Melatonin and oestrogen treatments were able to improve neuroinflammation and apoptotic processes in dentate gyrus of old ovariectomized female rats

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Abstract The aim of this study was to determine the outcomes of oestrogen and melatonin treatments following long-term ovarian hormone depletion on neuroinflammation and apoptotic processes in dentate gyrus of hippocampi. Forty-six female Wistar rats of 22 months of age were used. Twelve of them remained intact, and the other 34 were ovariectomized at 12 months of age. Ovariectomized animals were divided into three groups and treated for 10 weeks with oestrogens, melatonin or saline. All rats were killed by decapitation at 24 months of age, and dentate gyri were collected. A group of 2 month-old intact female rats was used as young control. The levels of pro-inflammatory cytokines and heat shock protein 70 (HSP 70) were analysed by ELISA. The expressions of TNF α , IL1 β , GFAP, nNOS, iNOS, HO-1, NF κ B, Bax, Bad, AIF, Bcl2 and SIRT1 genes

were detected by real-time (RT)-PCR. Western blots were used to measure the protein expression of NF κ B p65, NF κ B p50/105, I κ B α , I κ B β , p38 MAPK, MAP-2 and synapsin I. We have assessed the ability of 17 β -oestradiol and melatonin administration to downregulate markers of neuroinflammation in the dentate gyrus of ovariectomized female rats. Results indicated that 17 β -oestradiol and melatonin treatments were able to significantly decrease expression of pro-inflammatory cytokines, iNOS and HO-1 in the hippocampus when compared to non-treated animals. A similar age- and long-term ovarian hormone depletion-related increase in GFAP was also attenuated after both melatonin and oestradiol treatments. In a similar way to oestradiol, melatonin decreased the activation of p38 MAPK and NF κ B pathways. The treatments enhanced the levels of synaptic molecules synapsin I and MAP-2 and have been shown to modulate the pro-antiapoptotic ratio favouring the second and to increase SIRT1 expression. These findings support the potential therapeutic role of melatonin and oestradiol as protective anti-inflammatory agents for the central nervous system during menopause.

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Introduction

Dramatic changes in the endocrine activity of the female gonads ensue in ageing females, which are thought to

underlie the emergence of a spectrum of physiological and psychological health complications. Menopause, whether occurring naturally or induced surgically, is thereby affecting the functional capacity of certain brain regions involved in learning, memory, and cognitive ability (Buckler 2005).

Meng et al. (2010) showed that decreases in learning and memory functions in ovariectomized rats were associated with degenerative changes in hippocampal neurons. Oestrogen deprivation also enhanced apoptotic cell death and decreased expression of the anti-apoptotic protein Bcl2. How apoptosis causes neurons to die is still a matter of debate; however, the principal mechanism is by triggering the release of intercellular signals which induce phagocytic cells to take in the neurons. Astrocytes and microglia express membrane receptors that recognize molecules released by neurons, leading to phagocytosis of 'altered' cells and neuronal debris (Harrison et al. 1998; Noda et al. 2011). Chronic activation of microglia is believed to trigger and maintain an inflammatory response, which may ultimately lead to neuronal cell death (Tan et al. 1999).

In the past few years, it has been increasingly clear that neuroinflammation negatively impacted on neuronal plasticity and specifically LTP was decreased when microglial activation and/or inflammatory cytokine production were increased in hippocampus (Hall et al. 1998; Lyons et al. 2009; Lynch 2010). The loss of ovarian oestrogens in postmenopausal women may exacerbate central and peripheral inflammatory responses that occur with normal ageing. One of the most prominent changes in the brain's neuroinflammatory response during ageing is the increased and/or poorly regulated production of pro-inflammatory mediators (Franceschi et al. 2000).

Previous work has shown that oestrogens are able to repress the transcription of numerous pro-inflammatory cytokine genes, suggesting that these hormones could be used to prevent and/or treat inflammatory conditions associated with menopause (Cvoro et al. 2011). Furthermore, mechanisms by which oestrogens prevent neuronal death involve the inhibition of apoptosis by increasing the level of anti-apoptotic proteins or repressing the level of pro-apoptotic proteins (Meda et al. 2000; Amantea et al. 2005). In a previous publication, we have demonstrated that the number of neurons in the hilus of the dentate gyrus was significantly decreased during ageing and that 22-month-old ovariectomized females chronically treated with oestradiol

showed a significantly higher number of BrdU-immunoreactive cells in the GCL/SGZ than control animals treated with vehicle (Perez-Martin et al. 2005). Other groups have also confirmed that oestrogen treatments were able to enhance neurogenesis in the dentate gyrus of the hippocampus in adult animals (Brinton 2009; Pawluski et al. 2009).

Reiter et al. (1981) reported a marked decline in nocturnal pineal melatonin levels with ageing in female rats. Okatani et al. (2000) observed that although nocturnal serum melatonin levels in premenopausal women declined moderately from 17 to 45 years of age, it showed an increase during the period from 46 to 50 years of age. However, among postmenopausal women, a steep, age-related decline in nocturnal melatonin secretion was found for up to 15 years of postmenopause, followed by an extremely gradual decline thereafter. Some experimental and clinical studies have demonstrated positive effect of melatonin administration that was able to delay the characteristic endocrine changes that occur during the course of menopause (Diaz and Llaneza 2008) and that significantly increased serum levels of HDL cholesterol (Tamura et al. 2008). Ciortea et al. (2011) have shown that the addition of melatonin to oestrogen replacement treatment is associated with a decrease in endometrial proliferation and prevents the appearance of cellular atypia. These results suggested that melatonin supplementation might play an important role in the prophylaxis of endometrial cancer in menopause.

