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17α-Oestradiol-Induced Neuroprotection in the Brain of Spontaneously Hypertensive Rats

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17β-oestradiol is a powerful neuroprotective factor for the brain abnormalities of spontaneously hypertensive rats (SHR). 17α-Oestradiol, a nonfeminising isomer showing low affinity for oestrogen receptors, is also endowed with neuroprotective effects *in vivo* and *in vitro*. We therefore investigated whether treatment with 17α-oestradiol prevented pathological changes of the hippocampus and hypothalamus of SHR. We used 20-week-old male SHR with a blood pressure of approximately 170 mmHg receiving s.c. a single 800 μg pellet of 17α-oestradiol dissolved in cholesterol or vehicle only for 2 weeks Normotensive Wistar–Kyoto (WKY) rats were used as controls. 17α-Oestradiol did not modify blood pressure, serum prolactin, 17β-oestradiol levels or the weight of the testis and pituitary of SHR. In the brain, we analysed steroid effects on hippocampus Ki67+ proliferating cells, doublecortin (DCX) positive neuroblasts, glial fibrillary acidic protein (GFAP)+ astrocyte density, aromatase immunostaining and brain-derived neurotrophic factor (BDNF) mRNA. In the hypothalamus, we determined arginine vasopressin (AVP) mRNA. Treatment of SHR with 17α-oestradiol enhanced the number of Ki67+ in the subgranular zone and DCX+ cells in the inner granule cell layer of the dentate gyrus, increased BDNF mRNA in the CA1 region and gyrus dentatus, decreased GFAP+ astrogliosis in the CA1 subfield, and decreased hypothalamic AVP mRNA. Aromatase expression was unmodified. By contrast to SHR, normotensive WKY rats were unresponsive to 17α-oestradiol. These data indicate a role for 17α-oestradiol as a protective factor for the treatment of hypertensive encephalopathy. Furthermore, 17α-oestradiol is weakly oestrogenic in the periphery and can be used in males.

Key words: oestrogens, hippocampus, hypothalamus, hypertension

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The neuroprotective role of 17β-oestradiol has been increasingly recognised. At the cellular level, 17β-oestradiol increases neurogenesis, neuronal survival, and neurite outgrowth; promotes synaptogenesis and dendritic spine formation; stimulates growth factor expression; prevents excitotoxicity; modulates inflammatory responses; enhances mitochondrial dynamics; and regulates glial cell function and myelin formation (1–10). These effects are mediated by intracellular oestrogen receptors (OER) or by a variety of cell membrane effects including the prevention of oxidative damage (6,10–12).

However, adverse effects of 17β-oestradiol have been described (13). Unwanted effects can be minimised by employing selective oestrogen receptor modulators (SERMS) (14) or 17α-oestradiol, a nonfeminising isomer which, similar to SERMS, shows low affinity for OER (15). 17α-Oestradiol neuroprotection may be a result of the prevention of lipid peroxidation and oxidative damage (16–20). The beneficial effects of this isomer have been described in human neuroblastoma cells (21), muscimol and NMDA-induced toxicity of neuronal cultures (22,23), PC12 pheochromocytoma cells (17), dopaminergic neurones and spinal motoneurones exposed to toxic factors (24). *In vivo* protective effects of 17α-oestradiol have been shown in Alzheimer's disease mice (15,16), the hippocampus of oestrogen-depleted rats (25) and after brain ischaemia (26). 17α-Oestradiol and natural oestrogens also increase cell proliferation (neurogenesis) in the dentate gyrus of ovariectomised rats by a nonclassical-receptor mechanism (27).

We speculated that the encephalopathy of spontaneously hypertensive rats (SHR) could be a suitable model for studying 17α-oestradiol neuroprotection, considering that neuronal loss, astrogliosis,

blood–brain barrier disruption, low neurotrophic factor expression, decreased forebrain white matter and changes of hippocampal neurogenesis occur in this strain (28–31). Further evidence of degenerative changes in the brain of SHR include necrosis and apoptosis in the hippocampus (30) and atrophy of dendrites and a low spine number in the CA1 hippocampal region (32). In the hypothalamus, SHR show increased expression of the hypertensinogenic peptide arginine vasopressin (AVP) and its V1b receptor (33). We have already shown that treatment of SHR with 17β-oestradiol decreases blood pressure and precludes abnormalities of the hippocampus and hypothalamus (32–37). In the present study, we investigated whether: (i) 17α-oestradiol brings neuroprotection to SHR and (ii) blood pressure changes play a role in neuroprotection, considering that, although 17β-oestradiol elicits hypotension (31,38–40), the effects of 17α-oestradiol on blood pressure have not been reported.

Materials and methods

Experimental animals

Five-month-old male Wistar–Kyoto (WKY) rats and SHR (approximate body weight of 250 g), obtained from the Institute of Biology and Experimental Medicine animal facility, were used. Rats were kept under conditions of controlled humidity and temperature (22 °C) under a 12 : 12 h light/dark cycle (lights on 07.00 h) and were given food and water *ad lib*. Systolic mean blood pressure, as determined by an indirect tail-cuff method in conscious rats, demonstrated that all SHR were hypertensive (Table 1).

Animals were anaesthetised i.p. with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) and implanted s.c. with either a single 800-μg pellet of 17α-oestradiol (E8750 S; Sigma, St Louis, MO, USA) dissolved in cholesterol or a cholesterol pellet during the last 2 weeks of the experiment. 17α-Oestradiol-treated, cholesterol-implanted SHR and normotensive control WKY rats were used for immunocytochemistry and *in situ* hybridisation (ISH) procedures, as described below. After sacrifice, testicular and hypophysal weight was measured and blood was collected for prolactin and 17β-oestradiol determination by radioimmunoassay (Coat a Count RIA Kit; Diagnostic Products, Los Angeles, CA, USA).

Animal handling followed the Guide for the Care and Use of Laboratory Animals (NIH Guide, Instituto de Biología y Medicina Experimental Assurance Certificate # A5072-01) and was approved by our local Animal Care and Use Committee. Every effort was made to minimise animal suffering and to reduce the number of animals used to the minimum required for statistical accuracy.

