Journal of Steroid Biochemistry & Molecular Biology 146 (2015) 15–25

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Review

Estrogens are neuroprotective factors for hypertensive encephalopathy

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ARTICLE INFO

Article history:
Received 12 February 2014
Received in revised form 4 April 2014
Accepted 7 April 2014
Available online 13 April 2014

Keywords:
Hypertensive rats
Estradiol
Neurogenesis, Astrogliosis, Growth factor
Dendrites
Spinogenesis
Vasopressin
Neuroprotection

ABSTRACT

Estrogens are neuroprotective factors for brain diseases, including hypertensive encephalopathy. In particular, the hippocampus is highly damaged by high blood pressure, with several hippocampus functions being altered in humans and animal models of hypertension. Working with a genetic model of primary hypertension, the spontaneously hypertensive rat (SHR), we have shown that SHR present decreased dentate gyrus neurogenesis, astrogliosis, low expression of brain derived neurotrophic factor (BDNF), decreased number of neurons in the hilus of the dentate gyrus, increased basal levels of the estrogen-synthesizing enzyme aromatase, and atrophic dendritic arbor with low spine density in the CA1 region compared to normotensive Wistar Kyoto (WKY) ratsl. Changes also occur in the hypothalamus of SHR, with increased expression of the hypertensinogenic peptide arginine vasopressin (AVP) and its V1b receptor. Following chronic estradiol treatment, SHR show decreased blood pressure, enhanced hippocampus neurogenesis, decreased the reactive astrogliosis, increased BDNF mRNA and protein expression in the dentate gyrus, increased neuronal number in the hilus of the dentate gyrus, further increased the hyper-expression of aromatase and replaced spine number with remodeling of the dendritic arbor of the CA1 region. We have detected by qPCR the estradiol receptors ERα and ERβ in hippocampus from both SHR and WKY rats, suggesting direct effects of estradiol on brain cells. We hypothesize that a combination of exogenously given estrogens plus those locally synthesized by estradiol-stimulated aromatase may better alleviate the hippocampal and hypothalamic encephalopathy of SHR.

This article is part of a Special Issue entitled "Sex steroids and brain disorders".

Contents

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http://dx.doi.org/10.1016/j.jsbmb.2014.04.001
0960-0760/

1. The hippocampus in human and experimental hypertension

Hypertension is referred to as the "silent killer", because it gives no warning signs or symptoms of the incoming pathology. According to statistics from the World Health Organization, raised blood pressure causes 7.5 million deaths per year worldwide, about 12.8% of the total of all deaths. High blood pressure places a strong stress on target organs including the brain. Within the different brain structures, the hippocampus is highly vulnerable to the effects of hypertension. Patients suffering from chronic essential hypertension present atrophy of hippocampus and temporal lobe, with morphological evidence of remodeling of the microvascular wall, ischemia, cytotoxic edema, demyelination, micro infarcts, beta-amyloid deposits and tau pathology. These changes may be accompanied by cognitive decline [1–7]. Hippocampal dysfunction is frequent in elderly hypertensive subjects, leading to high incidence of small hippocampus volume and increased risk of dementia [8].

Animal models become valuable tools to study brain changes of hypertension. The spontaneously hypertensive rat (SHR), originally developed in 1960s by Okamoto and colleagues in Japan, is by far the most widely used rat model of primary hypertension. Sabbatini and coworkers [9,10] were pioneers in describing regressive changes and astroglial reaction in the hippocampus from SHR, similar to those occurring in neurodegenerative disorders with cognitive impairment. These authors have pointed out that SHR represent an animal model of vascular dementia and Alzheimer's disease, considering their flourished hippocampus pathology. Studies in the brain of SHR disclosed a range of disturbances, such as hydrocephalus, increased expression of the astrocytic marker glial fibrillary acidic protein (GFAP), blood–brain barrier disruption, cytoskeletal breakdown, decreased growth factor expression, decreased forebrain white matter volume, abnormal neurogenesis and hyperstimulation of the vasopressinergic and angiotensinergic systems [11–15]. Changes in learning and memory displayed by SHR made them also models of dementia and the attention-deficit hyperactivity syndrome [7]. Taking into consideration the high incidence of abnormalities in humans and rodents, the expression "hypertensive encephalopathy" has been coined as far back as 1928 to describe the brain damage caused by a persistent elevation of blood pressure [16].