Since melatonin and oestrogen are potent anti-oxidants, as well as anti-apoptotic and anti-inflammatory substances, the decrease in melatonin synthesis in the elderly, together with changes in hormone levels that occur with increased age, may account for the increased pathology in aged people, including neurodegenerative diseases (Harrod et al. 2005).

The aim of this study was to determine the outcomes of oestrogen and melatonin treatments following long-term ovarian hormone depletion in female rats on neuroinflammation and apoptotic processes in dentate gyrus of hippocampi.

Materials and methods

Animals and treatment

Forty-six female Wistar rats of 22 months of age were used in the present study. Twelve of them remained

intact, and the other 34 were ovariectomized at 12 months of age, according to the following procedure: Rats were anaesthetized with Equithesin, and two small incisions (8 mm) were made through the skin and the muscle back walls in parallel with the animal body line. The ovaries were then located, and a silk thread (Silkam®, Braun-Aesculap, Germany) was tightly tied around the oviduct, including the ovarian blood vessels. The oviduct was sectioned, and the ovary was removed, taking good care in leaving the knot intact. The muscle wall was then sutured with a synthetic absorbable thread (Safil®, Braun-Aesculap, Germany) and the skin with metallic clips. The animals were given a standard laboratory rat diet (A.04; Panlab, Barcelona, Spain) and water *ad libitum*, in a light and temperature controlled room. Ovariectomized animals were divided into three groups: non-treated, treated with oestrogens (oestradiol valerate, Sigma, St. Louis, Missouri, USA; 125 µg/week, s.c., diluted in sunflower seed oil) and treated with melatonin (melatonin, Sigma, St. Louis, Missouri, USA, was given in the drinking water at a dose of 1 mg/kg/day). A fresh melatonin solution was prepared three times per week, depending on the water consumption and the weight of the animals to obtain a daily melatonin dose of 1 mg/kg. Water bottles were covered with aluminium foil to be protected from light, and the drinking fluid was changed three times weekly. After 10 weeks of treatment, rats were sacrificed by decapitation, and dentate gyrus were collected as published before¹³ and processed as described later. All non-treated animals received the correspondent vehicle doses. Six of the intact rats were submitted to sham operation following the same procedure, without removing ovaries at 12 month of age. A group of 2-month-old female rats was used as young control ($n=12$). All the animals received humane care according to the Guidelines for Ethical Care of Experimental Animals of the European Union. The study was approved by the ethical committee of the Complutense University of Madrid.

Cytokine determination

Dentate gyrus were dissected and frozen in liquid nitrogen. Samples were homogenized in 0.05 M Tris-HCl buffer with protease inhibitors (0.1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin) for 30 s with an electrical homogenizer (Polytron; Brinkmann Instruments, Westminister, NY, USA) and later centrifuged at $7,000\times g$ (10 min, 4 °C). The supernatant was

collected and was stored at $-80\text{ }^{\circ}\text{C}$ until the determination of cytokines. Tumor necrosis factor alpha (TNF α), interleukin 1 beta (IL1 β) and interleukin 6 (IL6) were measured in dentate gyrus homogenates collected from aged, young controls and treated rats with an ELISA kit according to the manufacturer's instructions (Bio-NOVA Cientifica Ltd., Madrid, Spain).

Extraction of tissue samples and determination of HSP 70

Frozen dentate gyrus samples were transferred to 5-ml polypropylene tubes containing $1\times$ Extraction reagent (4 °C) with protease inhibitors (0.1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin). Samples were homogenized for 30 s with an electrical homogenizer (Polytron; Brinkmann Instruments, Westminister, NY, USA) and later centrifuged at $21,000\times g$ (10 min, 4 °C). The supernatant was collected and was stored at $-80\text{ }^{\circ}\text{C}$ until assayed for the quantitative presence of heat shock protein 70 (HSP 70).

HSP 70 was measured with an ELISA kit according to the manufacturer's instructions (Assay Designs, Stressgen, MI, USA, catalog number EKS-700B). HSP 70 concentrations from the sample are quantitated by interpolating absorbance reading from a standard curve generated with the calibrated HSP 70 protein standard provided.

Western blotting analysis

Western blots were used to measure the protein expression of NF κ B p65, NF κ B p50/105, I κ B α , I κ B β , mitogen-activated protein kinase (MAPK) p38, synapsin I and microtubule-associated protein 2 (MAP-2). Briefly, dentate gyrus samples after homogenization with lysis buffer were sonicated, boiled with gel-loading buffer (0.100 M Tris-Cl, 4 % sodium dodecyl sulphate (SDS), 20 % glycerol, 0.1 % bromophenol blue) in the ratio 1:1, and the concentration of protein solutions were measured using a BCA kit and a microplate reader. Total protein equivalents (25–30 µg) for each sample were separated by SDS-polyacrylamide gel electrophoresis (PAGE) by using 10 % acrylamide gels and were transferred onto nitrocellulose membrane in a semi-dry transfer system. The membrane was immediately placed into blocking buffer containing 5 % non-fat milk in 20 mM Tris, pH 7.5; 150 mM NaCl and 0.01 % Tween-20. The blot was allowed to block at 37 °C for

1 h. The membrane was incubated with rabbit polyclonal antibody (1:1,000) for 2 h at 25–27 °C, followed by incubation in an anti-rabbit horseradish peroxidase-conjugated antibody (1:4,000). After washing with T-TBS, the membranes were incubated with ECL Plus detection reagents (Amersham Life Science Inc., Buckinghamshire, UK), exposed to X-ray film. The films were scanned with densitometer (BioRad GS 800) to determine the relative optical densities. Pre-stained protein markers were used for molecular weight determinations. As an internal control, the expression of β -actin was also determined simultaneously by re-blotting the membranes with the antibody for β -actin (1:5,000) (Santa Cruz, CA). Expression levels were then normalized to the young control animals (controls were set to 100 %).