Immunocytochemistry of Ki67, doublecortin (DCX), glial fibrillary acidic protein (GFAP) and aromatase

Rats anaesthetised with isofluorane under a fume hood were transcardially perfused with 60 ml of 0.9% saline followed by 60 ml of 3% paraformaldehyde (PFA) in phosphate buffer (pH 7.4). After overnight incubation in 3% PFA, brains were transferred to Tris-buffered saline (TBS) (pH 7.4), sectioned frontally at 50 μm using a vibrating microtome and processed for free-floating immunocytochemistry. Briefly, sections were first treated for 45 min with a solution of methanol (50%) and hydrogen peroxide (5%) in TBS to quench endogenous peroxidase activity. To enhance antigen exposition, sections were washed three times in TBS (pH 7.4) in a shaker and incubated in 0.01 M citrate buffer (pH 6.0) at 90 °C during 40 min . Sections were left to cool at room temperature (RT) and further incubated with methanol : H_2O_2 (100 : 1) in TBS for 15 min at RT. For single immunocytochemistry, sections were then incubated with: (i) a polyclonal anti-Ki67 antibody made in rabbit (dilution 1 : 2000; NovaCastra, Newcastle, UK) to label proliferating cells; (ii) a polyclonal DCX antibody made in goat (dilution 1 : 250, sc-8066; Santa Cruz Labs, Santa Cruz, CA, USA) for staining of neuronal progenitors; (iii) a polyclonal anti-GFAP antibody made in rabbit (dilution 1 : 1000, G-9269; Sigma) to label quiescent and reactive astrocytes; or (iv) a polyclonal anti-aromatase antibody made in rabbit (dilution 1 : 500; generated at the Instituto Cajal, Madrid, Spain) (41), diluted in 0.5% Triton X-100 TBS 1% goat or rabbit serum for 24 h at 4 °C. After incubation with primary antibody, sections were exposed to biotinylated goat anti-rabbit immunoglobulin (Ig)G or a rabbit anti-goat IgG (dilution 1 : 200; Vector, Burlingame, CA, USA) and processed in accordance with the manufacturer's instructions (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA). Immunoreactions were visualised with 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 0.01% hydrogen peroxide. Nonspecific staining was assessed by omission of the primary antibodies. Furthermore, the specificity of the commercial antibodies (Ki67, DCX and GFAP) has been tested by the vending companies, whereas the aromatase antibody was verified by Western blotting at the Madrid laboratory. To obtain consistency of immunostaining, sections from the different experimental groups were stained simultaneously and sections from different animals were included on the same slide. This procedure eliminated any conflicts that may arise when using different batches. Sections were analysed in a double-blinded manner by different investigators.

Every eight 60-μm coronal brain sections throughout the entire rostrocaudal extension of the dentate gyrus corresponding to plates 26–40 from the stereotaxic atlas of Paxinos and Watson (43) were analysed for each rat. Cells positive for Ki67 were counted in the subgranular zone (SGZ), defined as a two-nucleus-wide band in the limit between the hilus and the granular cell layer (GCL). DCX-immunoreactive neuroblasts were counted in the inner GCL of the dentate gyrus. Both upper and lower blades of the dentate gyrus were considered for cell counting. The total number of Ki67+ cells and

Table 1. Weight of Tissues, Levels of Serum 17β-Oestradiol and Prolactin and Mean Arterial Pressure of Normotensive Wistar–Kyoto (WKY) Rats and Spontaneously Hypertensive Rats (SHR) With and Without Treatment with 17 α-Oestradiol (αE_2).

	WKY	WKY + αE_2	SHR	SHR + αE_2
Body weight (g)	334.20 ± 12.10	343.0 ± 14.6	339.60 ± 9.14	319.50 ± 11.32
Testicular weight (g)	1.25 ± 0.03	1.24 ± 0.03	1.49 ± 0.04	1.51 ± 0.04
Hypofisal weight (mg)	13.80 ± 0.21	14.64 ± 0.19	13.80 ± 0.38	13.05 ± 0.69
Serum 17β-oestradiol (pg/ml)	16.26 ± 4.29	13.71 ± 2.23	12.40 ± 2.29	18.71 ± 1.52
Serum prolactin (ng/ml)	18.89 ± 3.81	24.85 ± 1.54	14.02 ± 4.62	23.87 ± 6.36
Mean arterial pressure (mmHg)	115.6 ± 4.00	122.5 ± 4.62	166.8 ± 4.96***	166.6 ± 5.08***

***P < 0.001 versus WKY and WKY + αE_2.

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DCX+ cells was estimated according to Howart and Reed (42), as previously used for neurogenesis in our laboratory (36). Thus, immunoreactive bodies filled with DAB product that came into focus and focusing down through the thickness of the section were considered for the present study. The number of KI67+ cells counted in the SGZ and the number of DCX+ cells in the GCL cell layer was multiplied by a factor of 8 to estimate the total number of proliferating cells or neuronal progenitors in the dentate gyrus because every eighth section was used to estimate the total number of DCX or Ki67 positive cells in the dentate gyrus. Sections corresponding to five animals were studied for each experimental group. Data are expressed as the mean ± SEM number of Ki67-labelled cells or DCX-labelled cells per dentate gyrus.

For quantitative analysis of GFAP+ astrocytes, a computer-assisted image analysis system (OPTIMAS BIOSCAN IV; Optimas Bioscan Inc., Edmonton, WA, USA) was used. For all measures, counts were generated automatically by the software, and the investigators who processed the cell counting were blind to the treatment. The number of GFAP positive cells was counted in a previously measured area and expressed as the number of GFAP+ cells per mm^2. Astrocytes were determined in six to nine anatomically matched sections in two areas of white matter: the stratum radiatum of the CA1 region and the hilus of the dentate gyrus. Data were expressed as the mean ± SEM number of labelled cells/unit area.

To determine aromatase staining intensity, we followed a previously published procedure (37). Aromatase immunoreaction was measured in the CA1 region and dentate gyrus by computer-assisted image analysis. Digitised images of tissue sections (six sections per rat, five rats in each group) were processed simultaneously under identical operating conditions, such as light beam, wavelength and grey-scale threshold throughout the experiment. Images were acquired at the same magnification using a digital camera (GP-KR222; Panasonic, Osaka, Japan) connected to an BH2 microscope (Olympus, Tokyo, Japan) and using BIOSCAN OPTIMAS VI image analysis software. Results are expressed as the total immunoreactive area (μm^2).

