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Involvement of Brain-Derived Neurotrophic Factor and Neurogenesis in Oestradiol Neuroprotection of the Hippocampus of Hypertensive Rats

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The hippocampus of spontaneously hypertensive rats (SHR) and deoxycorticosterone (DOCA)-salt hypertensive rats shows decreased cell proliferation and astrogliosis as well as a reduced number of hilar cells. These defects are corrected after administration of 17β-oestradiol (E_2) for 2 weeks. The present work investigated whether E_2 treatment of SHR and of hypertensive DOCA-salt male rats modulated the expression of brain-derived neurotrophic factor (BDNF), a neurotrophin involved in hippocampal neurogenesis. The neurogenic response to E_2 was simultaneously determined by counting the number of doublecortin-immunopositive immature neurones in the subgranular zone of the dentate gyrus. Both hypertensive models showed decreased expression of BDNF mRNA in the granular zone of the dentate gyrus, without changes in CA1 or CA3 pyramidal cell layers, decreased BDNF protein levels in whole hippocampal tissue, low density of doublecortin (DCX)-positive immature neurones in the subgranule zone and decreased length of DCX+ neurites in the dentate gyrus. After s.c. implantation of a single E_2 pellet for 2 weeks, BDNF mRNA in the dentate gyrus, BDNF protein in whole hippocampus, DCX immunopositive cells and the length of DCX+ neurites were significantly raised in both SHR and DOCA-salt-treated rats. These results indicate that: (i) low BDNF expression and deficient neurogenesis distinguished the hippocampus of SHR and DOCA-salt hypertensive rats and (ii) E_2 was able to normalise these biologically important functions in the hippocampus of hypertensive animals.

Key words: 17β-oestradiol, brain-derived neurotrophic factor, doublecortin, spontaneously hypertensive rats, deoxycorticosterone hypertension.

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Studies in hypertensive humans have shown that high levels of blood pressure are associated with atrophy of the hippocampus and temporal lobe and an increased risk of cognitive decline, suggesting increased risk of Alzheimer's disease (1, 2). Along the same line, neurofibrillary tangles, senile plaques and neuronal lesions have been described in hypertensive patients (3). Therefore, a relationship appears to exist between hypertension, cerebrovascular disease and the decline of cognition, an index of hippocampal dysfunction (4).

Studies in spontaneously hypertensive rats (SHR) show a pronounced encephalopathy, distinguished by hippocampal neuronal loss, astrogliosis with increased expression of the marker glial fibrillary acidic protein, blood–brain barrier disruption, decreased white matter volume in forebrain regions and changes of hippocampal neurogenesis (5–9). Because these changes are similar to those occurring in mental disorders with cognitive impairment, SHR have been considered models of dementia and the attention deficit hyperactivity syndrome (5). The question remains whether hippocampal changes are the consequences of a pre-existing hypertension or whether hypertension and brain pathology reflect a central defect (5, 10–12).

In hypertension produced by the administration of deoxycorticosterone acetate (DOCA) plus high salt diet, hippocampal changes resemble SHR because both models present astrogliosis and reductions of hilar cell number and of cell proliferation (7, 8). It is likely that DOCA elicits hippocampal changes after activation of brain mineralocorticoid receptors (MR) (13–15). Mineralocorticoids as well as MR may also be involved in the encephalopathy of SHR (11, 16–19). This hypothesis is supported by demonstrations that SHR are hyper-responsive to mineralocorticoid administration (14), whereas

the administration of MR antagonists decreases blood pressure (20). Therefore, SHR may represent a form of endocrine hypertension as a result of MR overactivation. (11, 16, 19).

In is now accepted that sex steroids behave as neuroprotectants in cases of CNS trauma, ischaemia, ageing and neurodegeneration (21–23). In models of ageing, diabetes mellitus and, in SHR and DOCA-salt-treated male rats, oestradiol, normalises cell proliferation and the number of hilar cells in the dentate gyrus and attenuates hippocampal astrogliosis (8, 24). Because the available data suggest that oestradiol only enhances neurogenesis in normal female but not male rats (25), the results obtained in the above mentioned models using male rats may have some relevance regarding differential oestrogen effects under normal and pathological situations. Oestrogens also decrease blood pressure acting on the cardiovascular regulatory centres of the brain (26). In the brain, oestrogens increase the vasodilators nitric oxide and atrial natriuretic peptide, decrease angiotensin II-induced hypertension and catecholaminergic activity and attenuate damage as a result of ischaemia and stroke (22, 27, 28). In SHR and DOCA-salt hypertensive rats, oestradiol exposure reduces the expression of the hypertensinogenic peptide arginine vasopressin (AVP) in the hypothalamus (14). This is an important effect because the paraventricular nucleus is involved in the pathogenesis of hypertension (10), supporting the idea that neuroactive oestrogens decrease hypertension of central origin.

Effects of oestrogens on the hippocampus may the result of an interaction with neurotrophins such as brain-derived neurotrophic factor (BDNF), which could mediate some of the reported oestrogen neuroprotective mechanisms (29). The intermediary role of the growth factor is reinforced by the presence of an oestrogen receptor responsive element on the BDNF promoter, the stimulation of BDNF mRNA and protein in the hippocampus and the increased release of BDNF from the dentate gyrus promoted by oestrogens (30–32). Another interesting interaction between oestrogens and BDNF is the finding that both enhance dentate gyrus neurogenesis (29). There is also evidence that oestrogens enhance cell proliferation and survival in the hippocampus of adult animals (33, 34). In the subgranular layer of the dentate gyrus of ageing mice, oestrogens also stimulate labelling for doublecortin (DCX) (24), a microtubule-associated protein required for neuronal migration and axonal wiring, that is commonly used as a marker of immature neurones. Oestrogen effects on neurogenesis may be genomic because 80% of proliferating cells of the dentate gyrus express the oestrogen receptor (ER) isoforms ERα and ERβ (35), although nonclassical, nongenomic mechanisms may also be involved in oestrogen control of hippocampal cell proliferation (36). Similar to oestrogens, evidence obtained from *in vivo* and *in vitro* studies has shown that BDNF plays a key role in neurogenesis, by the enhancement of cell proliferation, progenitor survival and terminal differentiation of newborn neurones (29). Of great interest is that BDNF expression is down-regulated in a genetically hypertensive strain of Wistar rats, in which impaired learning and memory correlates with decreased BDNF expression in the dentate gyrus (37), and in SHR receiving carotid artery occlusion, in which BDNF mRNA and protein are decreased in the CA1 region and cortical areas (38). Finally, a mutation in the BDNF specific receptor TrkB gene in the stroke-prone SHR impairs neurotrophic function in this strain (39). Thus, cumulative evidence suggests that BDNF expression and hypertension are negatively inter-related.

Given that oestrogens promote BDNF expression and neurogenesis in the rodent hippocampus, we investigated changes with respect to both this neurotrophin and neurogenesis in oestrogenised-hypertensive rats. The experiments involved SHR and DOCA-salt-treated rats, in which a group were exposed to oestradiol for 2 weeks. The effects of oestrogen was then focused on: (i) an analysis of hippocampal BDNF mRNA by *in situ* hybridisation (ISH) and BDNF protein by enzyme-linked immunoadsorbent assay (ELISA) and (ii) the determination of DCX immunoreactive immature neurones and neurites in the dentate gyrus.