RNA isolation and RT-PCR

RNA was isolated from dentate gyrus samples of female rats using the TRI Reagent Kit (Molecular Research Center, Inc., Cincinnati, OH), following the manufacturer's protocol. The purity of the RNA was estimated by 1.5 % agarose gel electrophoresis, and RNA concentration was determined by spectrophotometry (260 nm). Reverse transcription of 2 μ g RNA for complementary DNA (cDNA) synthesis was performed using the Reverse Transcription System (Promega, Madison, WI, USA) and a pd(N)6 random hexamer. Real-time (RT)-PCR was performed in an Applied Biosystems 7300 apparatus using the SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and 300 nM concentrations of specific primers (Table 1). The thermocycling profile conditions used were 50 °C for 2 m, 95 °C for 10 m, 95 °C for 15 s, 60 °C for 1 m, 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. For the normalization of cDNA loading in the PCR reaction, the amplification the 18S rRNA for every sample was used. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method. Relative differences between groups were expressed as relative increases or decreases, with the young group set as 1.00.

Statistical analyses

The results were statistically analyzed with the ANOVA method, with a confidence level of 95 % (p 0.05) that was considered significant. Results were expressed as the mean $\pm$ SEM. Mean comparison was done by the

Table 1 Primers used in real-time PCR experiments

Primers	Sequence (5'-3')
18S	Forward GGTGCATGGCCGTTCTTA Reverse TCGTTCGTTATCGGAATTAACC
TNF α	Forward ATGAGAAGTTCCTCAAAATGGC Reverse CTCCACTTGGTGGTTTGCTA
IL1 β	Forward TGTGATGAAAGACGGCACAC Reverse CTTCTTCTTTGGGTATTGTTTGG
Bcl2	Forward CAGGTATGCACCCAGAGTGA Reverse GTCTCTGAAGACGCTGCTCA
Bad	Forward GCCCTAGGCTTGAGGAAGTC Reverse CAAACTCTGGGATCTGGAACA
Bax	Forward GTGAGCGGCTGCTGTCT Reverse GGTCCCGAAGTAGGAGAGGA
AIF	Forward AGTCCTATTGTGGGCTTATCAAC Reverse TTGGTCTTCTTAATAGTCTTGTAGGC
iNOS	Forward CTTTGCCACGGACGAGAC Reverse TCATTGTACTCTGAGGGCTGAC
nNOS	Forward AGATGAGGCACCCCAACTC Reverse CCTTTACGGGGAAGAAACG
eNOS	Forward CCAGTGCCCTGCTTCATC Reverse GCAGGGCAAGTTAGGATCAG
NF κ B1	Forward CAGCTCTTCTCAAAGCAGCA Reverse TCCAGGTCATAGAGAGGCTCA
NF κ B2	Forward TGGAACAGCCCAAACAGC Reverse CACCTGGCAAACCTCCAT
GFAP	Forward ACAGACTTTCTCCAACCTCCAG Reverse CCTTCTGACACGGATTGTTGGT
HO-1	Forward GTCAAGCACAGGGTGACAGA Reverse ATCACCTGCAGCTCCTCAAA
HO-2	Forward TACGGCACCAGAAAAGGAAA Reverse GTGCTTCCTTGGTCCCTTC
Sirtuin 1	Forward TCGTGGAGACATTTTAAATCAGG Reverse GCTTCATGATGGCAAGTGG

18S was used as a housekeeping gene to compare the samples

ANOVA analysis of variance followed by a Fisher's test.

Results

The gene expressions of TNF α and IL1 β were found to be increased in the dentate gyrus of old females as compared with young ones, and this elevation was more evident in ovariectomized animals. Treatments with

oestradiol and melatonin were able to downregulate the messenger RNA (mRNA) expression of these pro-inflammatory cytokines in the group of castrated old females (Fig. 1). As shown on Table 2, the levels of pro-inflammatory cytokines $\text{TNF}\alpha$, $\text{IL1}\beta$ and IL6 were also significantly elevated during ageing and ovariectomy. No statistically significant differences were observed between $\text{TNF}\alpha$ levels of intact and castrated old female rats. However, significant increases in $\text{IL1}\beta$ and IL6 levels in ovariectomized versus the old intact group were observed. Administration of oestradiol and melatonin to the ovariectomized groups significantly lowered the levels of $\text{TNF}\alpha$, $\text{IL1}\beta$ and IL6 (Table 2).

The dentate gyrus of aged rats showed an increase in mRNA expression of glial fibrillary acidic protein (GFAP) as compared with young females. Ovariectomy was able to significantly further increase the expression of this parameter. These changes were significantly blunted by melatonin and oestradiol

Fig. 1 Expression of mRNA $\text{TNF}\alpha$ and $\text{IL1}\beta$ in the dentate gyrus of young, old intact and ovariectomized female rats and effect of administration of melatonin and oestrogens. Data represent mean $\pm$ SEM. * $p < 0.01$ compared with 2 months, $\Delta p < 0.001$ compared with 2 months, ** $p < 0.01$ compared with 24 months, # $p < 0.05$, ## $p < 0.01$ compared with ovariectomized. Two-way ANOVA analysis for $\text{TNF}\alpha$ ($F = 13.2$, $p = 0.0001$ for age; $F = 12.48$, $p = 0.0014$ for treatments), for $\text{IL1}\beta$ ($F = 13.62$, $p = 0.0001$ for age; $F = 15.04$, $p = 0.0006$ for treatments)

treatments, and the expression of GFAP mRNA was also reduced (Fig. 2).