ISH for AVP mRNA

For ISH, isofluorane-anaesthetised rats were killed by decapitation and the brains were removed and frozen on dry ice. Cryostat sections were obtained at levels 1900–2000 μm containing the paraventricular nucleous (PVN) (43). AVP mRNA levels were determined by a non-isotopic EMBO (European Molecular Biology Organization) procedure (44). The 48 mer AVP probe 5′-G TA-GAC-CCG-GGG-CTT-GGC-AGA-ATC-CAC-GGA-CTC-TTG-TGT-CCC-AGC-CA G-3′ was complementary to the glycoprotein coding region of the AVP gene. Protocols for prehybridisation and hybridisation were described previously (32). The probe was labelled with d-ATP-digoxigenin (Behringer, Willich, Germany) at the 3′ end using the enzyme terminal transferase (Gibco, Gaithersburg, MD, USA). A second incubation was carried out with an alkaline phosphatase-conjugated anti-digoxigenin antibody (dilution 1 : 5000, Behringer). Development of the reaction was carried out by exposing the sections to the alkaline phosphatase substrates nitroblue tetrazolium and bromo-chloride-indolphosphate (Gibco). AVP mRNA was quantified within the lateral and medial magnocellular) division of the PVN using computerised densitometry (OPTIMAS BIOSCAN) previously validated for AVP mRNA (33). Digited images of tissue sections (six to nine sections per rat, five rats in each group) were displayed on the video screen under identical lighting conditions and the software calculated the average optical density (OD) of the pixels contained within the boundaries of the outlined area by using a 256-unit gray level scale. The densitometric reading of a comparable area outside the PVN but devoid of AVP mRNA was considered as background and subtracted from the readings of the magnocellular cells. The specificity of the hybridisation was determined by competition of the labelled probe with a 20-fold excess unlabelled probe, and by replacing the antidigoxigenin antibody by nonreactive serum. Under these conditions, the AVP signal was totally blunted. The results were expressed as the total immunoreactive area (μm^2) expressing AVP mRNA.

ISH for brain-derived neurotrophic factor (BDNF) mRNA

ISH for rat BDNF was carried out under RNAse-free conditions in accordance with a protocol described previously (36). Anaesthetised animals were killed by decapitation and the brains were removed and frozen on dry ice. Cryostat sections obtained at the level of the dorsal hippocampus according to plates 27–36 from the stereotaxic atlas of the rat brain (43) were fixed in 2% PFA, washed in × 0.5 sodium citrate/sodium chloride buffer (SCC; 1 × SCC: 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0), dried and acetylated with acetic anhydride. To detect BDNF-specific mRNA, we used a 48-mer synthetic oligonucleotide probe (Oligos Etc., Inc., Wilsonville, OR, USA) containing the complementary sequence to bp 562–609 of rat BDNF (45). The probe was end labelled with (^{35}S)dATP using the enzyme terminal transferase (Roche, Mannheim, Germany). Hybridisation was carried out using 10^6 c.p.m. of ^{35}S-labelled probe in 100 μl of hybridisation cocktail containing: 0.02% Ficoll 400, 0.02% polyvinylpirrolidone, 0.02% bovine serum albumin, 50% formamide, 3 × SCC buffer, 10 mM dithiothreitol, 0.1 mg/ml salmon sperm DNA, 1 mM ethylenediaminetetracetic acid, 4 μg/ml heparin, 0.4 mg/ml tRNA and 10% dextran sulphate. After overnight hybridisation at 42 °C, sections were washed several times in saline-sodium citrate, and dried under a cold air stream. Sections were then apposed against ^{35}S- sensitive film (Kodak BioMax MR; Kodak, Rochester, NY, USA) in the dark for 24 h. At the end of this period, film autoradiograms were analysed using a computer-assisted densitometer consisting of a CCD-X77 video camera coupled to a computer equipped with NIH IMAGE, version 1.5 (Dr W Rasband; NIMH, Bethesda, MD, USA). Relative optical density (OD; mean ± SEM) was expressed as a percentage of control (WKY) levels after background subtraction determined from a set of slides hybridised with 10^6 c.p.m. of ^{35}S-labelled oligonucleotide in the presence of a 20-fold excess of unlabelled probe.

Statistical analysis

Group differences in were determined by one-way ANOVA followed by Newman–Keuls post-hoc test. Results are expressed as the mean ± SEM number of immunoreactive cells in the dentate gyrus (Ki67 and DCX), number of immunoreactive cells/mm^2 (GFAP) or per μm^2 (aromatase or AVPmRNA), or percentage change in OD with respect to WKY control levels (BDNF mRNA). Statistical analyses were performed with GRAPHPAD PRISM, version 4 (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Measurement of blood pressure, tissue weights and serum hormones in WKY rats and SHR receiving vehicle or 17α-oestradiol

Table 1 shows the results for body, testicular and hypophysial weight, levels of serum prolactin and 17β-oestradiol, and the blood pressure of WKY rats and SHR with and without 17α-oestradiol treatment. Clearly, this compound did not modify body, testicular or hypophysial weight in WKY rats or SHR after 2 weeks of treatment. Serum prolactin and 17β-oestradiol were also similar in the

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four groups. All SHR remained hypertensive, regardless of whether they received vehicle or 17α-oestradiol treatment (Table 1). The lack of effect of the 17α-isomer on the weight of testis, pituitary and blood pressure contrasted with the effects described for 17β-oestradiol treatment of SHR (31,33,38–40).

Effects of 17α-oestradiol on neurogenesis

Previous results have shown a pronounced decrease of cell proliferation in SHR and its reversal after 17β-oestradiol treatment (35). To label proliferating cells in the SGZ of the dentate gyrus, we used KI67, a marker expressed during all active phases of the cell cycle. In WKY rats, clusters of Ki67+ cells were found in the SGZ of the dentate gyrus. The results show that Ki67+ cells were 50% lower in SHR compared to WKY ($F = 5.89$; $P < 0.05$) (Fig. 1A, top). After 17α-oestradiol treatment, cell proliferation levels of SHR reached those of WKY control rats (Fig. 1A, top). Light microscopy reflecting these changes is shown in the three photomicrographs in Fig. 1(A). To determine whether 17α-oestradiol stimulated a further step of neurogenesis, we labelled neural progenitors (neuroblasts) using DCX immunocytochemistry. Quantitative analysis (Fig. 1B, top) demonstrated a 50% reduction of DCX+ cells in steroid-naïve SHR compared to WKY ($F = 6.44$; $P < 0.05$), whereas a significant stimulatory effect on DCX+ cell number occurred after 17α-oestradiol treatment (SHR versus SHR + αE_2; $P < 0.05$). The photomicrographs in Fig. 1(B) show that bodies of DCX+ cells were mostly located in the inner GCL. Light microscope observations readily distinguished differences in DCX+ cell morphology between WKY rats, steroid-naïve SHR and SHR receiving 17α-oestradiol treatment. Thus, abundant DCX+ cells were detected in the inner GCL of the dentate gyrus of WKY rats, with staining of cell bodies and ramified cell processes. Instead, the staining profile of SHR showed a paucity of DCX+ cells of low staining intensity with fewer cell processes. After 17α-oestradiol treatment of SHR, the dystrophic morphology of DCX+ cells turned into highly branched DCX+ cells with enhanced staining intensity. Thus, quantification of Ki67 cell proliferation and the DCX immunopositive cell number indicated that 17α-oestradiol treatment stimulated both cell proliferation and neuroblasts in SHR.