Materials and methods

Experimental animals

Sixteen-week-old male Wistar-Kyoto (WKY) rats and SHR (approximately 250 g body weight, obtained from the University of La Plata, La Plata, Argentina) were housed under conditions of controlled humidity and temperature (22 °C) under a 12 : 12 h light/dark cycle (lights on 07.00 h). Animals were given food and water *ad lib*. At this age, the mean $\pm$ SD blood pressure of SHR measured 180 $\pm$ 2 mmHg. At the time of perfusion, SHR showed cardiac hypertrophy (SHR: 1.7 $\pm$ 0.025 g versus WKY rats: 1.09 $\pm$ 0.04 g; $P < 0.001$) that was attenuated by oestradiol treatment (1.4 $\pm$ 0.08 g; $P < 0.05$ versus SHR; n = 4 rats per group). Previous studies have shown that treatment of SHR with oestrogens produces antihypertensive effects and decreases cardiac hypertrophy (26, 40). For the DOCA-salt model, 2-month-old male Sprague–Dawley rats (250–280 g body weight; Instituto de Biologia y Medicina Experimental colony) were divided into two groups. The first group received s.c. injections of 10 mg of DOCA/rat every other day (Sigma, St Louis, MO, USA) dissolved in vegetable oil for 4 weeks and a solution of 1% NaCl as drinking water (DOCA + salt). The second group received s.c. injections of vegetable oil every other day for 4 weeks and a solution of 1% NaCl as drinking water. DOCA-treated rats developed hypertension (157 $\pm$ 11 mmHg), which was ameliorated by oestradiol administration (126 $\pm$ 5 mmHg; $P < 0.05$ versus steroid untreated DOCA-treated group). The development of cardiac hypertrophy in the DOCA-treated rats (1.9 $\pm$ 0.07 g) was also attenuated by oestradiol administration (1.57 $\pm$ 0.11 g; $P < 0.05$ versus DOCA only; n = 5 animals per group).

Hypertensive animals were anaesthetised using ketamine (50 mg/kg)/xylazine (10 mg/kg) and implanted s.c. with a single 14 mg pellet of 17β-oestradiol (E_2) (Sigma) during the last 2 weeks of the experiment. Steroid-naïve rats were sham-operated and implanted with cholesterol pellets. This hormone regimen effectively normalises hippocampal astrogliosis, cell proliferation and the neuronal density of the hilar region of SHR and DOCA-treated rats (8) and increases hippocampal glucocorticoid receptors and forebrain choline-acetyltransferase immunoreactive neurones of ageing rats (41). Levels of E_2 at the time of killing (i.e. 2 weeks after pellet implantation) were approximately 2500 pg/ml serum as measured by radioimmunoassay (8). Oestrogenised and steroid-untreated SHR, WKY rats and Sprague–Dawley rats with or without DOCA treatment were used for BDNF mRNA, BDNF protein measurement and DCX immunocytochemistry. Animal handling followed the Guide for the Care and Use of Laboratory Animals (NIH Guide, Instituto de Biologia y Medicina Esperimental Animal Facility Assurance Certificate # N-A5072-01) and was approved by the Institute's Animal Care and Use Committee. Every effort was made to minimise animal suffering and to reduce the number of animals used in the experiments.

BDNF ISH

ISH for rat BDNF was carried out under RNAse-free conditions in accordance with previously published protocols (42). Animals anaesthetised as described above were killed by decapitation and their brains were removed and frozen on dry ice. Coronal sections through the dorsal hippocampus corresponding to plates 27 to 36 from the stereotaxic atlas of the rat brain (43) were cut on a cryostat and collected on slides. The sections were fixed in 2% paraformaldehyde, 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 (44). 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 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 EDTA, 4 ug/ml heparin, 0.4 mg/ml tRNA and 10% dextran sulphate. After overnight hybridisation at 42 °C, sections were washed several times in SCC, and dried under a cold air stream. Sections were then apposed against ^{35}S- sensitive film (Kodak BioMax MR, Rochester, NY, USA) in the dark for 24 h. At the end of this period, film autoradiograms were analyzed using a computer-assisted densitometer consisting of a CCD-X77 video camera coupled to a Macintosh computer with NIH-Image software, version 1.5 (Dr W. Rasband, NIMH, Bethesda, MD, USA). Relative optical density (OD; mean ± SEM) was expressed as a percent of background levels, 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. In addition, the specific signal was absent when control tissue sections were preincubated with RNAse (20 μg/ml, 30 min at 37 °C) before ISH. To avoid bias in the interpretation of results, the experimenter was blind when calculating the OD of film autoradiograms.

BDNF protein assay

A set of rats different from the group used for BDNF mRNA studies was anaesthetised as described above, decapitated and their bilateral hippocampi were quickly dissected, frozen on dry ice and stored al −70 °C. At the time of analysis, samples were removed from the freezer and processed for BDNF total protein quantification by an ELISA (Promega BDNF Emax™ Inmunoassay System; Promega, Madison, WI, USA). Briefly, tissue was manually homogenised using a teflon pestle in lysis buffer containing protease inhibitors [137 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mM sodium vanadate]. Samples were then centrifuged at 10 000 *g* for 30 min at 4 °C and the supernatant was collected. An aliquot was removed from each sample to determine protein concentration using the colorimetric method of Bradford and 100 μl of the supernatant was diluted in 400 μl of DPBS (2.7 mM KCl, 0.137 M NaCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, pH 7.35). To maximise BDNF recovery, samples were acid treated with 20 μl of 1 N HCl to decrease the pH to 2.5, followed by incubation at room temperature for 15 min. Samples were then neutralised with 20 μl of 1 N NaOH. ELISA was in accordance with the manufacturer's instructions, and the optical density of the colorimetric reaction was measured at 450 nM using a plate reader. A standard curve, generated using BDNF standards (0–1000 pg/ml) was used to determine the concentration of BDNF in each tissue sample. BDNF levels were expressed as pg BDNF/mg total protein.

Doublecortin immunocytochemistry

Rats anaesthetised as described above were transcardially perfused with 60 ml of 0.9% saline followed by 60 ml 4% paraformaldehyde in phosphate buffer, pH 7.4. After an overnight incubation in 4% paraformaldehyde, brains were transferred to Tris-buffered saline (pH 7.4), sectioned frontally at 50 μm using a vibrating microtome and processed for free-floating immunocytochemistry. To study neurogenesis, we employed an antibody recognising DCX, a microtubule-associated protein expressed by immature neurones (45). Accordingly, coronal brain sections were first exposed to methanol: H_2O_2 (100 : 1) for 10 min at room temperature, washed and blocked for 30 min in phosphate-buffered saline containing 10% rabbit serum at 37 °C. Sections were incubated overnight with a goat polyclonal DCX antibody (dilution 1 : 250, sc-8066; Santa Cruz Labs, Santa Cruz, CA, USA) followed by a biotinylated anti-goat immunoglobulin G made in rabbit (dilution 1 : 200; Sigma) and processed in accordance with the ABC kit instructions (Vector Laboratories, Burlingame, CA, USA). For development, we used diaminobenzidine chloride 0.25 mg/ml, 0.05% H_2O_2 at room temperature. Nonspecific staining was assessed in the absence of primary antibody. The number of DCX-positive cells was determined in every eight section in a series of coronal sections comprising the subgranular zone of the dentate gyrus using stereological methods (46). The subgranular zone comprised a 50-μm band between the granular cell layer and the hilus, as defined by Sliwowska *et al.* (47). Six histological sections were assessed per rat using a × 60 objective of an Olympus BH-2 microscope (Olympus, Tokyo, Japan). All stained cells that came into focus in the subgranular zone were counted; thus, the couting frame was the entire subgranular zone The number of DCX+ cells counted was multiplied by 8 to estimate the total number of DCX+ cells in the dentate gyrus (46). Afterwards, each individual animal (n = 5 per group) contributed a single value generated by adding all the sections collected from the same animal. To obtain a consistency of immunostaining, sections from the three experimental groups in the SHR experiment (WKY rats, SHR and SHR + E_2) or the three groups in the DOCA experiment (control, DOCA and DOCA + E_2) were stained simultaneously. This procedure eliminated conflicts that may arise using different batches. Sections were analyzed in a double-blinded manner by different investigators.

In addition to quantitation of DCX cell number, we also determined the length of DCX immunopositive processes using Optimas Bioscan software for images in accordance with a previously described method (24). The software outlines the soma-associated cell processes giving the corresponding length in micrometre. Five soma-associated processes were counted per slice containing the dorsal blade of the dentate gyrus. From six slices per animal and five rats per group, we obtained the average length of cell processes per animal and per experimental group. The results were expressed as the length of the cell processes (μm; mean ± SEM).