Ageing significantly increased inducible nitric oxide synthase (iNOS) gene expression, and this increase was more marked in ovariectomized animals as compared to both intact old females and young controls. The enhanced expression of iNOS was significantly attenuated after oestradiol and melatonin replacement in the group of old castrated females (Fig. 3). However, mRNA expression of neuronal nitric oxide synthase (nNOS) did not change in the dentate gyrus during ageing and following long-term ovarian hormone depletion. Melatonin was able to significantly increase mRNA expression of nNOS in the group of ovariectomized females, but oestrogen treatment did not produce any effect on this parameter (Fig. 3). An elevation of the heme oxygenase-1 (HO-1) gene expression was induced by ageing and ovariectomy and was extremely high in the group of castrated animals as compared with young rats. Oestradiol and melatonin replacement reversed these enhancements in ovariectomized rats (Fig. 4). Expression of the constitutive enzymes HO-2 and endothelial NOS did not differ significantly between intact and ovariectomized females (data not presented).

HSP 70 levels were decreased in the dentate gyrus of intact females and ovariectomized rats as compared with young ones. When ovariectomized old females were treated with oestradiol or melatonin, the levels of HSP 70 were significantly increased as compared with untreated rats, with melatonin showing a more marked effect (Fig. 5).

The mRNA expression of $\text{NF}\kappa\text{B1}$ (p105/50) was increased in the dentate gyrus of 24-month-old animals as compared to 2-month female rats, but this was not the case in $\text{NF}\kappa\text{B2}$ (p100/52). Ovariectomy was able to further increase significantly the expression of both $\text{NF}\kappa\text{B1}$ and $\text{NF}\kappa\text{B2}$ genes as compared with intact old and with young animals. Following melatonin and oestradiol administration, a significant reduction in the expressions was observed (Fig. 6).

Ageing induced a significant elevation in the expression of $\text{NF}\kappa\text{B}$ p65 protein, and ovariectomy showed an additional increase. Treatment with oestradiol and melatonin significantly decreased this parameter in old castrated females (Fig. 7). Expression of $\text{NF}\kappa\text{B}$ p105 subunits did not change in the groups of intact and ovariectomized old females as compared with the young controls (data not presented). Expression of $\text{NF}\kappa\text{B}$ p50 was found to be increased in the dentate gyrus of

Table 2 Effect of ovariectomy and treatments with oestrogens and melatonin on the levels of pro-inflammatory cytokines in dentate gyrus of female rats

Cytokines pg/mg of proteins	Young (2 month)	Old intact (24 month)	Ovariectomized animals (24 month)		
			Without treatment	+EOS	+MEL
TNF α	8.6 $\pm$ 0.5	13.9 $\pm$ 1.4 ^a	14.1 $\pm$ 1.7 ^a	9.3 $\pm$ 0.7 ^d	7.7 $\pm$ 0.5 ^e
IL1 β	63.7 $\pm$ 5.9	120.8 $\pm$ 7.7 ^b	161.2 $\pm$ 8.2 ^{b,c}	89.5 $\pm$ 10 ^e	93.4 $\pm$ 13 ^e
IL6	20.6 $\pm$ 2.9	33.9 $\pm$ 4.2 ^a	44.5 $\pm$ 0.9 ^{b,c}	34.1 $\pm$ 3.5 ^e	37.6 $\pm$ 1.7 ^d

^a p 0.05, ^b p 0.01 compared with 2 months, ^c p 0.05 compared with 24 months (intact), ^d p 0.05, ^e p 0.01 compared with ovariectomized

ovariectomized old females, whereas this parameter did not show any change in the group of intact females as compared with the young controls. Treatment with oestradiol and melatonin to ovariectomized females was able to significantly reduce the NF κ B p50 protein expression values in the dentate gyrus (Fig. 7). Expressions of I κ B α and I κ B β proteins were dramatically decreased in the dentate gyrus of ovariectomized females as compared with young and old intact animals (Fig. 8). In the group of old intact females, we observed only a significantly decreased expression of I κ B α protein but not of I κ B β as compared with young animals. When old ovariectomized animals were treated with melatonin, a significant upregulation of both proteins could be observed. However, treatment with oestradiol to old ovariectomized females was only able to increase I κ B α but not I κ B β protein in the dentate gyrus (Fig. 8).

Western blotting analyses demonstrated activation of p38 MAPK in the dentate gyrus of aged intact and ovariectomized females. So, the ratio phospho-p38/p38 MAPK was higher in the groups of intact and castrated old female rats as compared with young animals. After chronic treatment with oestradiol and melatonin, a very marked decrease in the ratio phospho-p38/p38 MAPK was detected (Fig. 9).

The gene expressions of pro-apoptotic markers like Bcl-2-associated X protein (Bax) and Bcl-2-associated death promoter (Bad) were significantly upregulated in the dentate gyrus of old ovariectomized females (Fig. 10). In the group of old intact females, we could observe only an increase in the expression of Bad mRNA but not in Bax as compared with young animals. Treatment with oestradiol blocked Bad and Bax induction in the dentate gyrus of ovariectomized females (Fig. 10). However, melatonin administration was only able to downregulate the expression of Bad genes but did not affect the mRNA expression of Bax (Fig. 10). A

significant upregulation of apoptosis inducing factor (AIF) was also observed in the intact old and ovariectomized animals, and both oestradiol or melatonin therapy led to a significant downregulation of this parameter (Fig. 12). In the case of Bcl-2, we were not able to demonstrate any significant differences in its expression between young, intact old and ovariectomized females (Fig. 11). However, treatments with melatonin and oestradiol were able to significantly upregulate the expression of Bcl-2 in the group of ovariectomized animals (Fig. 11).