Effects of 17α-oestradiol on BDNF mRNA

Levels of BDNF mRNA in the there groups of rats was studied by ISH, followed by computer-assisted analysis of film autoradiograms. Compared with normotensive WKY rats, SHR showed a nonsignificant a reduction of BDNF mRNA in the gyrus dentatus (Fig. 2A) (WKY versus SHR; $P < 0.06$). However, impairment of BDNF mRNA was more accentuated in the CA1 region (WKY versus SHR; $F = 5.27$; $P < 0.05$) (Fig. 2B). Furthermore, 17α-oestradiol administration to SHR significantly increased the BDNF mRNA autoradiography signal in the dentate gyrus (Fig. 2A; SHR versus SHR + αE_2; $F = 4.94$; $P < 0.05$). In the CA1 region, steroid-untreated SHR showed lower levels of BDNF mRNA compared to WKY and SHR + αE_2 ($P < 0.05$). Levels of BDNF mRNA in the last two groups were undistinguishable.

17α-oestradiol and aromatase immunostaining

Previous results demonstrated increased basal levels of aromatase in the brain of SHR compared to WKY rats, with a further increase after exposure to 17β-oestradiol (37). To determine whether the 17α-isomer reproduced these effects, aromatase immunoreactivity was studied in the hilus of the dentate gyrus (Fig. 3A, top) and the CA1 hippocampus region (Fig. 3A, bottom) of the three experimental groups. Quantitative analysis by computerised densitometry demonstrated that both steroid naïve SHR and 17α-oestradiol-treated SHR presented higher aromatase immunoreactive area in the hilus and also the CA1 region compared to WKY rats (Fig. 3A, top for dentate gyrus; Fig. 3A, bottom for CA1 region; $F = 2.73$; $P < 0.05$ versus WKY rats). However, in contrast to previous results with 17β-oestradiol, the 17α-isomer did not enhance the already elevated staining for aromatase in the SHR group (Fig. 3A). Light microscopy observations of aromatase immunostaining in the CA1 region, showed that, in WKY rats, aromatase immunoreactivity was mainly localised in cell processes of pyramidal cells projecting to the stratum radiatum plus some scarce neuronal bodies (Fig. 3B). In SHR and SHR + 17α-oestradiol groups, stronger aromatase staining was detected in projection fibres found in the stratum radiatum in addition to some neurones found in the pyramidal cell layer and interneurones of the stratum radiatum and stratum lacunosum-moleculare (Fig. 3B).

Effects of 17α-oestradiol on hippocampus astrogliosis

Astrogliosis with a strong expression of GFAP has already been reported in the hypertensive rat brain (35,46). In the present study, we examined the response of GFAP-immunoreactive astrocytes to 17α-oestradiol in two subregions of the hippocampus, namely the dentate gyrus and CA1 region. In the normotensive WKY rat strain, density of GFAP immunoreactive cells varied from approximately 200 GFAP+ cells/mm^2 in the CA1 region (Fig. 4A, top) to approximately 450 GFAP+ cells/mm^2 in the hilus of the dentate gyrus (Fig. 4A, bottom). In the SHR group, GFAP immunopositive cells show a 1.8-fold higher density in the CA1 region compared to WKY rats (Fig. 4A, top; $F = 27.33$; $P < 0.001$) and a 1.3-fold higher density in the hilus compared to WKY rats (Fig. 4A, bottom; $F = 12.78$; $P < 0.001$). Treatment of SHR with 17α-oestradiol significantly decreased GFAP+ astrogliosis in the CA1 region (Fig. 4A, top; $P < 0.01$ versus SHR), whereas a nonsignificant reduction was found for the hilus (Fig. 4A, bottom). Morphologically, the GFAP+ astrocytes of the CA1 region showed a more reactive phenotype in SHR compared to WKY rats. The reactive astrogliosis subdued after treatment for 2 weeks with 17α-oestradiol (Fig. 4B).

Effects of 17α-oestradiol on AVP mRNA in the hypothalamus

Changes of AVP mRNA in the hypothalamus were assessed by a non-isotopic ISH method. Computerised analysis of the immunoreactive area (in μm^2) showed that AVP mRNA expression was two-fold higher in SHR versus WKY rats ($F = 5.99$; $P < 0.01$),