Statistical analysis

Group differences for BDNF mRNA and protein levels, number of DCX+ cells and the length of DCX-immunopositive neurites were determined by one-way analysis of variance followed by Bonferroni's post-hoc test. $P < 0.05$ was considered statistically significant.

Results

BDNF mRNA in control and hypertensive rats

To localise the neuroanatomical expression of BDNF mRNA, we employed a specific BDNF oligonucleotide probe hybridised to hippocampal sections from rats with experimental hypertension. As

shown in the film autoradiograms (Fig. 1), a strong signal for BDNF mRNA was found in the CA1–CA3 pyramidal areas and granule cell layer of the dentate gyrus. However, there was a region-specific reduction of the BDNF mRNA signal in both SHR and DOCA-treated rats (Fig. 1B,E) compared to their control strains, the WKY rats and Sprague–Dawley control rats (Fig. 1A,D). This reduction was preferentially found in the granule cell layer of the dentate gyrus, rather than in the CA1–CA3 hippocampal regions of control and hypertensive animals, as shown in the insets of Fig. 1, which depict the dorsal hippocampal formation. Furthermore, oestradiol administration to SHR and DOCA-treated rats (Fig. 1C,F), normalised the signal intensity of film autoradiograms to control levels.

These observations were substantiated by statistical comparison of the OD of films autoradiograms (Fig. 2). Significant reductions of BDNF mRNA were found for the granule cell layer of the dentate gyrus of the hypertensive groups, as shown in Fig. 2(A) for SHR ($F_{2,12} = 9.58$; overall $P < 0.05$) and Fig. 2(B) for DOCA-treated rats ($F_{2,12} = 9.32$; overall $P < 0.01$) ($P < 0.05$ for both hypertensive groups versus control strains). After oestradiol treatment, BDNF mRNA levels were significantly increased compared to the untreated groups for both the SHR and DOCA experiments (dark columns of Fig. 2(A,B); SHR + E_2 and DOCA + E_2; $P < 0.05$ versus respective steroid-naive hypertensive groups). However, when the same groups and treatment regime was compared for the hipocampal CA1 and CA3 regions (i.e. taken as representative of pyramidal cell regions), the changes did not replicate those of the subgranule cell layer of the dentate gyrus. Thus, levels were similar between WKY rats, SHR and SHR + E_2 (Fig. 2C,E) and between CTL, DOCA and DOCA + E_2-treated rats (Fig. 2D,F), reinforcing the hypothesis that pyramidal cell areas from hypertensive animals were spared from changes of BDNF mRNA levels.

BDNF protein levels in control and hypertensive rats

Figure 3 shows data for BDNF protein determined by a commercial ELISA method. Despite the fact that changes of BDNF mRNA were exclusive to the dentate gyrus, the BDNF protein content of whole hippocampus was in accord with the BDNF mRNA study. Thus, BDNF protein was reduced by half in oestrogen-free SHR (Fig. 3A) ($F_{2,12} = 5.78$; overall $P < 0.05$) and oestrogen-free DOCA-treated rats (Fig. 3B) ($F_{2,19} = 6,52$; overall $P < 0.01$) compared to their control strains. Similar to changes obtained for BDNF mRNA, oestradiol treatment normalised the BDNF protein levels of SHR to those of WKY rats (Fig. 3A) (SHR versus WKY rats and SHR + E_2; $P < 0.05$) and BDNF protein levels of DOCA-treated rat to those of the control Sprague–Dawley group (Fig. 3B) (DOCA versus control and DOCA + E_2; $P < 0.05$).

Doublecortin immunoreactive cells in control and hypertensive rats

The subsequent experiments report the effects of oestradiol on neurogenesis of hypertensive rats. We have already shown that oestradiol stimulates bromodeoxyuridine uptake in the subgranular zone and granular cell layer of SHR and DOCA-treated rats (8). To determine whether oestradiol stimulated a further step of neurogenesis, we used DCX immunocytochemistry to label immature neurones. Conventional microscopy readily distinguished differences in DCX+ cell morphology between control strains (WKY and Sprague–Dawley rats), hypertensive rats (SHR and DOCA-treated rats) and hypertensive rats receiving oestradiol treatment. Light microscopy observations (Fig. 4) demonstrated abundant DCX+ cells in the subgranular cell layer of the dentate gyrus of WKY rats at low (Fig. 4A) as well as at high magnification (Fig. 4D). This profile contrasted with the scarce density of DCX+ cells of SHR, as shown

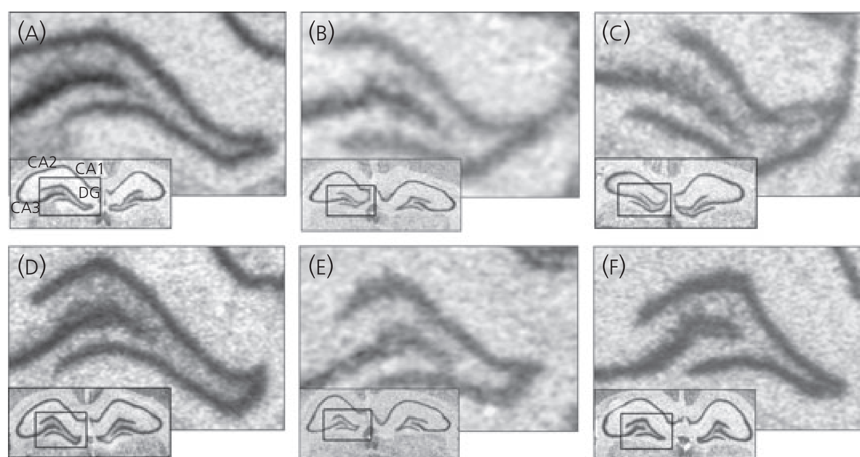

Fig. 1. Representative film autoradiograms of *in situ* hybridisation of brain-derived neurotrophic factor (BDNF) mRNA. (A) Normotensive Wistar-Kyoto (WKY) rat; (B) spontaneously hypertensive rat (SHR); (C) SHR treated with oestradiol; (D) Sprague–Dawley (SD) rat; (E) deoxycorticosterone (DOCA)-treated hypertensive SD rat; (F) DOCA-treated hypertensive rat treated with oestradiol. (A–F) High magnification views from the dentate gyrus; insets are low magnification views of dorsal hippocampus with dentate gyrus inside squares. BDNF mRNA signal is decreased in SHR (B) compared to WKY (A) and also in DOCA-treated rats (E) compared to SD control rats (D). In both strains, oestradiol treatment normalised BDNF mRNA (C, F). CA1, CA2 and CA3 in (A) correspond to hippocampus pyramidal cell layers; DG, dentate gyrus.

