcn1 are increased by E in females to a greater extent than in males.

Our data suggest that Dnm1l correlates with proliferation and Fis1 with apoptosis in both genders. Therefore, we attempted to correlate the ratios of Dnm1l/Fis1 and Pcn1/Bax transcription with viable/apoptotic cell number as shown in Fig. 5A (female) and B (male). The following gender-specific differences become obvious when comparing all three ratios: i) The Pcn1/Bax transcript level and viable/apoptotic cell ratios are >1 for female astrocytes treated with E or P, whereas these levels are <1 for E-/P-treated male astrocytes. ii) The Dnm1l/Fis1 ratios are decreased in P- versus E-treated male astrocytes, but slightly increased in females. For both genders, i) the ratios of Pcn1/Bax are decreased in P-treated compared with E-treated cells; ii) the ratios of viable/apoptotic cell numbers do not differ between the two steroid treatments.

**Discussion**

Astrocytes are essential for the support of neurons in the CNS (Pellerin et al. 2007, Schousboe et al. 2007). They are implicated in the regulation of growth, cell proliferation, and neuroprotection in the brain (Beyer 1999, Beyer et al. 2003, Garcia-Segura et al. 2003, Kajta & Beyer 2003, Kipp et al. 2006, McCarthy 2008). In the past years, the neuroprotective aspects of E and P effects in the CNS have been analyzed using *in vitro* and *in vivo*
approaches. Besides direct interactions of E and P with neurons (Kipp et al. 2006, Stein et al. 2008), there are indications that neurons are not always protected directly. However, E has consistently been demonstrated to be protective \textit{in vivo} and in organotypical cultures (Dhandapani & Brann 2002, Simpkins et al. 2005). This suggests the involvement of another non-neuronal brain cell type exerting a protective function. Of the non-neuronal brain cell types, astrocytes have the greatest potential for a possible involvement in the mediation of steroid-mediated neuroprotective effects. Astrocytes are the most abundant type of glial cells in the brain and are located juxtaposition to neurons, outnumbering them by a 10:1 ratio in the cortex. This cell type has the greatest potential for a critical role in steroid-mediated protection of neurons in the brain, since ablation of astrocytes \textit{in vivo} results in a significant decrease in neuronal survival (Cui et al. 2001). Indeed, astrocytes are well-described target cells for E and P expressing the corresponding nuclear steroid receptors (Jung Testas et al. 1992, Santagati et al. 1994, Beyer 1999, Quadros et al. 2007). Confirming previously published data (Pawlak et al. 2005), we observed transcripts for ER\textsubscript{a}, ER\textsubscript{b}, and PR in cortical astrocytes from both genders.

Besides the above described supportive function, astrocytes play a major role for the energy homeostasis in the brain pointing at astrocyte mitochondria as an interesting compartment involved in neuroprotection. However, there is only limited information available on interactions between sex steroids and mitochondria. In recent studies, we and others have shown that E promotes the expression of mitochondria-encoded subunits of the respiratory chain, thereby influencing mitochondrial respiratory activity and enhancing functional efficiency (Araújo et al. 2008, Irwin et al. 2008). Mitochondrial functions are not only affected by regulation of nuclear and mitochondrial gene expression and activity of the respiratory chain (Zheng & Ramirez 1999, Araújo et al. 2008, Irwin et al. 2008), but