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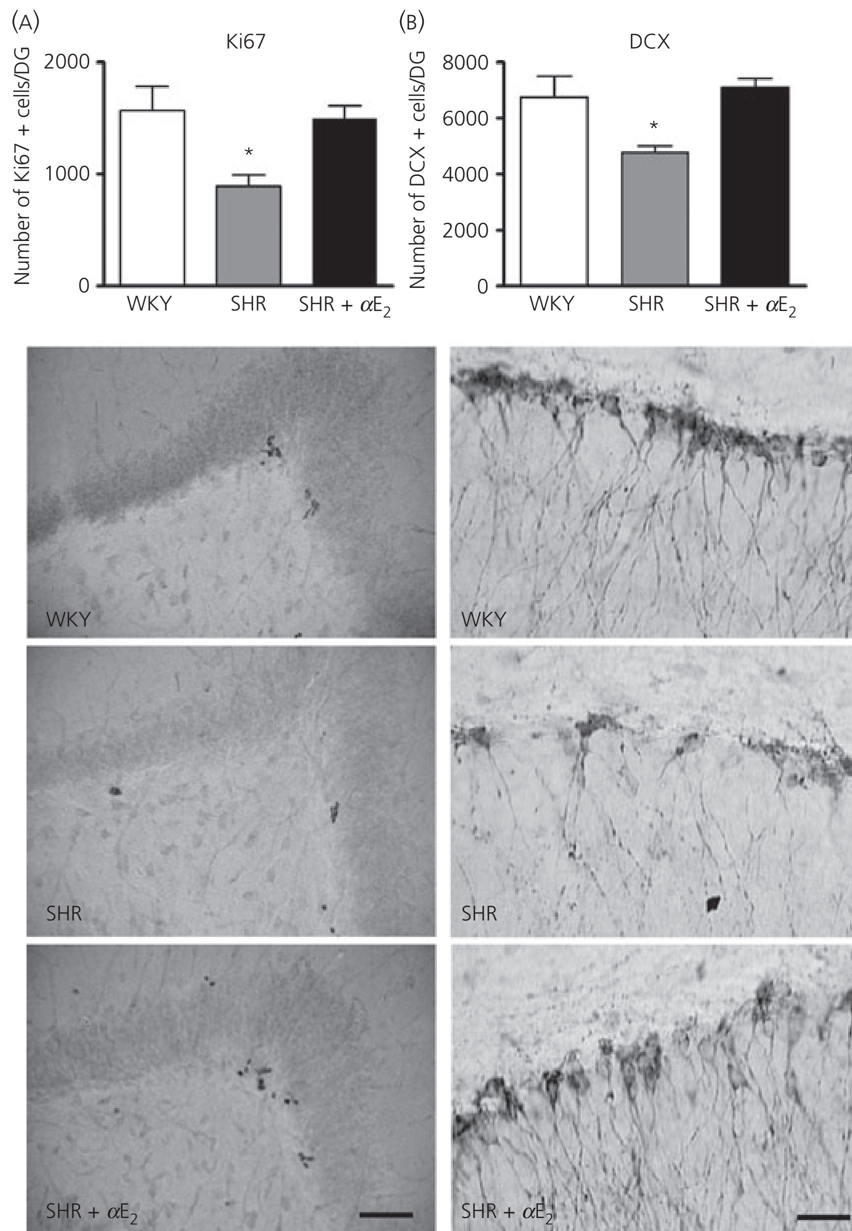

Fig. 1. Effects of 17α-oestradiol (αE_2) on hippocampus neurogenesis. (A) Top: Quantitative analysis of the number of Ki67+ proliferating cells in the subgranular zone of the dentate gyrus (DG). The graph shows a smaller amount of proliferating cells in spontaneously hypertensive rats (SHR) versus the Wistar–Kyoto (WKY) rats and SHR + αE_2 groups (*$P < 0.05$). The lower three photomicrographs denoted fewer Ki67+ cells in SHR than in WKY and SHR + αE_2 groups. Inside bar: 100 μm. (B) Top: Quantification of doublecortin (DCX) immunoreactive neuronal progenitors in the inner granular cell layer of the dentate gyrus. Analysis of DCX+ cell number showed a reduction in SHR compared to both WKY rats and SHR + αE_2 groups (*$P < 0.05$). The lower three photomicrographs showed profuse DCX staining of cell bodies and processes in WKY, in contrast to the low staining of cell bodies and processes in SHR. 17α-Oestradiol treatment of SHR returned DCX staining to levels of WKY rats. Inside bar = 100 μm. Data represent the mean ± SEM of six sections per animal, five animals per group. For statistical comparisons, we used one-way ANOVA followed by the Newman–Keuls post-hoc test.

whereas, in 17α-oestradiol-receiving SHR, the AVP mRNA signal returned to normal levels (Fig. 5A). Neuroanatomically, AVP mRNA-expressing neurones of WKY rats were moderately abundant in the posterior part of the magnocellular cells of the PVN (Fig. 5B, left). In agreement with previous results obtained in SHR (35), AVP mRNA was enhanced in the posterior magnocellular region of the PVN, with some AVP mRNA expressing cells spreading out to the medial portion of the magnocellular region (Fig. 5B, centre). When SHR received 17α-oestradiol for 2 weeks, the AVP mRNA signal was attenuated both in the posterior and the medial magnocellular region of the PVN (Fig. 5B, right). Therefore, changes of the vasopressinergic system induced by 17α-oestradiol in SHR were in

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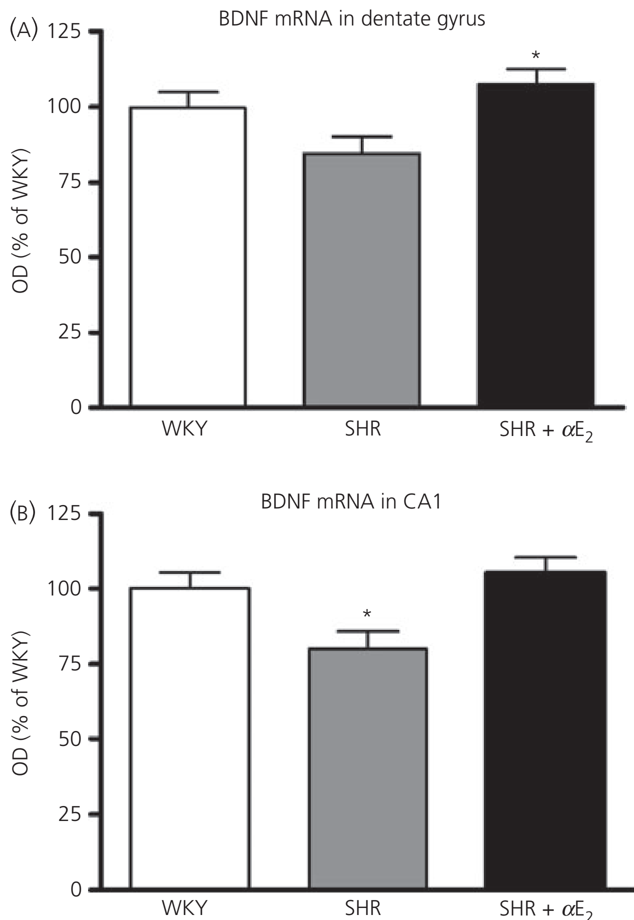

Fig. 2. Effects of 17α-oestradiol (αE_2) on brain-derived neurotrophic factor (BDNF) mRNA determined by *in situ* hybridisation. The graphs represent the optical density (OD) of film autoradiograms taken as % of Wistar–Kyoto (WKY) rat values. ODs were analysed on films obtained from the combined right and left dorsal hippocampus. (A) Top: BDNF mRNA in the dentate gryus of spontaneously hypertensive rats (SHR) shows a slight reduction of borderline significance compared to WKY rats. 17α-oestradiol treatment of SHR, however, significantly enhanced the BDNF mRNA signal compared to untreated hypertensive rats (*$P < 0.05$). (B) Bottom: BDNF mRNA in the CA1 hippocampal region. Results showed a significant decreased expression of BDNF mRNA in SHR compared to WKY and SHR + αE_2 groups (*$P < 0.05$ for both groups versus untreated SHR). Data represent the mean ± SEM of six sections per rat from five rats per group. For statistical comparisons, we used one-way ANOVA followed by the Newman–Keuls post-hoc test.

agreement with the effects of 17β-oestradiol obtained for this parameter (35).