In addition, we also demonstrated that the expression of sirtuin 1 (SIRT1) was dramatically decreased in the dentate gyrus of old intact and castrated rats and that treatments with oestradiol and melatonin were able to significantly increase this parameter (Fig. 13). MAP-2 immunoreactivity was not influenced by ageing but was significantly decreased in the group of ovariectomized

Fig. 2 Expression of mRNA GFAP in the dentate gyrus of young, old intact and ovariectomized female rats and effect of administration of melatonin and oestrogens. Data represent mean $\pm$ SEM. ** p <0.01 compared with 2 months, Δ p <0.001 compared with 2 months, ** p <0.01 compared with 24 months, # p <0.05, ## p <0.01 compared with ovariectomized. Two-way ANOVA analysis for GFAP (F =12.99, p =0.0001 for age; F =12.18, p =0.0016 for treatments)

Fig. 3 Expression of mRNA iNOS and nNOS in the dentate gyrus of young, old intact and ovariectomized female rats and effect of administration of melatonin and oestrogens. Data represent mean±SEM. $\Delta p < 0.001$ compared with 2 months, $## p < 0.01$ compared with ovariectomized. Two-way ANOVA analysis for iNOS ($F = 10.47$, $p = 0.0005$ for age; $F = 15.7$, $p = 0.0005$ for treatments), for nNOS ($F = 0.87$, $p = 0.4303$ for age; $F = 4.18$, $p = 0.0501$ for treatments)

females. The concentration of synapsin I declined with age and was more markedly decreased in ovariectomized animals (Fig. 14). The expression of MAP-2 was significantly upregulated after oestrogenic

Fig. 4 mRNA expression of HO-1 in the dentate gyrus of young, old intact and ovariectomized female rats and effect of administration of melatonin and oestrogens. Data represent mean±SEM. $**p < 0.01$ compared with 2 months, $\Delta p < 0.001$ compared with 2 months, $**p < 0.01$ compared with 24 months, $##p < 0.01$ compared with ovariectomized. Two-way ANOVA analysis for HO-1 ($F = 13.69$, $p = 0.0001$ for age; $F = 16.27$, $p = 0.0003$ for treatments)

Fig. 5 Level of HSP 70 in the dentate gyrus of young, old intact and ovariectomized female rats and effect of administration of melatonin and oestrogens. Data represent mean±SEM. $*p < 0.05$, $**p < 0.01$ compared with 2 months, $#p < 0.05$, $##p < 0.01$ compared with ovariectomized. Two-way ANOVA analysis for HSP 70 ($F = 1.83$, $p = 0.182$ for age; $F = 14.64$, $p = 0.0008$ for treatments)

replacement, but melatonin did not produce any effect on this parameter in the group of ovariectomized rats. Synapsin I was significantly increased in the group of

Fig. 6 Expression of mRNA NFκB 1 (p105/50) and NFκB 2 (p100/52) in the dentate gyrus of young, old intact and ovariectomized female rats and effect of administration of melatonin and oestrogens. Data represent mean±SEM. $**p < 0.01$ compared with 2 months, $\Delta p < 0.001$ compared with 2 months, $*p < 0.05$ compared with 24 months, $##p < 0.01$ compared with ovariectomized. Two-way ANOVA analysis for NFκB1 ($F = 13.47$, $p = 0.0001$ for age; $F = 15.7$, $p = 0.0005$ for treatments), for NFκB2 ($F = 13.71$, $p = 0.0001$ for age; $F = 20.27$, $p = 0.0001$ for treatments)

Fig. 7 NFκB p65 and NFκB p50 expressions in the dentate gyrus of young, old intact and ovariectomized female's and effect of administration of melatonin and oestrogens. The *upper part* of figures illustrates expression of NFκB p65 and NFκB p50 by representative Western blots from each group compared to β-actin. The *columns* show the mean±SEM from each group. ** $p<0.01$ compared with 2 months, $\Delta p<0.001$ compared with 2 months, ** $p<0.01$ compared with 24 months, # $p<0.05$, ## $p<0.01$ compared with ovariectomized

castrated females that received oestrogenic and melatonin treatments (Fig. 14).

Discussion

In this study, we have investigated the possibility that oestrogen and melatonin may reduce in a similar way neuroinflammation and apoptosis and its molecular signalling pathways, which may, in turn, prevent neuronal loss and/or connectivity, during ageing and long-term

Fig. 8 IκBα and IκBβ expressions in the dentate gyrus of young, old intact and ovariectomized female's and effect of administration of melatonin and oestrogens. The *upper part* of figures illustrates expression of IκBα and IκBβ by representative Western blots from each group compared to β-actin. The *columns* show the mean±SEM from each group. ** $p<0.01$ compared with 2 months, $\Delta p<0.001$ compared with 2 months, ** $p<0.01$ compared with 24 months, # $p<0.05$, ## $p<0.01$ compared with ovariectomized

ovarian hormone depletion in the dentate gyrus of female rats.

The levels of pro-inflammatory cytokines and the gene expressions were increased in the dentate gyrus of old females as compared with young animals. These data confirm previous studies, which showed that ageing led to increases in IL1β, TNFα, IL6, MHCII, CD68, and CD11b mRNA in hippocampal tissue (Murphy et al. 2012). As we had previously demonstrated, ageing by itself was able to induce alterations in hippocampus, but when females with long-term oestrogen deprivation

Fig. 9 Intensities of p38 MAPK phosphorylation in the dentate gyrus of young, old intact and ovariectomized female rats and effect of administration of melatonin and oestrogens are presented. The intensity of phosphorylation is expressed by the ratio of p-MAPK/MAPK (see also the representative Western blots above the columns). ** $p < 0.01$ compared with 2 months, $\Delta p < 0.001$ compared with 2 months, ** $p < 0.01$ compared with 24 months, # $p < 0.05$, ## $p < 0.01$ compared with ovariectomized