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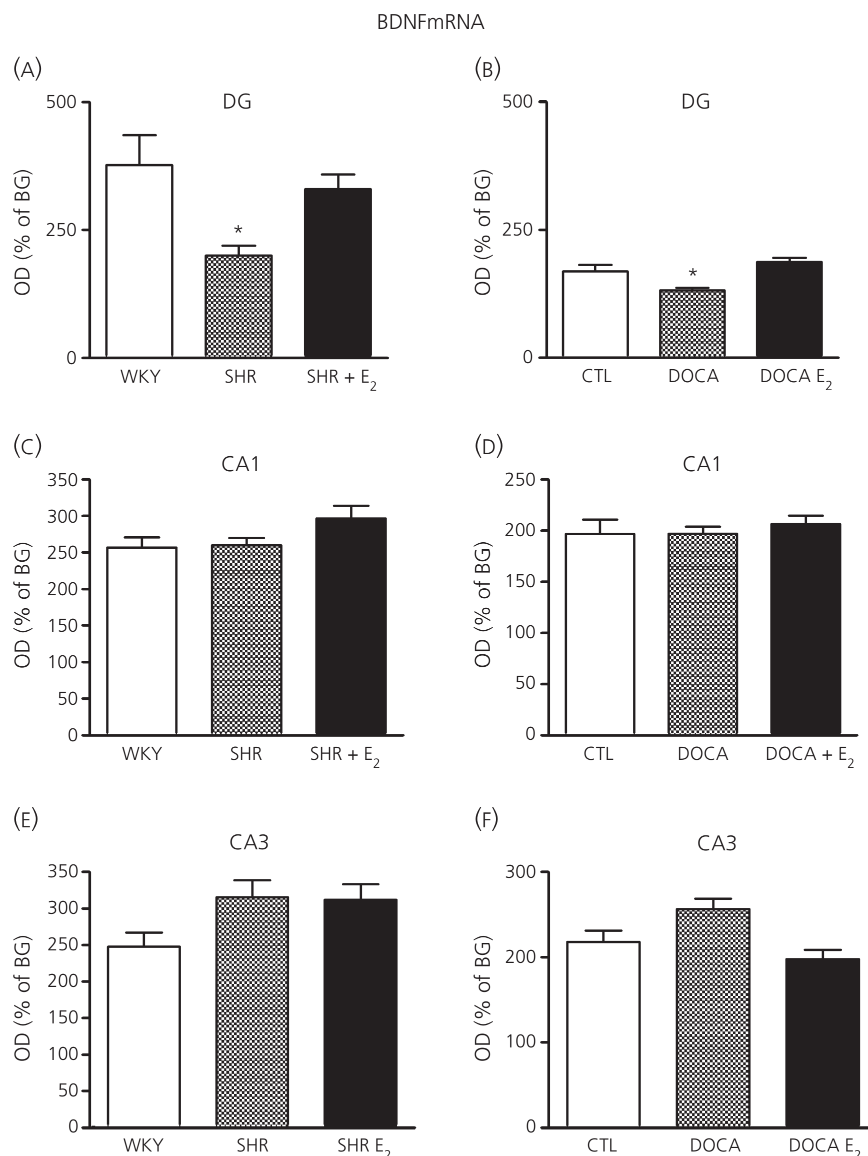

Fig. 2. Optical density (OD) of brain-derived neurotrophic factor (BDNF) mRNA measured on film autoradiograms corresponding to the experimental groups of Fig. 1. (A, C, E) Data from the granule cell layer of the dentate gyrus (DG) (A), the CA1 pyramidal cell layer (B) and the CA3 pyramidal cell layer (E) for the spontaneously hypertensive rat (SHR) experiment. (B, D, F) Data from the same hippocampal regions for the deoxycorticosterone (DOCA) experiment: in the granule cell layer of the dentate gyrus (A), OD for SHR was significantly lower than the Wistar-Kyoto (WKY) rat and SHR plus oestradiol (*P < 0.05). In the dentate gyrus (B), OD for DOCA was significantly lower than control (CTL) and DOCA + oestradiol (E_2) (*P < 0.05). BDNF mRNA in the CA1 or CA3 pyramidal cell layers showed no significant differences between the three groups of animals studied in the SHR or DOCA experiments. Results correspond to the mean ± SEM % of background (BG) of n = 5 animals per group. Statistical analysis was performed by analysis of variance followed by the Bonferroni post-hoc test.

under low and high magnifications (Fig. 4B,E). The reduced number and atrophic morphology of DCX+ cells of SHR was normalised following oestradiol treatment, as shown in the low and high magnification views of the subgranule cell layer (Fig. 4C,F). Analogous images were obtained for the DOCA experiment (Fig. 4G–L). Thus, low and high magnification views showed that DOCA-treated rats presented few DCX-labelled cells (H,K), in sharp contrast to the numerous DCX-immunopositive cells of the control Sprague–Dawley rat (Fig. 4G,J) and DOCA-salt-treated rat receiving oestradiol (Fig. 4I,L).

Quantitative analysis (Fig. 5A) determined a 50% reduction of DCX+ cells in SHR compared to WKY rats ($F_{2,10}$ = 12.68; overall P < 0.01). A significant stimulation of DCX+ cell number followed oestradiol treatment (Fig. 5A) (SHR versus SHR plus oestradiol and WKY rats; P < 0.05), although the magnitude of the change of oestradiol-treated SHR remained below the WKY rat level. Figure 5(C) shows that DOCA-treated rats contained fewer DCX+ cells in the subgranular cell layer of the dentate gyrus compared to Sprague–Dawley control rats ($F_{2,10}$ = 12.69; overall P < 0.01). Oestradiol treatment during 2 weeks normalised the number of DCX+ cells of

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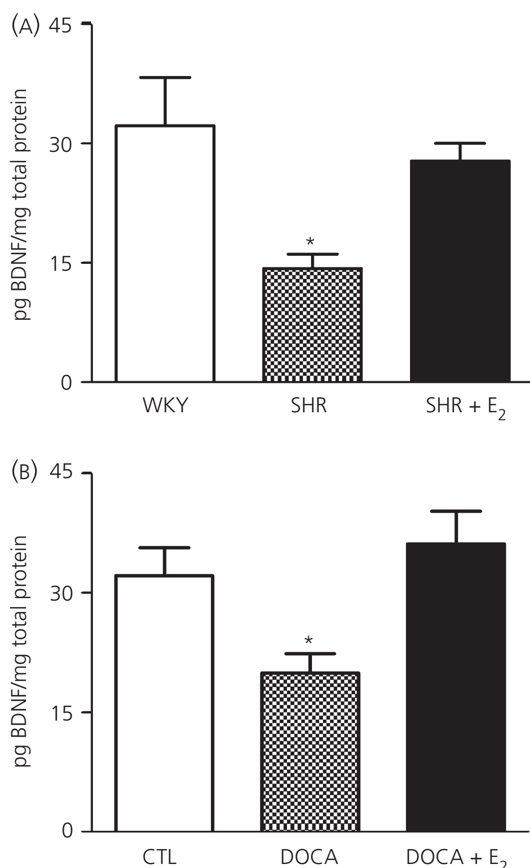

Fig. 3. Determination of brain-derived neurotrophic factor (BDNF) protein (pg/mg hippocampal protein) by enzyme-linked immunosorbent assay in the spontaneously hypertensive rat (SHR) experiment (A) and deoxycorticosterone (DOCA) experiment (B). (A) Levels of BDNF protein were significantly lower in SHR (*$P < 0.05$) compared to the Wistar-Kyoto (WKY) rat and SHR + oestradiol (E_2). (B) DOCA-treated rats showed reduced BDNF protein levels compared to control (CTL) and DOCA + E_2 (*$P < 0.05$). Results expressed as the mean ± SEM of n = 5 animals per group. Statistical analysis was performed by analysis of variance followed by the Bonferroni post-hoc test.

DOCA-treated rats to the level of Sprague–Dawley rats (Fig. 5C) (DOCA versus DOCA plus oestradiol; $P < 0.05$).

We also observed that changes of DCX immunostaining induced by oestradiol treatment of hypertensive animals also comprised the length of DCX immunopositive neuronal processes besides the neuronal soma. Thus, in contrast to the short neurites of steroid-naïve SHR, oestradiol treatment during 2 weeks of SHR significantly enhanced the DCX+ neurite length in this group (Fig. 5B) ($F_{2,9} = 85{,}28$, overall $P < 0.0001$) (SHR versus SHR + E_2; $P < 0.001$). In an analogous manner, the extent of neurites in the DOCA-treated oestrogenised rats was enhanced compared to DOCA-treated rats without steroid treatment, approaching control values (Fig. 5D) ($F_{2,10} = 31.22$; overall $P < 0.0001$) (DOCA versus DOCA + E_2; $P < 0.001$). Thus, quantitative analysis reinforced morphological observations that oestradiol treatment increased the density of DCX+ immature neurones and the length of neurites in the hippocampus of hypertensive rat models.