Discussion

In the present study, we studied the neuroprotective potential of a nonfeminising oestrogen in the brain of hypertensive rats. Measurements of testicular weight, pituitary weight and serum prolactin showed that 17α-oestradiol did not modify the weight of testis or pituitary, or serum prolactin, when given for 2 weeks to male WKY rats or SHR. These findings are opposite to those shown after 17β-oestradiol, which causes testicular atrophy, pituitary hypertrophy and hyperprolactinaemia in male control rats and SHR (31,33). Thus, 17α-oestradiol lacked typical oestrogenic effects in male rats. Analysis of circulating steroids revealed the lack of interconversion between the isomers because levels of 17β-oestradiol were identical in rats receiving vehicle or the 17α-isomer. Interestingly, 17α-oestradiol did not decrease the blood pressure of SHR, suggesting that hypotension caused by 17β-oestradiol is not a requirement for neuroprotection (33,38–40).

Thus, present and past results support direct neuroprotective effects of both 17β- and 17α-isomers in the brain from rats with hypertensive encephalopathy. However, it should be noted that, although the time of steroid treatment was similar (s.c. pellet implantation for 2 weeks), the dose was different for both isomers. Whereas a pellet containing 800 μg of 17α-isomer was implanted in the present study, 12 mg 17β-oestradiol was used in previous studies (35,37). The reason for decreasing the oestrogen dose was to avoid the undesirable side effects obtained with the natural hormone. Hence, when implanted for 2 weeks, 17β-oestradiol increased its serum levels by approximately 60-fold (37) and significantly decreased the testis weight of normotensive rats (WKY rats: 2.87 ± 0.17 g, WKY rats + 17β-oestradiol: 1.98 ± 0.30 g; $P < 0.01$ versus WKY) and hypertensive rats (SHR: 3.00 ± 0.04 g, SHR+ 17β-oestradiol: 1.96 ± 0.12 g; $P < 0.05$ versus SHR). By contrast, 17α-oestradiol neither increased serum 17β-oestradiol, nor decreased testis weight. Therefore, the procotol used for 17α-oestradiol is possibly safe for neuroprotection.

17α-Oestradiol restores neurogenesis in SHR

The neuroprotective activity of 17α-oestradiol in SHR was first confirmed by measuring neurogenesis that takes place in the dentate gyrus. Thus, 17α-oestradiol restored the density of Ki67-labelled cells in the SGZ of the dentate gyrus of SHR compared to the low neurogenesis in vehicle-treated SHR. Because Ki67 is detected during all active phases of the cell cycle: G1, S, G2 and mitosis (47), our data support the idea that 17α-oestradiol as an inducer of cell proliferation Second, after receiving 17α-oestradiol, SHR showed an increased density of DCX+ cells in the inner border of the GCL of the dentate gyrus. DCX is a microtubule-associated protein involved in cellular migration and expressed by neural progenitors (48). In steroid-naïve SHR, most DCX+ cells showed a type-2b or type-3 phenotype in accordance with the terminology of Kempermann *et al.* (49). This phenotype, characterised by staining of cell bodies but not processes, was modified after 17α-oestradiol treatment, with DCX+ cells resembling the CR type of Kempermann *et al.* (49) with enlarged cell bodies and processes. These results support the idea that 17α-oestradiol also induced progenitor maturation. However, although decreased neurogenesis of SHR was found in the present study, as well as in a previous study (36), an increased number of Ki67+ cells in the dentate gyrus of SHR has also been reported (28). Among other factors, both studies differ with respect to the age of the rats and the blood pressure levels (3 months old and 153 mmHg in the previous study) (28) compared to our present cohort of SHR (5 months old and 166 mmHg).

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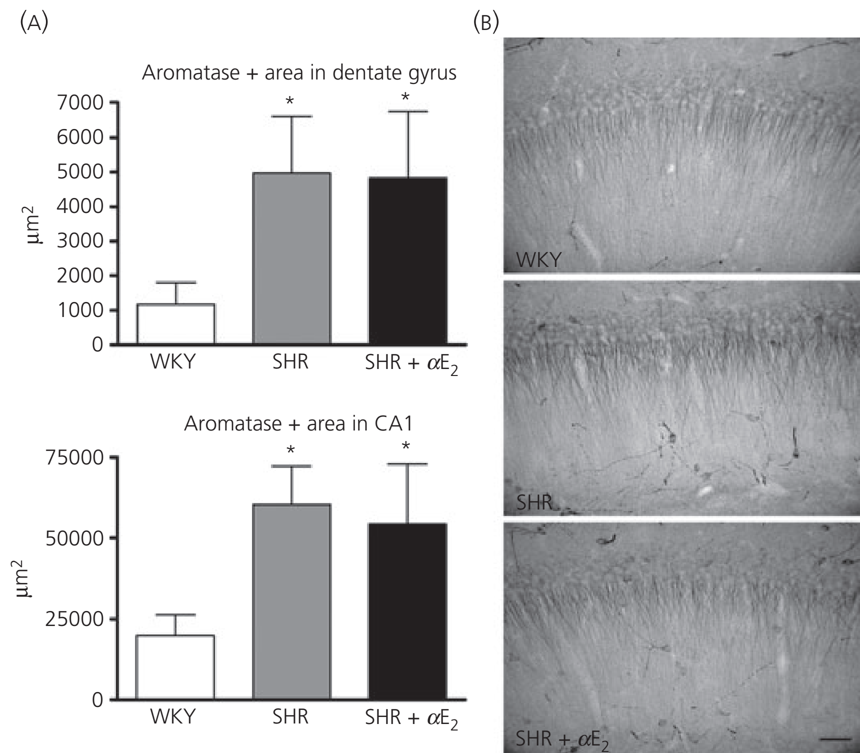

Fig. 3. Effects of 17α-oestradiol (αE_2) on aromatase immunostaining. Aromatase immunoreactive area (μm^2) in the hilus of the dentate gyrus (A, top) and the CA1 region (A, bottom) of Wistar–Kyoto (WKY) rats, spontaneously hypertensive rats (SHR) and SHR + αE_2-treated rats. SHR and SHR + αE_2 groups showed higher aromatase immunoreactivity in both hilus and CA1 region versus WKY (*$P < 0.05$) but there was no difference between steroid-untreated and steroid-treated SHR. (B) Example of aromatase immunoreactivity in the CA1 region showed more intense immunopositive fibres in SHR and SHR + αE_2 groups compared to WKY rats. In addition, some aromatase immunoreactive interneurones were present in the stratum radiatum and the stratum lacunosum-moleculare of steroid-untreated and steroid-treated SHR. Inside bar = 150 μm. Data represent the mean ± SEM of six sections per rat, five animals in each group. For statistical comparisons, we used one-way ANOVA followed by the Newman–Keuls post-hoc test.