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Effect of estrogen (E) and progesterone (P) on the transcription of mitochondrial fusion and fission genes in cortical astrocytes from female and male brains. Primary cortical astrocytes from (A and B) female and (C and D) male mouse brains were incubated in the presence of 10^{-7} M E or P for 24 h. Antagonists of estrogen and progesterone receptors (ICI and Rti respectively) were added simultaneously to the respective steroid hormone treatment. Specific quantification of fusion genes, \textit{Mfn1} (A and C; grey columns) and \textit{Mfn2} (A and C; black columns), and fission genes, \textit{Dnm1l} (B and D; grey columns) and \textit{Fis1} (B and D; black columns), was performed by RT-PCR. Data were normalized to 18S rRNA and \textit{Hprt} and related to the transcription levels in untreated control astrocytes set 100\% (dotted lines). Data represent the percentage of control as means ± S.E.M. of triplicates of five independent experiments. *\textit{P}<0.05, **\textit{P}<0.01, ***\textit{P}<0.001, E-/P-treated samples versus untreated controls; *\textit{P}<0.05, **\textit{P}<0.01, inhibitor plus E-/P-treated samples versus E-/P-treated samples.}
\end{figure}
also by the morphology of these organelles. It is noteworthy that E influences mitochondria not only through its nuclear receptors, but acts also directly by binding of E to mitochondria in vitro and in situ (Yang et al., 2004, Stirone et al., 2005, Yager & Chen 2007, Milner et al., 2008). Interestingly, we observed that some effects, in particular those exerted by E but not by P, were not subject to inhibition by classical nuclear ER antagonists. Proliferation and fission gene transcription were obviously mediated by E via non-classical mechanisms in male and female astrocytes. The context of mitochondrial fission and cell division additionally supports the role of one for the other. Such a proliferative E action that is intracellularly transmitted through, for instance, the MAP kinase system has already been reported for non-neural cells (Bouskine et al., 2008). This is in line with a previous observation of a rapid activation of ERK in astroglia (Ivanova et al., 2001). Intriguingly, a recent report suggests a mitochondrial ER-mediated pathway in stimulation of cell division (Chen et al., 2008). Besides proliferation, the ER antagonist ICI did not abolish any of the E effects on female astrocytes studied, whereas in male astrocytes, ICI prevented the E effects by decreasing apoptotic cell death and increasing Bcl2 and Mfn1 transcription. This indicates gender-specific E actions in the context of anti-apoptotic processes and an involvement of Mfn1 therein.

It is known for several years and for a wide range of neurodegenerative disorders that they show distinct sex-specific differences in course and incidence. Thus, a female prevalence is known for Alzheimer’s disease and multiple sclerosis, whereas a male prevalence was observed for Parkinson’s disease and stroke. In particular, intact adult female rodents sustain lower mortality and less neuronal damage when compared with age-matched male rodents following middle cerebral artery occlusion (Alkayed et al., 1998). Parkinson’s disease reveals a clear gender disparity (Shulman 2007). Interestingly, E appears to exert opposite effects in females and males in an experimental animal model for Parkinson’s disease being neuroprotective only in females (Gillies et al., 2004). Estrogen replacement therapy leads to an improvement of Parkinsonian symptoms of women in the menopause (Shulman 2007). Both disorders, stroke and Parkinson’s disease, and also Alzheimer’s disease are

Figure 4: Correlation analysis between (A and C) Mfn1 and Bcl2 gene transcription as well as (B and D) Dnm1l and Pcna gene transcription in (A and B) female and (C and D) male astrocytes. Data used for this graph derived from Figs 1–3. Gene transcription levels for Mfn1 (A and C; grey columns) and Bcl2 (A and C; black columns) as well as for Dnm1l (B and D; grey columns), and Pcna (B and D; black columns) after E/P-treatment in the absence or presence of ICI/Rti for 24 h are presented as percentage of control set 100% (dotted lines). Note that P exerted opposite effects in female and male astrocytes. Treatment of astrocytes with E caused higher transcription levels of all four genes in (A and B) females compared with (C and D) males.
Regulation of mitochondrial fusion and fission

characterized by a disruption of mitochondrial function (Singh et al. 2006, Thomas & Beal 2007, Poole et al. 2008, Schapira 2008). This raised the question whether steroid-mediated regulation of mitochondrial morphology and function could be involved in the gender specificity of neurodegenerative and neuroprotective processes. Besides functional characteristics, such as energy production, the morphology of mitochondria including the expression of fusion and fission genes are indicators of mitochondrial vitality. Elevated fusion processes cause an elongation of mitochondria, whereas an increase in fission leads to mitochondrial fragmentation (Chan 2007). Generally, an imbalance of mitochondrial fusion and fission causes mitochondrial dysfunction and cellular dysfunction, indicating that fusion and fission processes are essentially linked to cell function and protection/apoptosis. In an attempt to better understand the protective action of E and P, we have focused on fusion and fission processes in astrocytes and have tried to correlate their regulation with cell viability and apoptosis.