Fig. 10 Expression of mRNA Bax and Bad in the dentate gyrus of young, old intact and ovariectomized female rats and effect of administration of melatonin and oestrogens. Data represent mean $\pm$ SEM. $\Delta p < 0.01$ compared with 2 months, * $p < 0.05$ compared with 24 months, # $p < 0.05$, ## $p < 0.01$ compared with ovariectomized. Two-way ANOVA analysis for Bax ($F = 9.7$, $p = 0.0008$ for age; $F = 3.29$, $p = 0.0818$ for treatments), for Bad ($F = 16.40$, $p = 0.00001$ for age; $F = 28.37$, $p = 0.00001$ for treatments)

Fig. 11 mRNA expression of Bcl-2 in the dentate gyrus of young, old intact and ovariectomized female rats and effect of administration of melatonin and oestrogens. Data represent mean $\pm$ SEM. # $p < 0.05$; ## $p < 0.01$ compared with ovariectomized. Two-way ANOVA analysis for Bcl-2 ($F = 0.51$; $p = 0.6078$ for age; $F = 17.32$; $p = 0.0003$ for treatments)

were investigated, the damages observed were due to the combination of ovariectomy and ageing combined and could be regarded as a model for menopause. The present study demonstrates also that long-term ovariectomy further negatively affects the already altered neuroinflammatory status of the dentate gyrus in old intact female rats. So, castration induced additional significant increases of IL1 β and TNF α gene expression and in IL6 and IL1 β levels as compared to old intact animals. Other authors have also demonstrated that ovariectomy was able to further increase the already enhanced age-dependent accumulation of mRNAs encoding inflammatory mediators (TNF α , IL1 β and macrophage inflammatory protein-2) and to induce changes in the morphology of astroglia and microglia (Benedusi et al. 2012). Santizo and Pelligrino (1999) reported that chronic oestrogen depletion did enhance

Fig. 12 mRNA expression of AIF in the dentate gyrus of young, old intact and ovariectomized female rats and effect of administration of melatonin and oestrogens. Data represent mean $\pm$ SEM. $\Delta p < 0.01$ compared with 2 months, # $p < 0.05$, ## $p < 0.01$ compared with ovariectomized. Two-way ANOVA analysis for AIF ($F = 4.87$, $p = 0.0153$ for age; $F = 9.57$, $p = 0.0045$ for treatments)

Fig. 13 mRNA expression of SIRT1 in the dentate gyrus of young, old intact and ovariectomized female rats and effect of administration of melatonin and oestrogens. Data represent mean $\pm$ SEM. $**p < 0.01$ compared with 2 months, $\Delta p < 0.001$ compared with 2 months, $**p < 0.01$ compared with 24 months, $\#p < 0.05$, $##p < 0.01$ compared with ovariectomized. Two-way ANOVA analysis for SIRT1 ($F = 10.19$, $p = 0.0004$ for age; $F = 33.66$, $p = 0.00001$ for treatments)

leucocyte adhesion in rat cerebral circulation, and this has been suggested to underlie the more marked brain damage observed in ovariectomized females as compared to intact animals of the same age.

In our data, the administration of oestradiol or melatonin was accompanied by an improvement in the inflammatory status of ovariectomized female rats. As a result, oestradiol and also melatonin treatments were able to decrease pro-inflammatory cytokine (TNF α , IL1 β and IL6) levels and to induce also reductions in the gene expressions of TNF α and IL1 β . In different experimental models, other authors have also demonstrated protective effects of melatonin and oestrogen treatments. So, melatonin reduced in a concentration-dependent manner IL6 secretion in amyloid β peptide-treated brain slices (Clapp-Lilly et al. 2001). Administration of melatonin in doses of 5 and 10 mg/kg were able to decrease lipopolysaccharide (LPS)-induced pro-inflammatory cytokines (TNF α , IL1 β) and oxidative stress in different brain regions, including the hippocampus (Tyagi et al. 2010). Choi et al. (2008) reported that oestrogen-induced protection was associated with a decrease in IL1 β and an increase in interleukin-1 receptor antagonist (IL1ra) expression in the ischemic hippocampus during early reperfusion periods, which suggests that the modulation of IL1 β /IL1ra might be part of the anti-inflammatory effects of oestrogens. Oestrogen were also able to increase IL-10 levels while decreasing TNF α and IFN γ release from both basal and LPS-stimulated N9 (murine microglial) cells (Dimayuga et al. 2005).

Fig. 14 MAP-2 and synapsin-I expressions in the dentate gyrus of young, old intact and ovariectomized female's and effect of administration of melatonin and oestrogens. The upper part of figures illustrates expression of MAP-2 and synapsin-I by representative Western blots from each group compared to β -actin. The columns show the mean $\pm$ SEM from each group. $**p < 0.01$ compared with 2 months, $\Delta p < 0.001$ compared with 2 months, $**p < 0.01$ compared with 24 months, $\#p < 0.05$ compared with ovariectomized

Like microglia, astrocytes become activated with age—a process known as 'astrogliosis', which is characterized by altered gene expression, increased expression of gliosis marker molecules (i.e. GFAP and vimentin) and also by hypertrophy and proliferation (Ridet et al. 1997) of this tissue. Activated astrocytes release also a wide array of immune mediators such as cytokines, chemokines, and growth factors, contributing either to the neuroprotective or to the neurotoxic effects raised by microglia (Farinas et al. 2007). In the current study, the activated astrocytes were identified by the enhanced expression of GFAP and were also

demonstrated by the positive correlations between this marker, the ageing process and ovarian hormone depletion. It has been also shown that with increasing age, the staining for GFAP showed a rise (e.g. labelling for astrocytes), and age-related enhancement in GFAP mRNA content has also been demonstrated in the mouse brain (Hayakawa et al. 2007). Previous studies have shown that pharmacological doses of oestrogens can suppress ageing-associated reactive gliosis in 20 to 24-month-old mice (Lei et al. 2003). Interestingly, oestradiol significantly was able to suppress GFAP expression by 49 days following ovariectomy but not during the period between 5 and 28 days (McAsey et al. 2006) after castration. The present results showed that the pharmacological administration of melatonin and oestradiol for 10 weeks prevented the elevation of GFAP content in the dentate gyrus of old chronically ovariectomized female rats.