Oestrogenic effects on neurogenesis are considered genomic because 80% of proliferating cells of the dentate gyrus express oestrogen receptor isoforms OERα and OERβ (50). However, alternative factors may operate for the stimulation of neurogenesis in SHR receiving 17α-oestradiol because, mechanistically, this compound was considered to be a free radical scavenger (16). Barha *et al.* (27) have already demonstrated the regulation of cell proliferation in the dentate gyrus of ovariectomised rats by 17α-oestradiol and postulated a nongenomic mechanism for this effect.

17α-Oestradiol stimulates BDNA mRNA expression in SHR

In parallel with the stimulatory effect on neurogenesis, we found that 17α-oestradiol enhanced BDNF expression in the CA1 region and dentate gyrus of SHR. Normally, BDNF exerts a robust stimulation of neurogenesis (51); thus, the finding that neurogenesis and BDNF expression was similarly reduced in the hippocampus of SHR was not unexpected. Down-regulation of BDNF in SHR may be a consequence of hypertension because high blood pressure shows a negative influence on BDNF expression in the hippocampus of SHR and in control rats with hypertension as a result of arterial occlusion (52,53). Previously, we reported that BDNF depletion of SHR was prevented by treatment with 17β-oestradiol. This steroid induces BDNF transcription and protein levels in the hippocampus (8), an effect that is considered to be primarily genomic as a result of the localisation of an oestrogen-response element on the BDNF promoter (54). However, Sato *et al.* (55) have shown that 17β-oestradiol stimulates BDNF release from dentate granule cells in an oestrogen-receptor independent manner. Therefore, it remains possible that the stimulatory effects of oestrogens on BDNF in the dentate gyrus and CA1 region of SHR may include both genomic and indirect mechanisms. This dual hypothesis has been already considered (51).

17α-Oestradiol down-regulates astrogliosis in the brain of SHR

Astrogliosis in the brain of SHR has already been described by Tomassoni *et al.* (46) and later confirmed by our laboratory (35). In the present study, reactive astrocytes with a high expression of GFAP were detected by immunocytochemistry in the CA1 region and dentate gyrus of SHR. 17α-Oestradiol treatment significantly decreased astrogliosis in the CA1 region but not the dentate gyrus, whereas, in a previous study, 17β-oestradiol decreased GFAP+ astrocyte density in both regions (35). The astroglial activation is a hallmark of neuronal damage. Regarding this event, neuronal apoptosis determined by the Tunnel method has been described in SHR (56).

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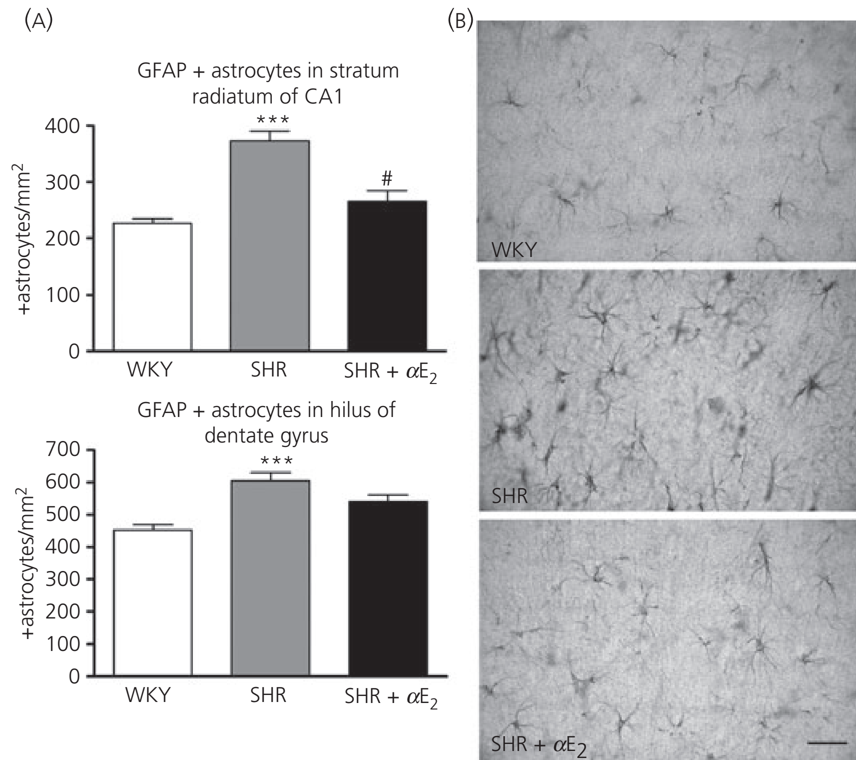

Fig. 4. Effects of 17α-oestradiol (αE_2) on glial fibrillary acidic protein (GFAP)+ immunoreactive astrocytes per unit area (mm^2) in the stratum radiatum below the CA1 subfield of the dorsal hippocampus and in the hilus of the dentate gyrus. (A) In the CA1 region (top), astrogliosis was significantly higher in spontaneously hypertensive rats (SHR) versus Wistar–Kyoto (WKY) rats (***$P < 0.001$), although it was significantly reduced in SHR + αE_2 versus the SHR group (#$P < 0.05$). In the dentate gyrus (bottom), astrogliosis was also present in SHR (SHR versus WKY rats: ***$P < 0.001$) but αE_2 had no reducing effect. (B) GFAP+ astrocytes in the CA1 region of a WKY rat, SHR and SHR + αE_2 rat. Astrocytes immunopositive for GFAP showed a more reactive profile in the untreated SHR compared to WKY rats and SHR + αE_2 groups. Inside bar: 50 μm. Thus, 17α-oestradiol treatment downregulated the number of GFAP+ astrocytes and decreased their reactive phenotype in the CA1 region of hypertensive rats. Data represent the mean ± SEM from six sections per rat, five animals per group. For statistical comparisons, we used one-way ANOVA followed by the Newman–Keuls post-hoc test.