Cultured cortical astrocytes were treated with E concentrations known to be effective in the brain (Luconi et al. 1999, Ivanova et al. 2001, Pawlak et al. 2005, Rune et al. 2006, von Schassen et al. 2006, Araújo et al. 2008). Under basal (physiological) conditions, cultured cortical astrocytes from males and females did not differ in their characteristics for mitochondria morphology and cell viability. E appeared to stimulate proliferation in females, whereas P facilitates cell death in males. The age-related drop of E levels in females may, thus, play a role for the vulnerability of cortical neurons in late-onset neurodegenerative disorders, such as Alzheimer’s disease, due to reduced astrocyte stimulation and survival. We might speculate that the prevalence for Alzheimer’s disease observed for females is causally linked to decreased E levels and astrocyte activation. Notwithstanding the importance of our observations, we did not provide a pathological model with our experimental set-up.

For E-treated astrocytes of both genders, we observed a correlation between Mfn1 and anti-apoptotic marker Bcl2 transcription as well as Dnm1l and proliferation marker Pcnα transcription. This indicates a role of Mfn1 in anti-apoptotic processes and Dnm1l in cell proliferation and in agreement with the generally accepted role of fusion genes in anti-apoptotic processes and fission genes in proliferation and apoptosis (Yaffe 1999, James et al. 2003, Karbowski & Youle 2003, Olichon et al. 2003, Yoon et al. 2003, Lee et al. 2004, Stojanovski et al. 2004, Chen & Chan 2005, Chen et al. 2005, Chan 2007). Our studies also revealed gender differences in the transcription of Mfn1 and Dnm1l showing higher levels in females that were accompanied by lower apoptotic cell death and elevated number of viable cells. The role of Dnm1l in mitochondrial fragmentation was previously supported by the discoveries that Dnm1l mutations blocked and Dnm1l overexpression increased mitochondrial proliferation (Bleazard et al. 1999, Labrousse et al. 1999). Furthermore, Dnm1l was co-localized with constriction of mitochondria that can coincide with actual division events (Bleazard et al. 1999, Labrousse et al. 1999). During mitosis, Dnm1l is involved in fusion of mitochondria, a phenomenon that might facilitate the
partitioning of fragmented mitochondria to daughter cells during cytokinesis (Taguchi et al. 2007). This makes Dnm1l a factor of controlling mitochondrial division and a potential candidate for steroid-mediated regulation of cell proliferation, although the involvement of Dnm1l in apoptosis cannot completely be ruled out (Frank et al. 2001). We have deliberately chosen the Dnm1l/Fis1 ratio to describe proliferative and apoptotic events in astrocyte cultures. This enables us to discriminate both opposite mechanisms in cultures of equal numbers of viable cells. A constant number of cells does not allow to conclude whether there is an effect or no effect on both processes by hormones.

Comparing the anti- and pro-apoptotic marker gene transcription with apoptotic cell death, it becomes obvious that an increase or the absence of differences in Bax compared with Bcl2 is related to increased or unchanged apoptosis respectively. The apparent discrepancy between the effect of P on male astrocytes showing a reduced Bax transcription and increased apoptotic cell death can be clarified by determination of the Bax/Bcl2 ratio which is elevated. This points at an increase of apoptotic cell death, although the individual values of Bax and Bcl2 in males are below the basal levels found in controls.