Cytokines released from microglia and astrocytes activated MAPKs and NF κ B (McCoy and Tansey 2008), and these pathways were important in the production of many inflammatory proteins (O'Neill 2006) and were able to increase NADPH oxidase activation (Kozuka et al. 2005). Our data have shown for the first time that ageing together with long-term ovarian hormone depletion induced phosphorylation of p38 MAPK in the dentate gyrus. On the other hand, we have also demonstrated that mRNA expression of NF κ B 1 and 2 and levels of p50 and p65 proteins were also increased in the dentate gyrus of ovariectomized females when compared with old intact animals. Elevated levels of NF κ B were accompanied with low expression of I κ B α and I κ B β proteins. These data closely correlated with high levels of pro-inflammatory cytokines in the group of ovariectomized animals. Upstream MAPK signalling mediated both the transcriptional and post-transcriptional regulation of iNOS in activated microglia and astrocytes (Bhat et al. 1998; Marcus et al. 2003). On the other side, NF κ B responded to p38 signalling and was also involved in iNOS induction (Bhat et al. 2002), suggesting that there was an interplay between signalling pathways, transcription factors and cytokine production in determining the neuroinflammatory response of the CNS. Oestrogen receptor (ER) and NF κ B family members have been shown to influence each other's transcriptional activity. Much work has been done to delineate the multiple mechanisms by which ER could repress NF κ B action to exert an anti-inflammatory effect (Kalaitzidis and Gilmore 2005; Cerciati et al. 2010).

Similarly, there were several pieces of evidence indicating that NF κ B could repress ER expression and transcriptional activity (Feldman et al. 2007; Mahmoodzadeh et al. 2009). In the present investigation, administration of oestradiol and melatonin to ovariectomized females significantly abolished iNOS expression, by decreasing expression of NF κ B genes and proteins and by inhibiting the p38 mitogen-activated kinase. In an animal model of transient cerebral ischemia, administration of pharmacological doses of oestrogens produced strong neuroprotective effects by reducing the negative elements associated with inflammatory responses, namely I κ B phosphorylation, NF κ B activation and iNOS over-expression in the cortex during the reperfusion phase (Wen et al. 2004). In vitro studies showed that pre-treatment with oestradiol was able to prevent neuronal death by inhibiting the activation of p38 MAPK after the treatment of cells with A β (Valles et al. 2008). Eijo et al. (2011) described that oestradiol was able to decrease TNF α -induced, NF κ B p65 and p50 nuclear translocation in primary cultures of anterior pituitary cells from ovariectomized rats. Inhibition of NF κ B by melatonin has been also previously reported in other experimental models that showed how melatonin prevented NF κ B activation by oxidative stress (Veneroso et al. 2009). Previous studies also showed that melatonin inhibited phosphorylation of p38 MAPK and to a lesser extent of ERK1/2 thus reducing iNOS mRNA expression and rescuing neurons from apoptosis after acute traumatic SCI (Xu et al. 2006).

During ageing (Rastogi et al. 2012) and ovariectomy (Martins et al. 2012), the enhanced generation of ROS induced chronic oxidative stress in the brain. This condition caused gradual accumulation of damaged proteins leading to a functional decline in the brain's endogenous defence system (Rumora et al. 2007; Min et al. 2008) and leading to the activation of stress kinases (JNK and p38 MAPK) (Gabai et al. 1997). Hou et al. (2010) demonstrated that oestrogen deficiency induced by ovariectomy was able to exacerbate the impairment of heat stress-induced brain HSP 70 expression in female mice during ageing and resulted in reduced thermotolerance. Oestrogen supplementation restored HSP 70 expression by increasing the binding activity of heat shock transcriptional factor (HSF1) and heat shock element (HSE) and subsequently enhanced thermotolerance thus attenuating heat stress-induced brain damage. Our results clearly indicated that levels of HSP 70 were

decreased in the dentate gyrus of old and ovariectomized female rats and that the administrations of oestrogens or melatonin were able to increase its levels. Previous studies also suggested that oestrogen could regulate the expression of heat shock proteins in neuronal cells (Manthey and Behl 2006) and oestrogen deficiency might accelerate brain ageing in (postmenopausal) oestrogen-deprived or castrated animals (Sherwin and Henry 2008; Shuster et al. 2008). Since ageing and menopause are accompanied with increased oxidative stress, induction of HO-1 may have a compensatory action caused by its antioxidant feature. On the other hand, free iron and CO released during heme degradation might, under certain circumstances, perpetuate intracellular oxidative stress and predispose the mitochondrial compartment to free radical damage (Zhang and Piantadosi 1992; Frankel et al. 2000). Interestingly, increased hippocampal astroglial HO-1 expression was associated with lower scores for global cognition, semantic memory and perceptual speed (Schipper et al. 2006). Our results clearly indicated that HO-1 expression was significantly increased in both old intact and castrated females, as compared to young animals, and these data correlated with a parallel increase in the level of pro-inflammatory cytokines and expression of iNOS. These findings are in consonance with the results obtained by Lee et al. (2005), in which HO-1 protein expression in heart was induced by ovariectomy and was extremely high 2–6 weeks after castration when compared with the sham-operated group. 17β -Oestradiol replacement after ovariectomy was able to reverse these changes in rats.