Gender differences in the development of cardiovascular diseases and hypertension have suggested that female steroids exert a protective function in humans and rodent models. For example, it is widely recognized that during menopause transition, declining estrogen levels in women constitutes a risk factor for hypertension [17,18]. Female SHR suffer from alterations of the reproductive axis, including defects of ovarian development, decreased steroidogenesis and poor responsiveness to gonadotropins [19]. In fact, loss of gonadal hormones due to ovariectomy aggravates the hypertension of SHR [20]. Several reports have given proof that treatment with natural or synthetic estrogens decrease blood pressure and show protective effects on the brain and the cardiovascular system of SHR [21–24]. In women, however, the situation is less clear and conflicting results have been reported. The Women Health Initiative trial employing estrogen alone has shown increased risk of dementia and cerebrovascular disease [25]. However, these data have been reinterpreted based on the selection of patient cohort and the timing of estrogen replacement therapy after cessation of menses. Recent reports have accentuated that early initiation of steroid treatment after loss of ovarian hormones may prove beneficial, suggesting the existence of a window of opportunity for estrogen neuroprotection [26–28].

In this review we describe our contributions to unravel 17β-estradiol effects in the brain of SHR, emphasizing the pharmacological relevance of estrogens for the prevention of hypertensive encephalopathy. To avoid the confounding issue of ovarian cyclicity, we routinely used male normotensive Wistar Kyoto rats and male SHR. Our experiments demonstrated that hypertensive – but not normotensive – rats were highly sensitive to estrogen treatment, supporting that tissue microenvironment plays an important role for steroid responsiveness and for neuroprotection.

2. Neuroprotective effects of estrogens in the hippocampus

Estrogen modulation of hippocampus functions has strong implications for the management of inflammation, trauma, ischemia, neurodegeneration and changes of higher cognitive functions caused by aging and neurological disorders. There is unanimous support for the view that estrogens qualify as hippocampus "neuroprotectans". The pleiotropic effects of 17β-estradiol in the hippocampus involves the prevention of excitotoxicity, inflammation and oxidant injury, the inhibition of apoptosis with stimulation of the anti-apoptotic gene Bcl_2, increased neuronal survival, the regulation of dendritic remodeling, synaptogenesis and spinogenesis and the regulation of a plethora of neurotransmitters: acetylcholine, dopamine, serotonine, catecholamines, glutamate and GABA [28–34]. Although these effects may be genomically-mediated, extranuclear sites of estrogen action involving membrane, synaptic and mitochondrial sites are also likely players in neuroprotection. In molecular terms, estrogen actions via activation of nuclear or extranuclear binding sites regulate the phosphatydylinositol 3-kinase, a Ca^{2+} independent protein kinase C isoform, Src kinase, mitogen-activated kinase, phosphorylation of AKT, the LIM kinase and also regulate Ca^{2+} influx and the ERK 1/2 pathways [35]. In response to 17β-estradiol, mitochondrial sequestration of Ca^2 play an important role for ion homeostasis and cell survival [27,28]. Estrogens also increase dendritic spine formation and synaptic density in CA1 pyramidal cells, an effect probably mediated by estrogen receptors (ER). Gould et al. [36] and Herrick et al. [37] have detected extranuclear ERβ immunoreactivity in doublecortin (DCX) positive, newly born cells of the dentate gyrus.

Further information on estrogen neuroprotection has been provided by culture studies. In hippocampal neurons in culture, estrogens protect against glutamate toxicity, glucose deprivation, FeSO toxicity and amyloid-peptide toxicity, the hallmark of Alzheimer's disease [30]. According to Azcoitia et al. [26] and Garcia Segura et al. [38], 17β-Estradiol interaction with IGF1 is an important neuroprotective mechanism. Some of the estrogen effects could be genomically mediated, after interaction of ligand with ER. Estrogen binding has been reported in the hippocampal pyramidal cells and the hilus of the dentate gyrus. Of the two isoforms of the estrogen receptor, ERα and ERβ the β isoform is abundantly expressed in hippocampus, whereas ERα is found in CA1 interneurons and a subset of pyramidal and granule cells [35,39].