Discussion

The results obtained in the present study show that oestradiol treatment normalised BDNF mRNA and protein expression of SHR and DOCA-treated rats and enhanced DCX labelling of immature neurones in the subgranular cell layer of the dentate gyrus. In the absence of oestradiol treatment, both parameters were severely compromised in hypertensive strains compared to WKY and Sprague–Dawley control rats. Previous studies have shown that oestrogenisation of SHR and DOCA-treated rats increases cell proliferation and hilar cell number in the dentate gyrus, reduces hippocampal astrogliosis and attenuates the synthesis in the hypothalamus of AVP, a peptide endowed with hypertensinogenic and adrenocorticotrophin-releasing properties (7, 8, 14). Taken together, these studies strengthen the neurotrophic and neurogenic properties of oestrogens in animals suffering from genetic or mineralocorticoid-induced hypertension.

There is evidence that oestrogens *per se* could ameliorate hypertension and normalise brain parameters by decreasing blood pressure acting at the periphery (26, 40). Indeed, oestrogen treatment of SHR and DOCA-treated rats decreases blood pressure and attenuate cardiac hypertrophy, as shown in previous studies (26, 40) and confirmed by the present study. One way to discern between the central and peripheral effects of oestrogen on blood pressure would involve injecting oestrogens directly into the brain. Although this approach is lacking for SHR, central injection of oestrogens prevent the development of high blood pressure in rats with mineralocorticoid/salt-induced hypertension (48). Furthermore, the fact that oestrogens reduce central peptides involved in the pathogenesis of hypertension (i.e. AVP expression in the paraventricular nucleus) in an analogous manner in SHR and DOCA-treated rats (8) also supports the possibility of a central effect of oestrogen in both models.

It is important to discuss the etiopathogenic factors involved in the hippocampal damage of hypertension. Among them, microvascular damage and ischaemia are liable factors (4–6). In addition, there may be enhanced hippocampal vulnerability to overactivation of MR in a hypertension context (11, 15–19), considering the similarity of changes in SHR and rats receiving a mineralocorticoid load. Several studies have assigned a role to the mineralocorticoid system in the brain of SHR and DOCA-treated rats, concerning an abnormal aldosterone hormonal system, hypersensitivity to mineralocorticoids and the effectiveness of MR antagonists with respect to decreasing blood pressure and ischaemic damage (11, 14, 20, 49, 50). Some studies have shown increased MR binding capacity in the hippocampus and hypothalamus of male SHR (16), whereas others have found normal levels of MR and GR mRNA (51). Increased MR levels have been detected in the microcirculation, kidney and heart of SHR (19, 52). We have also found increased MR mRNA and protein in hippocampus and hypothalamus of SHR, employing ISH, real-time polymerase chain reaction and immunocytochemistry (L. Pietranera and A. F. De Nicola unpublished data).

It is presently accepted that hippocampal MR show some peculiarities with respect to the receptor expressed in peripheral organs. Thus, hippocampal MR is considered to be a high affinity receptor

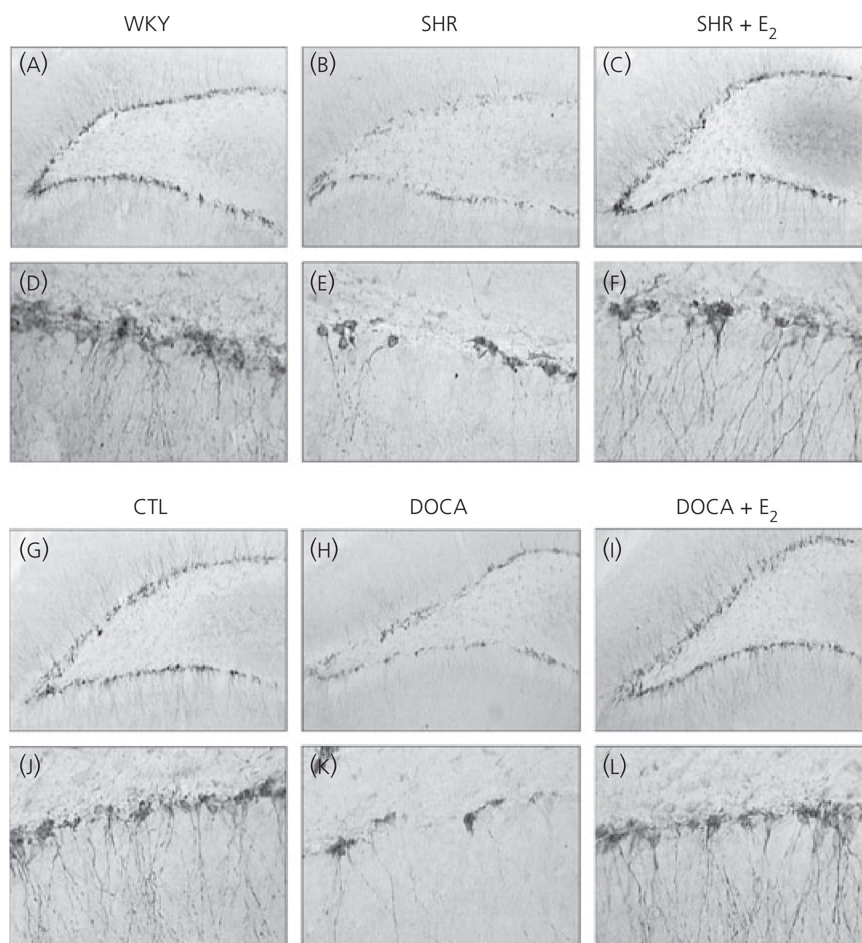

Fig. 4. Morphology of doublecortin (DCX) immunopositive cells (immature neurones) in the dentate gyrus of control and hypertensive rat models with or without oestradiol treatment. (A, B, C) Low magnification of DCX+ cells in Wistar-Kyoto (WKY) rat (A), spontaneously hypertensive rat (SHR) (B) and SHR plus oestradiol-treated rats (C). (D, E, F) High magnification of DCX+ cells in WKY, SHR and SHR plus oestradiol (E_2) groups, respectively. (G, H, I) Low magnification of DCX+ cells in Sprague–Dawley control rat (G), deoxycorticosterone (DOCA) (H) and DOCA plus oestradiol-treated rat (I). (J, K, L) High magnification of DCX+ cells in control, DOCA and DOCA plus oestradiol-treated rat, respectively. Fewer DCX-labelled cells were observed in SHR compared to WKY and SHR plus oestradiol rats. Similarly, DOCA-treated rats expressed fewer DCX+ cells compared to Sprague–Dawley control and DOCA plus oestradiol-treated rats. (A–C, G–I) magnification × 100; (D–F, J–L) magnification × 400.

binding both glucocorticoids and mineralocorticoids. However, hippocampal MR prefers corticosterone, which circulates in high amounts, rather than aldosterone, which circulates in minor amounts. This irregularity is a result of the low hippocampal expression of the enzyme 11β-hydroxysteroid dehydrogenase type 2 that oxidises corticosterone to inactive metabolites (53). Plasma corticosterone levels measured under basal conditions have been reported to be both high or normal in SHR compared to WKY rats (51, 54), although the adrenocortical response to stress is exaggerated in hypertensive rats (10). In this case, stress levels of corticosterone can activate glucocorticoid receptors (GR), an event generally related to detrimental neuronal effects in the hippocampus. However, the literature is not concise on the role of GR in SHR. There are reports of enhanced GR density in the mesenteric microcirculation and pituitary, decreased GR binding capacity in the hypothalamus and normal GR mRNA in hippocampus of SHR (51, 52). Therefore, the likelihood that GR occupation by corticosterone leads to hippocampal abnormalities deserves further appraisal aiming to better understand the role of GR in the SHR strain.