Down-regulation of astrogliosis by oestrogens may be neuroprotective because reactive astrocytes change their gene expression, release pro-inflammatory mediators and cytokines that damage neurones and attract macrophages and microglia (57–59). Therefore, reducing astrogliosis in the CA1 region could attenuate neurodegeneration. Rozovsky *et al.* (60) reported that 17β-oestradiol inhibits GFAP expression *in vivo* and *in vitro* in a transcriptionally mediated manner. This effect could be a result of the expression of OER in astrocytes and the presence of an oestrogen-responsive element on the GFAP promoter (60,61). Therefore, although the effects of 17β-oestradiol on glial effects may be genomically mediated, the question remains as to why 17α-oestradiol reduces astrogliosis. In cultured glioma cells, 17β-oestradiol action occurs in a nontranscriptional manner (62), and it would be interesting in future experiments to determine whether a similar mechanism accounts for 17α-oestradiol reduction of astrogliosis in the CA1 region.

17α-Oestradiol modulates AVP mRNA in hypothalamus of SHR

Enhanced levels of AVP mRNA in the PVN of the hypothalamus have been reported in SHR (35,63). In agreement with these findings, higher mRNA levels were obtained in the magnocellular division of the PVN compared to WKY rats. AVP mRNA overexpression was reduced by treatment with 17α-oestradiol (present study) and also by 17β-oestradiol (35). There is some discrepancy towards the effects of oestrogen on the PVN. For example, activation of OERβ with a specific ligand decreased AVP transcripts in the hypothalamus of mice (64). Instead, in SH-SY5Y neuroblastoma cells, as well as in ovariectomsied rats, 17β-oestradiol stimulated via OERα and inhibited via OERβ AVP mRNA (65). It is likely that the hypertension environment and endocrine conditions prevailing in SHR conditioned the vasopressinergic response in the basal state and after oestrogenic stimulation. For example, it is known that depletion of oestrogens in SHR increases hypertension (40,66), whereas 17β-oestradiol administration lowers blood pressure in this strain (33,38,39). Therefore, an unexpected finding was that hypotension did not follow 17α-oestradiol treatment of SHR, considering that the steroid decreased the expression of this powerful hypertensinogenic peptide (32,67). One possibility would be that higher doses of oestrogens, which are sufficient to induce endothelial-relaxing effects in the periphery, are needed to reduce blood pressure.

However, modulation of AVP may not reconcile in appearance with the neuroprotection hypothesis because decreased AVP

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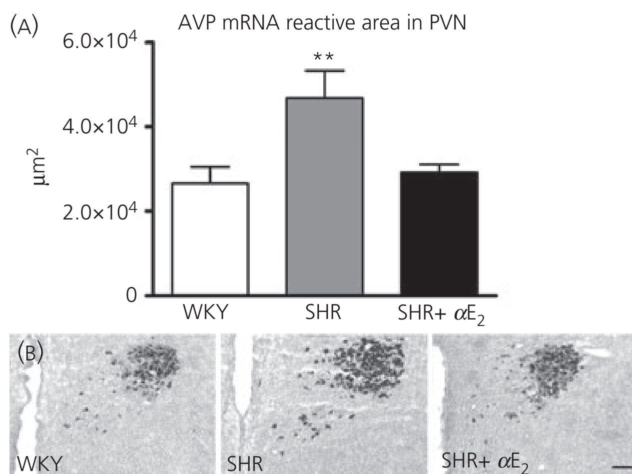

Fig. 5. Effects of 17α-oestradiol (αE_2) on arginine vasopressin mRNA in the paraventricular nucleus (PVN) of the hypothalamus measured by *in situ* hybridisation. (A) Quantitative analysis in hypertensive rats showed a higher arginine vasopressin (AVP) mRNA reactive area (μm^2) versus Wistar–Kyoto (WKY) rats and versus spontaneously hypertensive rats (SHR) + αE_2-treated rats (**$P < 0.01$). (B) AVP mRNA was restricted to dorsal magnocellular cells in WKY rats. SHR showed higher AVP mRNA expression in the dorsal, as well as the medial, magnocellular PVN. The expression pattern of AVP mRNA returned to the profile of WKY rats after 17α-oestradiol treatment. Inside bar = 100 μm. Data represent the mean ± SEM: of six to nine sections per rat, five animals per group. For statistical comparisons, we used one-way ANOVA followed by the Newman–Keuls post-hoc test.

synthesis as a result of 17α-oestradiol was not followed by changes of blood pressure. Excess production of AVP in the brain exerts pro-inflammatory effects by increasing local inflammatory mediators (68). Therefore, down-regulation of AVP by oestrogens, which are potent anti-inflammatory factors (2,5,14), could additionally benefit the brain of SHR.

17α-Oestradiol lacks effects on aromatase

The present study also included aromatase, the enzyme catalyzing oestrogen synthesis in the brain from androgenic precursors. We have already shown increased basal levels of aromatase expression in the hippocampus of SHR, which are further stimulated by exogenous 17β-oestradiol administration (37). The present study confirmed that basal levels of immunoreactive aromatase are elevated in the hippocampus of SHR but, in contrast to 17β-oestradiol, the 17α-isomer was unable to further modulate the enzyme. It is likely that increased basal levels of aromatase are beneficial for SHR because this enzyme plays a protective role by enhancing the local synthesis of neurooestrogens in the hippocampus (2,69). It is possible that genomic mechanisms are involved in the effects of 17β-oestradiol with respect to the expression of aromatase. Two activator protein 1 elements in the aromatase promoter are essential for inducing the transcriptional activity of the Cyp19a1 (aromatase) gene by 17β-oestradiol (70). These elements may not be activated by 17α-oestradiol, as suggested in the present study.

Concluding remarks

In summary, the data obtained in the present study show that 17α-oestradiol is endowed with neuroprotective potential in the hippocampus and hypothalamus of SHR. This proposal was supported by analyses of neurogenesis, growth factor expression, astrocyte counting and vasopressin expression. The present study also showed for the first time that a nonfeminising oestrogen could arrest hypertensive encephalopathy. The lack of an effect of 17α-oestradiol on body and tissue weights, serum 17β-oestradiol and prolactin levels of either WKY rats and SHR suggests that pharmacotherapy with this compound may be therapeutically safe.

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