Besides the classical concept that ERα and ERβ are the predominant nuclear receptors involved in many effects of estrogen, modulation of cell-signaling pathways also occurs via membrane estrogen receptors, such as the G protein coupled receptor (GPER). This receptor participates in the control of several hippocampal functions, including neuritogenesis [40]. It is also known that ligand-bound GPER regulates vasomotor tone, delays development of hypertension [41], and plays a protective function in the cardiovascular system of SHR [42]. Therefore, a relevant endeavor would be to establish a link between nuclear and extranuclear sites with the estrogen positive stimulation of neurochemical parameters in hypertensive encephalopathy.

Functionally, estrogens play an important role in the hippocampus related to limbic-associated behaviors, learning and memory. One way estrogens modulate these processes is by enhancement of neurogenesis: the proliferation, migration and differentiation of new neurons in the dentate gyrus of adult animals [43]. In this region, and in the subventricular zone, neurogenesis continues into adulthood. For instance, uptake of the thymidine analog bromodeoxyuridine (BrdU) by proliferating cells of the dentate gyrus is higher in adult proestrus than in estrus rats, suggesting the participation of endogenous hormones. In this sense, BrdU+ cells are more abundant in ovariectomized-estrogen replaced rats than in ovariectomized-vehicle treated rats. [45]. A gender difference has been suggested, because in males the response of hippocampal neurogenesis to 17β-estradiol is attenuated. In our experience, a ceiling effect probably accounts for the failure of 17β-estradiol to increase granule cell proliferation in normal male mice. The effect on neurogenesis seems ER-mediated, since both ERα and ERβ mRNA are found in 80% of proliferating cells of the dentate gyrus labeled with the Ki67 antibody, and in an important proportion of cells showing a more mature phenotype.

From the point of view of neuroprotection, it is highly rewarding that estrogen beneficial effects are now expanded to models of diabetes mellitus, stroke, aging, ischemia, multiple sclerosis, neuroinflammation, Parkinson's disease and Alzheimer's disease [45–52]. The pronounced steroid responsiveness offer promise for treating different diseases of the CNS.

3. Levels of 17β-estradiol and estradiol receptor isoforms (ERα and ERβ) in normotensive Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR)

To study estrogens effects in the hippocampus of SHR, our treatment protocol consisted of implanting under the skin of the neck a single pellet containing 12 mg pure 17β-estradiol diluted in cholesterol or cholesterol vehicle. This protocol was initially used to preserve the cholinergic system of aging rats [53]. Measurements of serum 17β-estradiol by radioimmunoassay 2 weeks after pellet implantation showed substantial elevations of 17β-estradiol in the steroid-treated WKY or SHR groups compared to the cholesterol implanted rats. Statistical comparisons showed that basal 17β-estradiol levels of steroid-untreated male rats were similar between WKY rats (39.8 ± 11.6) and SHR (35.2 ± 2.8 pg/ml serum). A large increase in serum 17β-estradiol levels was found in steroid-treated rats, without significant differences between WKY rats (1501 ± 171.8) and SHR (983.4 ± 91.6) pg/ml serum ($p < 0.001$ vs. respective untreated group). However, estrogen levels in the hippocampus can be higher than plasma levels, due to local synthesis of estrogens [51]. Analysis of several parameters in hippocampus and hypothalamus demonstrated that pharmacological estrogen levels effectively arrested hypertensive encephalopathy.