Nevertheless, there is also evidence for MR being overactive in SHR, and this abnormality in receptor function may additionally participate in the neuropathology of SHR and DOCA-treated rats. Three situations indicate a distinctive role of brain MR in SHR. First, it is known that prevailing stress conditions lead to the activation of hippocampal MR by glucocorticoids (53) and, as already pointed out, SHR are hyper-responsive to stress (10). Second, oxidative stress could initiate the pathological ligand-independent activation of MR (55). Third, there is enhanced sensitivity to exogenous mineralocorticoid treatment in SHR (14). The involvement of mineralocorticoids and MR is more obvious in the hypertension of DOCA-treated rats. DOCA-derived deoxycorticosterone can occupy and activate brain MR, eliciting typical mineralocorticoid-responses. These include induction of salt appetite, increased AVP synthesis, increased angiotensin II binding and the development of hyperten-

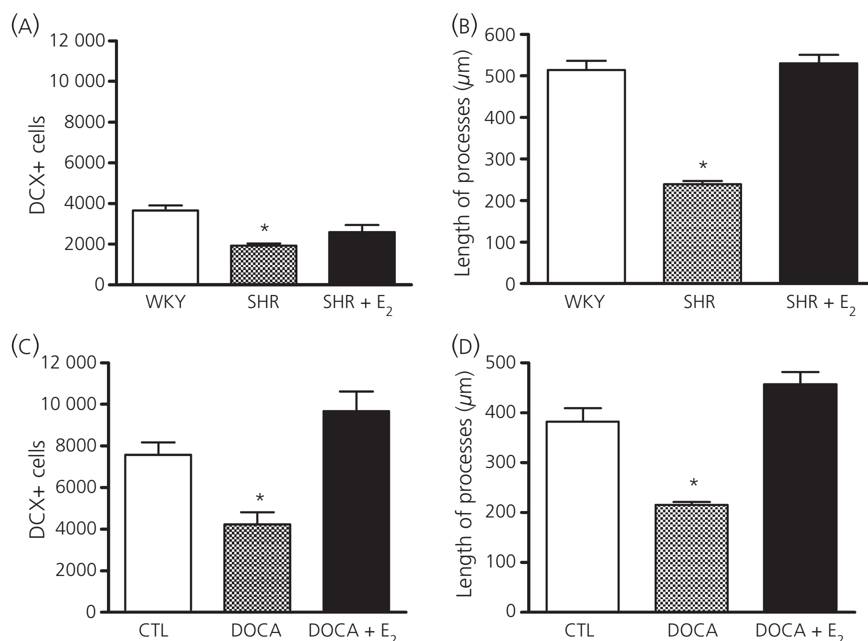

Fig. 5. Morphometric analysis of the number of doublecortin (DCX)-immunopositive cells and the length of DCX-immunoreactive processes in the dentate gyrus of the spontaneously hypertensive rat (SHR) experiment (A, B) and deoxycorticosterone (DOCA) experiment (C, D). (A) DCX+ cell number per dentate gyrus was lower in SHR (*P < 0.05) compared to Wistar-Kyoto (WKY) rat and SHR + oestradiol (E_2). (C) DOCA-treated rats showed the lowest DCX-positive cells compared to Sprague–Dawley control (CTL) and DOCA plus oestradiol (E_2)-treated rats (*P < 0.05) (n = 5 animals per group). (B, D) Length of DCX-immunoreactive cell processes (neurites) was reduced in SHR (B) compared to WKY (P < 0.001) and in DOCA-treated rats (D) compared to control Sprague–Dawley rats (P < 0.01). Oestradiol treatment enhanced the length of neurites in both hypertensive models compared to steroid-naïve SHR (P < 0.001) and DOCA-treated rats (P < 0.001). Data correspond to the mean of six sections per animal and five rats per group. Statistical analysis was performed by analysis of variance followed by the Bonferroni post-hoc test.

sion (13, 56). Furthermore, i.c.v. administration of the antagonists prorenone and RU-28318 block hypertension produced by systemic mineralocorticoid loading (18, 50). However, our hypothesis that MR may be linked to the hippocampus abnormalities of SHR is in apparent conflict with studies showing that MR-mediated actions preserve the integrity and stability of the hippocampus (57). As an alternative, it is possible that MR changes its properties from protective to damaging in a context of hypertension, oxidative stress and ischaemia (11, 15, 18, 19, 55). In a different scenario, corticosterone activation of both MR and GR may contribute to the encephalopathy of SHR.

The neuroendocrine interactions taking place in the brain of SHR and DOCA-treated rats could prime the reduction of BDNF expression in the dentate gyrus. As mentioned in the Introduction, BDNF is down-regulated in the dentate gyrus of hypertensive Wistar rats and SHR with carotid artery occlusion (37, 38). In addition to hypertension, stress and high levels of glucocorticoids are risk factors for BDNF mRNA and protein expression in the hippocampus (58). Therefore, a role for corticosterone in the reduction of BDNF and cell proliferation in SHR cannot be discounted. On the other hand, our experimental evidence suggests that female steroids powerfully stimulate BDNF expression in hypertensive animals. This is in context with the modifications of hippocampal BDNF that occur during the oestrous cycle and the demonstration that oestradiol replacement after ovariectomy increases the levels of BDNF mRNA and protein (29). Oestrogens may induce BDNF transcription as a result of the localisation of an ER element in the BDNF promoter (30). In the present study, the stimulatory effect of oestradiol in the hippocampus was obtained both at the mRNA as well as the protein level. This was an interesting finding because the effect on BDNF mRNA was observed in the granule cell layer of the dentate gyrus, whereas the increase of BDNF protein, as determined by ELISA, was obtained in whole hippocampal homogenates. We propose that oestradiol treatment of hypertensive rats produced two effects on BDNF: one at the level of gene transcription in the dentate gyrus, and the other on translation and secretion of the growth factor from nerve terminals. According to the 'anterophin' hypothesis, locally synthesised BDNF is stored into presynaptic terminals and later released post-synaptically (59). In connection with this event, it has been recently shown that oestrogen stimulates BDNF release from the dentate gyrus (32). This positive interaction between oestradiol and BDNF could result in trophic effects for the hippocampus involving neurogenesis.

Thus, changes of BDNF expression in oestrogen-receiving hypertensive rats may contribute to changes of neurogenesis, considering the intimate association between these events (29). Similar to the effects on BDNF, stress and adrenal steroids could have a direct impact on cell proliferation in the dentate gyrus, considering that neural cell precursors of the hippocampus express MR and GR (60, 61). As a result, changes of circulating adrenal steroids or an

enhanced response to stress could mediate the changes of neurogenesis observed in the hypertensive rats. BDNF expression and cell proliferation in the subgranular and granular zones of the dentate gyrus are highly responsive to oestrogens (29, 33, 34). In certain circumstances, oestradiol also enhances the differentiation of progenitors into immature neurones, according to the increased number of DCX immunolabelled cell bodies and cell processes found in oestradiol-treated middle age mice (24) and presently observed for SHR and DOCA-treated rats receiving oestradiol. The influence of oestradiol on maturation and survival of cells committed to a neural lineage could be BDNF-mediated, because terminal differentiation of newborn neurones in the adult hippocampus is under BDNF control (62). Therefore, middle-aged mice and hypertensive rats become useful tools for unveiling the interactions between oestradiol, growth factors and neurogenesis under pathological conditions. In this regard, it is worth noting that the neuroprotective effects of oestrogens may require special pathophysiological conditions prevailing in SHR because the effects of oestrogen on hippocampal or hypothalamic parameters have not been observed in WKY control rats (7, 8, 14). We are aware that, in the present study, plasma oestradiol levels of rats receiving steroid treatment were in the supraphysiological range. However, this steroid regime normalises the stress response, increases hippocampal glucocorticoid receptors, enhances immunoreactive levels of choline acetyltransferase and stimulates mRNA for the growth-associated protein 43 in the brain of ageing rats (41). Considering that the normal oestradiol content of the rodent hippocampus is already six-fold higher than that in plasma (63), it is possible that high amounts may be required to normalise grossly altered hippocampal parameters.