A clear gender-specific difference in the apoptosis rate of steroid-treated astrocytes was observed and could be correlated with fusion/fission gene transcription. Female astrocytes showed a mainly balanced increase of both, fusion and fission gene transcription. This can be considered as a reason for a balanced transcription of anti- and pro-apoptotic marker genes. On the contrary, fusion and fission gene transcription in males was imbalanced. Consequently, this imbalance caused a disturbance of the balance between anti- and pro-apoptotic marker genes, thus promoting apoptotic processes specifically in male astrocytes treated with gonadal steroids. A higher level of fusion gene transcription supports the increased astrocyte viability in females (Zuchner et al. 2004, Kijima et al. 2005). This could be, at least in part, due to the role of mitochondrial fusion processes in mixing mitochondrial DNA copies, thereby keeping high loads of mutated mitochondrial DNA below the threshold of developing diseases (Chen & Chan 2005, Chan 2007).

The increased apoptosis in male astrocyte cultures could only be compensated by an elevation of proliferation resulting in an unchanged level of viable cells after steroid hormone treatment. Indeed, E-treated male astrocytes exhibited a higher Pcna/Bax ratio than P-treated cells. A ratio of Pcna/Bax > 1 together with an increased proliferation marker transcription at constant levels of apoptosis in female astrocytes after steroid treatment caused an elevated number of viable cells. In contrast, male astrocytes demonstrated an increased proliferation after E treatment that compensated for the increased apoptosis, thereby demonstrating a similar number of viable cells as in untreated controls. However, a decreased proliferation after P treatment of male astrocytes was not able to counterbalance the increased apoptotic cell death leading to a net decrease in viable cells. Other authors have similarly demonstrated E to increase glial cell proliferation (Jung Testas et al. 1992, Dhandapani & Brann 2002), whereas no effect of P on cell proliferation was described. This could be due to the fact that gender-specific differences observed for P-treated astrocytes are counterbalanced in cell cultures of mixed sexes.

In conclusion, our data show for the first time that sex steroids are capable of influencing fusion and fission processes in astroglia mitochondria. Importantly, this effect occurred in a gender-specific way and revealed distinct differences in the effectiveness between E and P. Under physiological conditions, sex steroids may be important for the balance of mitochondrial morphology and function in the brain. After toxic events and under neuropathological conditions, sex steroids may differently contribute to the homeostasis and integrity of cell function, thus providing a basis for the well-known gender differences in neuronal vulnerability and cell death. Our findings suggest a pronounced sensitivity of male astroglia for apoptosis. This may serve as an explanation for the higher incidences of neurodegenerative diseases in males (Miller et al. 1998, Roof & Hall 2000, van den Eeden et al. 2003, Dluzen & McDermott 2004, Kenchamma et al. 2004, Baba et al. 2005, Marchetti et al. 2005).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Emmy-Noether-Programm of the Deutsche Forschungsgemeinschaft (S A), the START-Programm (S A) and IZKF BIOMAT (S A, C B) of the Faculty of Medicine, RWTH Aachen University.

Acknowledgements

We thank Petra Ilbold for excellent technical support.

References


Arnold S 2005 Estrogen suppresses the impact of glucose deprivation on astrocytic calcium levels and signaling independently of the nuclear estrogen receptor. Neurobiology of Disease 20 82–92.


James DI, Parone PA, Mattenberger Y & Martinou JC 2003 Drp1, a novel component of the mammalian mitochondrial fission machinery. Journal of Biological Chemistry 278 36373–36379.


Lee VJ, Jeong SY, Karbowski M, Smith CL & Youle RJ 2004 Roles of the mammalian mitochondrial fusion and fission mediators Fis1, Drp1, and Opa1 in apoptosis. Molecular Biology of the Cell 15 5001–5011.


Received in final form 25 July 2008
Accepted 27 August 2008
Made available online as an Accepted Preprint 27 August 2008