A further step consisted in determining if the response to 17β-estradiol was conditioned by differential expression of ER in hippocampus between normotensive and hypertensive rats. In this regard, the hippocampus expresses the two isoforms of the classical intracellular estrogen receptors, ERα and ERβ [35,39]. Using real time PCR and primer-specific isoforms, we compared the mRNA expression of ERα and ERβ in whole hippocampus tissue from WKY rats and SHR with and without 17β-estradiol treatment. Without steroid treatment, the hippocampus of normotensive and hypertensive rats showed similar levels of Erα mRNA (WKY: 0.2623 ± 0.0007; SHR: 0.2772 ± 0.044 arbitrary units (a.u.); $n = 5$ animals per goup). Likewise, no differences were found regarding Erβ mRNA (WKY: 0.2486 ± 0.052; SHR: 0.2340 ± 0.090 a.u., $n = 5$). Furthermore, after receiving the 2 week 17β-estradiol treatment, statistical analysis did not show differences in hippocampus Erα mRNA levels between normotensive and hypertensive rats (WKY: 0.2272 ± 0.033; SHR: 0.2394 ± 0.0173 a.u., $n = 5$) or in Erβ mRNA levels (WKY: 0.3338 ± 0.659; SHR: 0.359 ± 0.1354 a.u., $n = 5$).

It should be recognized, however, that other potential mechanisms were not studied, and may also account for differences in estrogen effects in the hippocampus of WKY rats and SHR. The role of these alternative mechanisms of estrogen action in hypertension remains an important field of investigation, as discussed above for GPER. So far, available data suggest that potential changes of estrogen responsiveness between WKY and SHR could not be ascribed to differences in circulating 17β-estradiol levels, or to discrepancies in the hippocampus expression of ERα or ERβ between normotensive and hypertensive animals.

4. Effects of 17β-estradiol on brain derived neurotrophic factor and restoration of hippocampal neurogenesis in SHR

Evidence has been presented that BDNF could mediate some estrogen effects in the brain. BDNF is a potent modulator of neuronal functions in the hippocampus, including neurotransmission, memory formation and neurogenesis [55]. In many ways, BDNF effects resemble the neuroprotective effects of estrogens in the hippocampus [56,57]. An estrogen receptor response element has been found on the BDNF gene promoter, supporting that estrogens directly regulate BDNF gene expression [58–62]. Estrogens also increase the release of BDNF from the dentate gyrus, one of the two neurogenic zones of the adult brain. Of great interest is that BDNF expression is down-regulated in hypertension and ischemia. This has been shown in a genetically hypertensive Wistar rats, in which impaired learning and memory correlates with decreased BDNF expression in the dentate gyrus [63] and in SHR receiving carotid artery occlusion, in which BDNF mRNA and protein are further decreased in the CA1 region and cortical areas [64]. Finally, a mutation in the BDNF specific receptor TrkB gene in the stroke-prone SHR [65], impairs its neurotrophic function. Thus, cumulative evidence suggests that BDNF expression and hypertension are negatively interrelated.

To analyze the effects of 17β-estradiol on the expression of BDNF, the response of this neurotrophic factor was measured in the hippocampus of normotensive and hypertensive rats [66]. WKY rats and SHR were exposed to 17β-estradiol for 2 weeks, following our standard protocol. Levels of BDNF mRNA were studied by radioactive in situ hybridization with a ^{35}S-labeled oligoprobe, and its expression was quantified by computerized image analysis of film autoradiograms. The signal for BDNF mRNA was very strong 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 the autoradiograms of SHR compared to those of WKY rats (Fig. 1). This reduction was more accentuated in the granule cell layer of the dentate gyrus, whereas the mRNA signal of the CA1-CA4 regions of the hippocampus remained slightly attenuated in control and hypertensive animals. Furthermore, 17β-estradiol administration to SHR rescued the signal intensity of film autoradiograms to levels of WKY rats, as shown by statistical comparison of the optical density of films autoradiograms (Fig. 1, lower graph). When the same groups and treatment regime were compared for the hippocampus CA1 region (taken as representative of the pyramidal cell area), changes did not replicate those of the granule cell layer of the dentate gyrus, reinforcing that pyramidal cell areas from hypertensive animals were spared from changes of BDNF mRNA levels. The BDNF mRNA studies were complemented with determinations of BDNF protein levels in hippocampus by ELISA. We found that BDNF protein content was reduced by half in steroid naïve SHR compared to WKY rats. In agreement with changes obtained for BDNF mRNA, 17β-estradiol treatment