We propose that modulation of BDNF expression could underlie oestradiol neuroprotection of the hippocampus of SHR and DOCA-salt hypertensive rats. These effects may have important functional and behavioural outcomes for the encephalopathy of hypertensive rats. Thus, further evidence is needed to link changes in the hippocampus with reductions in blood pressure and the behavioural effects provided by natural and synthetic oestrogens in hypertensive rat models.

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References

1 Skoog I, Lernfelt B, Landahl S, Palmertz B, Andreasson LA, Nilsson L, Persson G, Oden A, Svanborg A. 15-year longitudinal study of blood pressure and dementia. *Lancet* 1996; **347**: 1141–1145.

2 Korf ES, White LR, Scheltens P, Launer LJ. Midlife blood pressure and the risk of hippocampal atrophy: the Honolulu Asia Aging Study. *Hypertension* 2004; **44**: 29–34.

3 Petrovitch H, White LR, Izmirilian G, Ross GW, Havlik RJ, Markesbery W, Nelson J, Davis DG, Hardman J, Foley DJ, Launer LJ. Midlife blood pressure and neuritic plaques, neurofibrillary tangles, and brain weight at death: the HAAS. Honolulu-Asia aging Study. *Neurobiol Aging* 2000; **21**: 57–62.

4 Paglieri C, Bisbocci D, Caserta M, Rabbia F, Bertello C, Canade A, Veglio F. Hypertension and cognitive function. *Clin Exp Hypertens* 2008; **30**: 701–710.

5 Sabbatini M, Catalani A, Consoli C, Marletta N, Tomassoni D, Avola R. The hippocampus in spontaneously hypertensive rats: an animal model of vascular dementia? *Mech Ageing Dev* 2002; **123**: 547–559.

6 Tomassoni D, Avola R, Di Tullio MA, Sabbatini M, Vitaioli L, Amenta F. Increased expression of glial fibrillary acidic protein in the brain of spontaneously hypertensive rats. *Clin Exp Hypertens* 2004; **26**: 335–350.

7 Pietranera L, Saravia F, Gonzalez Deniselle MC, Roig P, Lima A, De Nicola AF. Abnormalities of the hippocampus are similar in deoxycorticosterone acetate-salt hypertensive rats and spontaneously hypertensive rats. *J Neuroendocrinol* 2006; **18**: 466–474.

8 Pietranera L, Saravia FE, Roig P, Lima A, De Nicola AF. Protective effects of oestradiol in the brain of rats with genetic or mineralocorticoid-induced hypertension. *Psychoneuroendocrinology* 2008; **33**: 270–281.

9 Kronenberg G, Lippoldt A, Kempermann G. Two genetic rat models of arterial hypertension show different mechanisms by which adult hippocampal neurogenesis is increased. *Dev Neurosci* 2007; **29**: 124–133.

10 Imaki T, Naruse M, Harada S, Chikada N, Nakahuma N, Yoshimoto T, Demura H. Stress-induced changes of gene expression in the paraventricular nucleus is enhanced in spontaneously hypertensive rats. *J Neuroendocrinol* 1998; **10**: 633–643.

11 Rahmouni K, Barthelmebs M, Grima M, Imbs JL, deJong W. Involvement of brain mineralocorticoid receptor in salt-enhanced hypertension in spontaneously hypertensive rats. *Hypertension* 2001; **38**: 902–906.

12 Saavedra JM. Opportunities and limitations of genetic analysis of hypertensiverat strains. *J Hypertens* 2009; **27**: 1129–1133.

13 Vallée S, Grillo C, Gonzalez S, Cosen-Binker L, De Kloet ER, McEwen BS, De Nicola AF. Further studies in DOCA-treated rats: brain content of mineralocorticoid and glucocorticoid receptors and effect of steroid antagonists on salt intake. *Neuroendocrinology* 1995; **61**: 117–124.

14 Pietranera L, Saravia F, Roig P, Lima A, De Nicola AF. Mineralocorticoid treatment upregulates the hypothalamic vasopressinergic system of spontaneously hypertensive rats. *Neuroendocrinology* 2004; **80**: 100–110.

15 Dorrance AM, Rupp NC, Nogueira EF. Mineralocorticoid receptor activation causes cerebral vessel remodeling and exacerbates the damage caused by cerebral ischemia. *Hypertension* 2006; **47**: 590–595.

16 Koch B, Sakly M, Lutz-Bucher B. Specific mineralocorticoid receptors in the hippocampus of spontaneously hypertensive rats. I. Evidence for a sex difference. *Horm Metab Res* 1982; **14**: 166.

17 Hashimoto K, Makino S, Hirasawa R, Takao T, Sugawara M, Murakami K, Ono K, Ota Z. Abnormalities in the hypothalamic-pituitary-adrenal axis in spontaneously hypertensive rats during development of hypertension. *Endocrinology* 1989; **125**: 1161–1167.

18 Gomez-Sanchez EP. Brain mineralocorticoid receptors: orchestrators of hypertension and end-organ disease. *Curr Opin Nephrol Hypertens* 2004; **13**: 191–196.

19 Mirshahi M, Nicolas C, Agarwal MK. Enhanced activation of the mineralocorticoid receptor in genetically hypertensive rats. *Biochem Biophys Res Commun* 1998; **244**: 120–125.

20 Susic D, Varagic J, Ahn J, Matavelli LC, Frohlich ED. Beneficial cardiovascular actions of eplerenone in the spontaneously hypertensive rat. *J Cardiovasc Pharmacol Ther* 2005; **10**: 197–203.

21 Garcia-Segura LM, Azcoitia I, DonCarlos LL. Neuroprotection by oestradiol. *Prog Neurobiol* 2001; **63**: 29–60.

22 Wise PM. Oestrogen therapy: does it help or hurt the adult and aging brain? Insights derived from animal models. *Neuroscience* 2006; **138**: 831–835.

23 Spencer JL, Waters EM, Romeo RD, Wood GE, Milner TA, McEwen BS. Uncovering the mechanisms of oestrogen effects on hippocampal function. *Front Neuroendocrinol* 2008; **29**: 219–237.

24 Saravia F, Beauquis J, Pietranera L, De Nicola AF. Neuroprotective effects of estradiol in hippocampal neurons and glia of middle age mice. *Psychoneuroendocrinology* 2007; **32**: 480–492.

25 Barker JM, Galea LA. Repeated estradiol administration alters different aspects of neurogenesis and cell death in the hippocampus of female, but not male, rats. *Neuroscience* 2008; **152**: 888–902.

26 Jazbutyte V, Arias-Loza PA, Hu K, Widder J, Govindaraj V, Poser-Klein C, Bauersachs J, Fritzemeier KH, Hegele-Hartung C, Neyses L, Ertl G, Pelzer T. Ligand-dependent activation of ER{beta} lowers blood pressure and attenuates cardiac hypertrophy in ovariectomized spontaneously hypertensive rats. *Cardiovasc Res* 2008; **77**: 774–781.

27 Cherney A, Edgell H, Krukoff TL. NO mediates effects of oestrogen on central regulation of blood pressure in restrained, ovariectomized rats. *Am J Physiol Regul Integr Comp Physiol* 2003; **285**: R842–R849.

28 Belo NO, Silva-Barra J, Carnio EC, Antunes-Rodrigues J, Gutkowska J, Dos Reis AM. Involvement of atrial natriuretic peptide in blood pressure reduction induced by oestradiol in spontaneously hypertensive rats. *Regul Pept* 2004; **117**: 53–60.

29 Scharfman HE, MacLusky NJ. Oestrogen and brain-derived neurotrophic factor (BDNF) in hippocampus: complexity of steroid hormone-growth factor interactions in the adult CNS. *Front Neuroendocrinol* 2006; **27**: 415–435.

30 Sohrabji F, Miranda RC, Toran-Allerand CD. Identification of a putative oestrogen response element in the gene encoding brain-derived neurotrophic factor. *Proc Natl Acad Sci USA* 1995; **92**: 11110–11114.

31 Zhou J, Zhang H, Cohen RS, Pandey SC. Effects of oestrogen treatment on expression of brain-derived neurotrophic factor and cAMP response element-binding protein expression and phosphorylation in rat amygdaloid and hippocampal structures. *Neuroendocrinology* 2005; **81**: 294–310.