IL1 β and TNF α caused cell death by the activation of a signal transduction pathway leading to apoptosis in hippocampal and neocortical cells (Kajta et al. 2006). Cerbai et al. (2012) demonstrated that astrocytes and microglia in the hippocampus of aged and adult LPS-treated rats participated in the clearance of neuronal debris associated with programmed cell death and the phagocytosis of apoptotic neurons. We have demonstrated that Bcl-2 gene did not change its expression in the dentate gyrus of female Wistar rats, neither during ageing nor with ovariectomy. However, the Bax/Bcl-2 ratio was significantly higher in the group of ovariectomized animals as compared with intact ones, indicating a higher level of apoptosis. Oestradiol and melatonin administrations were able to increase the expression of Bcl2 and decreased pro-apoptotic markers like Bad and/or Bax. Results obtained in our research were in

accordance with data demonstrating that chronic administration of E2 prevented the ovariectomy-induced downregulation of Bcl-2 and upregulation of Bax expression while restoring the Bcl-2/Bax ratio as observed in the hippocampus of intact rats. Furthermore, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay demonstrated a decline in the percentage of TUNEL positive cells in E2-treated groups (Sharma and Mehra 2008). Sales et al. (2010) also found that ovariectomy decreased Bcl-2 expression and increased Bax expression and the also the number of apoptotic cells. Replacement with 17β -oestradiol (21 days) throughout the post-ovariectomy period reduced the number of apoptotic cells to control levels and prevented the enhancing effects of ovariectomy on Bax expression but only partially restored Bcl-2 expression. It has been shown that oestradiol reduced mRNA levels of the pro-apoptotic Bad in PC12 cells transfected with ER α (Gollapudi and Oblinger 1999). Nopparat et al. (2010) have demonstrated, in the SK-N-SH dopaminergic cell line, a novel role of melatonin in protecting cells from autophagic cell death triggered by the Bcl-2/Beclin 1 pathway, by inhibiting the activation of the JNK1 (c-Jun amino-terminal kinase), Bcl-2 upstream pathway. Melatonin increased Bcl-2 and augmented expression of XIAP in ethanol-treated HN2-5 (mouse hippocampal neuron-derived) cells (Shetha et al. 2009). Our results also demonstrated an increase in the mRNA expression of AIF in the dentate gyrus of old and ovariectomized rats, and this could suggest that this brain area was following a different apoptotic pathway. In addition, we have also observed that treatment with melatonin or oestrogens was able to decrease mRNA expression of AIF. Our data were in agreement with previous reports in which melatonin was able to prevent the insult-related release of AIF from mitochondria (Liang et al. 2012). Bethea et al. (Bethea et al. 2009) showed that AIF gene expression was significantly decreased by hormone therapy (E and E+P-treated animals) in laser-captured serotonergic neurons.

One of the most important changes that we have observed for the first time is that ageing and more markedly ovariectomy decreased the expression of SIRT1 in the dentate gyrus. Previous results have shown that SIRT1 is associated with a reduction in apoptosis and could regulate other pathways. So, Kolthur-Seetharam et al. (2006) showed that SIRT1 modulates poly(ADP-ribose)polymerase-1 (PARP-1) activity following DNA damage. Indeed, these authors showed that

SIRT1-null cells exposed to H₂O₂ undergo AIF-mediated programmed cell death due to a dysregulation in PARP-1 expression. Yeung et al. (2004) demonstrated that SIRT1 can physically interact with the p65/RelA protein in the NFκB complex, specifically to deacetylate the lysine-310 of p65 protein. The cleavage and removal of the acetyl group thereafter inhibited the transactivation efficiency of the NFκB-dependent complex since acetylated lysine-310 is a powerful promoter of its transactivation. The absence of SIRT1 also impaired cognitive abilities, including immediate memory, classical conditioning, and spatial learning. Michan et al. (2010) found that the cognitive deficits in SIRT1 knock-out (KO) mice were associated with defects in synaptic plasticity without alterations in basal synaptic transmission or NMDA receptor function.

The expression of SIRT1 gene was significantly up-regulated after oestrogen or melatonin replacement therapy in the dentate gyrus of ovariectomized females. In addition, we also observed that both melatonin and oestradiol induced an enhancement of protein levels of MAP-2 and synapsin I that might have been partially responsible for the improved learning and memory performance in ageing rats, since they were end-stage elements in synaptic plasticity.

Summing up, our study indicated that long-term ovarian hormone depletion and ageing induced a neuroinflammatory process (with increased levels of pro-inflammatory cytokines, and enhanced expression of iNOS, HO-1) in the dentate gyrus. Increased production of pro-inflammatory cytokines is associated with the activation of stress kinases and NFκB. Using ovariectomy model in old rats, we have found that reduced levels of synaptic plasticity markers (synapsin I and MAP-2) in the dentate gyrus were associated with low levels of SIRT1 expression and high levels of neuroinflammation. Treatments with oestradiol or melatonin have been shown to modulate the pro/anti-apoptotic ratio favouring the second and to increase SIRT1 expression. Other neuroprotective effects after both melatonin and oestradiol administrations were clearly demonstrated by the normalization of the levels of pro-inflammatory cytokines and by the decrease of p38 MAPK phosphorylation and gene and protein expressions of NFκB. Oestradiol or melatonin treatments were also able to increase the expression of synapsin I and MAP-2 in dentate gyrus of old ovariectomized females. Since these proteins might have been an index of synaptic integrity, this implied that melatonin might be

beneficial in the preservation of synaptic performance during senescence and that melatonin might also have applications in the clinical practice for menopausal women.

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Conflict of interest The authors declare no conflict of interest.

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