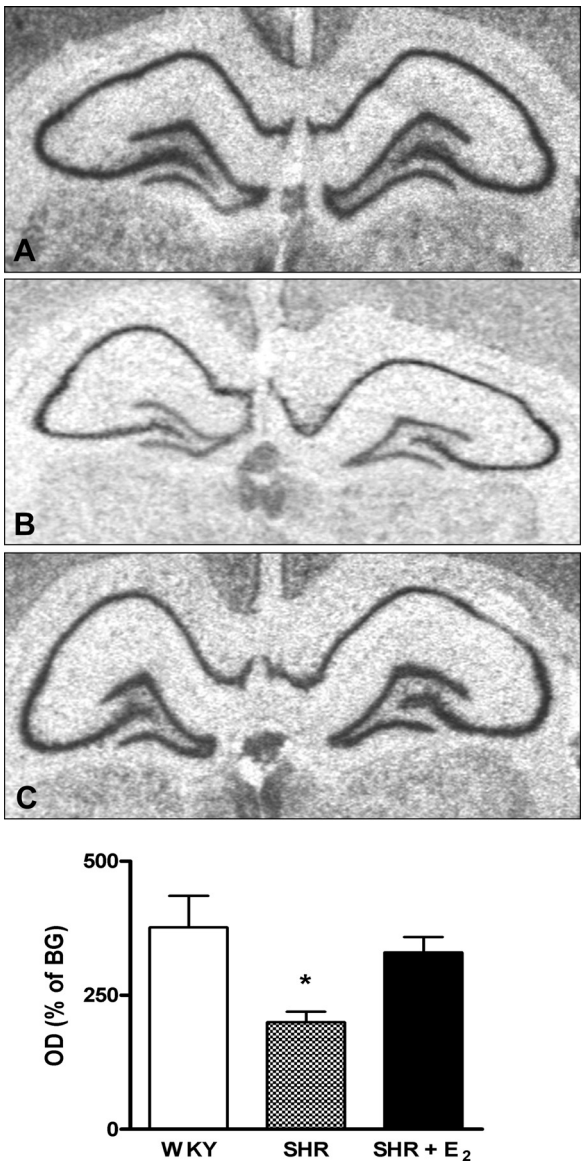

Fig. 1. *Upper images*: Representative film autoradiograms of in situ hybridization of BDNF mRNA in a normotensive WKY rat (A); SHR (B) and SHR treated with 17β-estradiol (C). All animals were 5 months-old at the time of treatment. In the SHR, 17β-estradiol treatment preferentially increased BDNF mRNA in the dentate gyrus. *Lower graph*: Optical density (OD) expressed as % of background (BG) of BDNF mRNA measured on film autoradiograms of the dentate gyrus. Group labeling as in the legend to figure. In the granule cell layer of the dentate gyrus, OD for SHR was significantly lower than WKY rats and SHR plus 17β-estradiol (*$p < 0.05$). Results correspond to the mean ± S.E.M. of $n = 5$ animals per group. Statistical analysis was performed by ANOVA followed by the Bonferroni post hoc test.
Modified from Ref. [66] with permission.

up-regulated BDNF protein of SHR to levels similar of those of WKY normotensive rats.