32 Sato K, Akaishi T, Matsuki N, Ohno Y, Nakazawa K. Beta-Oestradiol induces synaptogenesis in the hippocampus by enhancing brain-derived neurotrophic factor release from dentate gyrus granule cells. *Brain Res* 2007; **1150**: 108–120.

33 Tanapat P, Hastings NB, Gould E. Ovarian steroids influence cell proliferation in the dentate gyrus of the adult female rat in a dose- and time-dependent manner. *J Comp Neurol* 2005; **481**: 252–265.

34 Ormerod BK, Lee TT, Galea LA. Estradiol enhances neurogenesis in the dentate gyrus of adult male meadow voles by increasing the survival of young granule neurons. *Neuroscience* 2004; **128**: 645–654.

35 Isgor C, Watson SJ. Oestrogen receptor alpha and beta mRNA expressions by proliferating and differentiating cells in the adult rat dentate gyrus and subventricular zone. *Neuroscience* 2005; **134**: 847–856.

36 Barha CK, Lieblich SE, Galea LA. Different forms of oestrogens rapidly upregulate cell proliferation in the dentate gyrus of adult female rats. *J Neuroendocrinol* 2009; **21**: 155–156.

37 Hennigan A, Callaghan CK, Kealy J, Rouine J, Kelly AM. Deficits in LTP and recognition memory in the genetically hypertensive rat are associated with decreased expression of neurotrophic factors and their receptors in the dentate gyrus. *Behav Brain Res* 2009; **197**: 371–377.

38 Lee TH, Yang JT, Kato H, Wu JH. Hypertension downregulates the expression of brain-derived neurotrophic factor in the ischemia-vulnerable hippocampal CA1 and cortical areas after carotid artery occlusion. *Brain Res* 2006; **1116**: 31–38.

39 Kageyama H, Nemoto K, Nemoto F, Sekimoto M, Nara Y, Nabika T, Iwayama Y, Fukamachi K, Tomita I, Senba E, Forehand CJ, Hendley ED, Ueyama T. Mutation of the trkB gene encoding the high-affinity receptor for brain-derived neurotrophic factor in stroke-prone spontaneously hypertensive rats. *Biochem Biophys Res Commun* 1996; **229**: 713–718.

40 Si H, Liu D. Genistein, a soy phytooestrogen, upregulates the expression of human endothelial nitric oxide synthase and lowers blood pressure in spontaneously hypertensive rats. *J Nutr* 2008; **138**: 297–304.

41 Ferrini M, Piroli G, Frontera M, Falbo A, Lima A, De Nicola AF. Oestrogens normalize the hypothalamic-pituitary-adrenal axis response to stress and increase glucocorticoid receptor immuno-reactivity in hippocampus of aging male rats. *Neuroendocrinology* 1999; **69**: 129–137.

42 Gonzalez SL, Labombarda F, Gonzalez Deniselle MC, Guennoun R, Schumacher M, De Nicola AF. Progesterone up-regulates neuronal brain-derived neurotrophic factor expression in the injured spinal cord. *Neuroscience* 2004; **125**: 605–614.

43 Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*, 3rd edn, Sydney: Academic Press, 1997.

44 Maisonpierre PC, Le Beau MM, Espinosa RIII, Ip NY, Belluscio L, de la Monte SM, Squinto S, Furth ME, Yancopoulos GD. Human and rat brain-derived neurotrophic factor and neurotrophin-3: gene structures, distributions, and chromosomal localizations. *Genomics* 1991; **10**: 558–568.

45 He J, Nixon K, Shetty AK, Crews FT. Chronic alcohol exposure reduces hippocampal neurogenesis and dendritic growth of newborn neurons. *Eur J Neurosci* 2005; **21**: 2711–2720.

46 Howard V, Reed M. Unbiased stereology. *Three-Dimensional Measurement in Microscopy*. Oxford: BIOS, 1998.

47 Sliwowska JH, Barker JM, Barha CK, Lan N, Weinberg J, Galea LA. Stress-induced suppression of hippocampal neurogenesis in adult male rats is altered by prenatal ethanol exposure. *Stress* 2010; **13**: 301–313.

48 Xue B, Badaue-Passos D Jr, Guo F, Gomez-Sanchez CE, Hay M, Johnson AK. Sex differences and central protective effect of 17beta-estradiol in the development of aldosterone/NaCl-induced hypertension. *Am J Physiol Heart Circ Physiol* 2009; **296**: H1577–H1585.

49 Kumar NN, Goodchild AK, Li Q, Pilowsky PM. An aldosterone-related system in the ventrolateral medulla oblongata of spontaneously hypertensive and Wistar-Kyoto rats. *Clin Exp Pharmacol Physiol* 2006; **33**: 71–75.

50 van den Berg DT, De Kloet ER, de Jong W. Central effects of mineralocorticoid antagonist RU-28318 on blood pressure of DOCA-salt hypertensive rats. *Am J Physiol Endocrinol Metab* 1994; **30**: E927–E933.

51 Sutanto W, Oitzl MS, Rots NY, Schobitz B, Van den Berg DT, Van Dijken HH, Mos J, Cools AR, Tilders FJ, Koolhaas JM, de Kloet ER. Corticosteroid receptor density in the central nervous system of various rat models. *Endocr Regul* 1992; **26**: 111–118.

52 DeLano FA, Schmid-Schonbein GW. Enhancement of glucocorticoid and mineralocorticoid receptor density in the microcirculation of the spontaneously hypertensive rat. *Microcirculation* 2004; **11**: 69–78.

53 De Kloet ER, Karst H, Joels M. Corticosteroid hormones in the central stress response: quick-and-slow. *Front Neuroendocrinol* 2008; **29**: 268–272.

54 Djordjevic J, Vuckovic T, Jasnic N, Cvijic G. Effect of various stressors on the blood ACTH and corticosterone concentration in normotensive Wistar and spontaneously hypertensive Wistar-Kyoto rats. *Gen Comp Endocrinol* 2007; **153**: 217–220.

55 Nagase M, Fujita T. Mineralocorticoid receptor activation in obesity hypertension. *Hypertens Res* 2009; **32**: 649–657.

56 Gutkind JS, Kurihara M, Castren E, Saavedra JM. Increased concentration of angiotensin II binding sites in selected brain areas of spontaneously hypertensive rats. *J Hypertens* 1988; **6**: 79–84.

57 Joëls M, Karst H, DeRijk R, de Kloet ER. The coming out of the brain mineralocorticoid receptor. *Trends Neurosci* 2008; **31**: 1–7.

58 Schaaf MJ, de Jong J, de Kloet ER, Vreugdenhil E. Downregulation of BDNF mRNA and protein in the rat hippocampus by corticosterone. *Brain Res* 1998; **813**: 112–120.

59 Altar CA, Cai N, Bliven T, Juhasz M, Conner JM, Acheson AL, Lindsay RM, Wiegand SJ. Anterograde transport of brain-derived neurotrophic factor and its role in the brain. *Nature* 1997; **389**: 856–860.

60 Cameron HA, Gould E. Adult neurogenesis produces is regulated by adrenal steroids in the dentate gyrus. *Neuroscience* 1994; **61**: 203–209.

61 Wong EY, Herbert J. Roles of mineralocorticoid and glucocorticoid receptors in the regulation of progenitor proliferation in the adult hippocampus. *Eur J Neurosci* 2005; **22**: 785–792.

62 Lee E, Son H. Adult hippocampal neurogenesis and related neurotrophic factors. *BMB Rep* 2009; **42**: 239–244.

63 Hojo Y, Hattori TA, Enami T, Furukawa A, Suzuki K, Ishii HT, Mukai H, Morrison JH, Janssen WG, Kominami S, Harada N, Kimoto T, Kawato S. Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons. *Proc Natl Acad Sci USA* 2004; **101**: 865–870.