An important function of BDNF in the hippocampus is the regulation of neurogenesis. It is known that cell proliferation, progenitor survival and terminal differentiation of newborn neurons is under BDNF control [55,67–69]. Because 17β-estradiol increased BDNF mRNA and protein levels in SHR, it was likely that steroid treatment also modulated hippocampal neurogenesis. Of the steps of adult neurogenesis, it has been reported that estrogens increase cell proliferation [44], whereas doublecortin (DCX)-labeled neuronal progenitors are also stimulated by estrogen treatment of aging animals [49]. Estrogen effects on neurogenesis may be genomic, because 80% of proliferating cells of the dentate gyrus express the ERα and ERβ isoforms [70]. The presence of extranuclear ER also suggests non-genomic mechanisms [71]. Initially, *we* studied if there were differences in cell proliferation between normotensive and hypertensive rats by using bromodeoxyuridine uptake by cells located in the subgranular zone and granular cell layer of the dentate gyrus. Quantitation of BrdU+ cell number demonstrated low uptake of this thymidine analog in SHR compared to WKY rats, which was increased in the hypertensive group receiving estrogen [72]. To elucidate if 17β-estradiol stimulated a further step of neurogenesis, we analyzed the population of neural progenitors (neuroblasts), that express the doublecortin (DCX) antigen [73]. Differences in DCX+ cell morphology were readily distinguished between WKY rats and SHR without and with 17β-estradiol treatment (Fig. 2). Thus, abundant DCX+ cells were observed in the subgranular cell layer of the dentate gyrus of WKY rats, with evidences of strongly stained cell bodies and cell processes (Fig. 2A). This pattern contrasted with the 50% reduction of DCX+ cell number in SHR, which showed an atrophic morphology, reduced staining intensity and fewer cell processes (Fig. 2B). Following 17β-estradiol treatment, cells of SHR modified this profile, with the appearance of highly branched DCX+ cells of enhanced staining intensity in the subgranule cell layer (Fig. 2C). These morphological observations were validated by computerize esterology demonstrated that 17β-treatment increased two steps of neurogenesis in SHR, i.e., cell proliferation and neuronal progenitors (Fig. 2, lower graph). In conclusion, the hippocampus of SHR was distinguished by a low BDNF expression and deficient neurogenesis, whereas both parameters were normalized in the hippocampus of hypertensive animals receiving 17β-estradiol.

5. Effects of 17β-estradiol on hypothalamic arginine-vasopressin (AV) expression and blood pressure levels of SHR

The brain maintains a balance between the factors that decrease or increase blood pressure. The central control of blood pressure is under tight regulatory control by different factors, including those of endocrine origin. Among the latter, estrogens decrease blood pressure by favoring vasodilator agents such as nitric oxide (NO), atrial natriuretic peptide and vascular endothelial growth factor (VEGF), whereas hypertensive mechanisms including the central renin-angiotensin system and the activity of the sympathetic nervous system are down-regulated [74–77] This selective regulation explains the reduction of blood pressure and attenuated damage caused by ischemia and stroke in the presence of estrogens [32,78,79]. Binding of estrogens to ERβ may play a substantial protective role in these effects. The ERβ subtype is abundantly expressed in the hypothalamic paraventricular nucleus (PVN) that synthesizes arginine vasopressin (AVP) [39]. In the hypothalamus, this hypertensinogenic peptide is under negative regulation by estradiol [80]. The work of Nomura et al. [80] has arrived at this conclusion because ERβ activation in wild mice decreases AVP gene expression in the paraventricular nucleus, but it is not effective in ERβ KO mice [80].

Therefore, it is important to underline that increased expression of AVP, which may aggravate hypertension, has been found in the hypothalamus of SHR [11,81]. In our studies, we employed non isotopic in situ hybridization to compare if 17β-estradiol controls the expression of AVP mRNA in the hypothalamus of SHR and WKY. We found that AVP mRNA was moderately abundant in cells located in the magnocellular posterior portion of the PVN of normotensive WKY rats, whereas stronger expression of AVP mRNA was found in SHR [11]. The AVP transcript was also expressed by cells located not only in the posterior magnocellular portion but also in the medial portion of the magnocellular PVN of SHR. After receiving

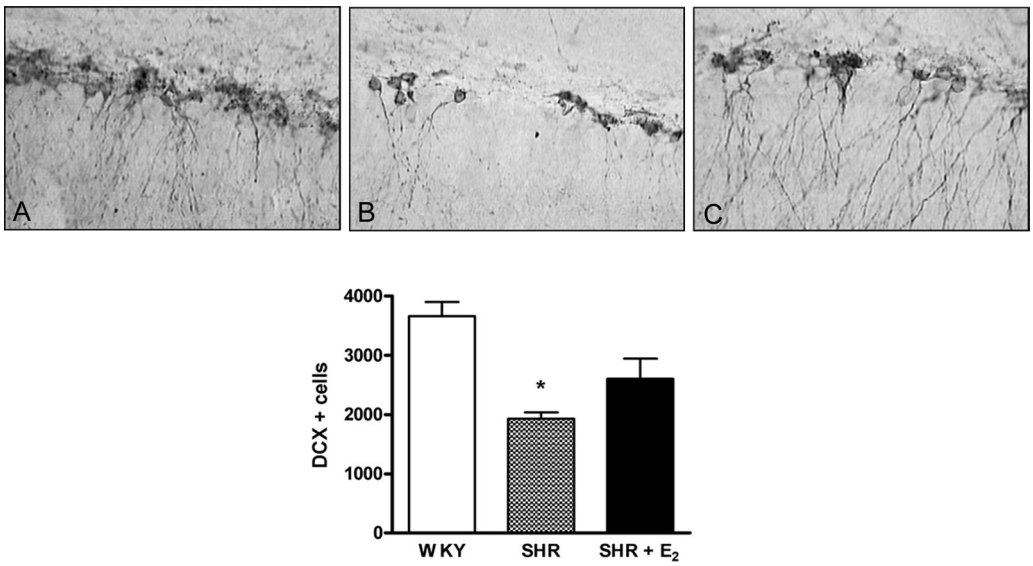

Fig. 2. Morphology of doublecortin (DCX) positive cells in the subgranular zone of the dentate gyrus in a WKY rat (A), SHR (B) and SHR plus 17β-estradiol treated rat (C). Fewer DCX-labeled neuroblasts were present in SHR respect of WKY and SHR plus estradiol. Magnification 400×. Lower graph Quantitative analysis of the number of DCX-immunopositive cells resulted lower in SHR ($^*p < 0.05$) respect of WKY and SHR + 17β-estradiol. Results expressed as mean number of stained cells per dentate gyrus ± S.E.M. of $n = 5$ animals per group. Statistical analysis was performed by ANOVA followed by the Bonferroni post hoc test.
Modified from Ref. [66] with permission.

17β-estradiol for 2 weeks, the AVP mRNA signal was considerable attenuated in SHR, changes validated by quantitative image analysis. Although increased AVP synthesis may have been involved in the hypertension of SHR, the issue is still debatable [82]. However, blockade of AVP receptors with a V1 receptor antagonist attenuates genetic hypertension [81]. Thus, estrogen reduction of AVP mRNA expression could have a negative impact on hypertension., and be part of the neuroprotective mechanism of the steroid in the brain of SHR.

The hypotensive potential of 17β-estradiol was studied in WKY rats and SHR by measuring blood pressure of non-anesthetized animals using a non-invasive tail cuff method. Basal blood pressure measured 180.4 ± 4.7 in SHR and 118 ± 3.2 mm Hg in WKY rats. In both groups, blood pressure was decreased after 17β-estradiol treatment, although SHR remained moderately hypertensive (151.5 ± 6.2 mmHg). A two-way ANOVA of blood pressure levels revealed a main effect of strain ($p < 0.0001$) and steroid treatment ($p < 0.01$). However, the hypotensive effects of 17β-estradiol may be explained only in part by down-regulation of AVP [69], because Firmes et al. [83] recently reported that the decrease of blood pressure of SHR by 17β-estradiol was associated to changes of several components of the natriuretic peptide system. In summary, hypertension under ovarian steroid deprivation could be due to several contributing factors [84], whereas estrogen-induced decrease of blood pressure may also involve several central and peripheral mediators.

6. 17β-Estradiol restores length of dendrites and spine density in the hippocampus of SHR

A pronounced regional heterogeneity exists for changes of the dendritic arbor and spines of the hippocampus in response to physiological and pathologic stimuli. In pathological conditions, pyramidal neurons of the CA1 subfield show high vulnerability associated to excitotoxic hyperactivation of n-methyl-d-aspartate (NMDA) receptors [85]. In this regard, NMDA agonists added to organotypic cultures of hippocampus causes a larger increase of intracellular calcium in the CA1 rather than in the CA3 region [86]. This susceptibility explains why a selective death of hippocampus CA1 neurons occurs after ischemia [87]. Increased vulnerability of CA1 neurons has also been reported in stroke-prone spontaneously hypertensive rat (SHRSP) [88]. In a recent study, decreased spine density from hippocampal pyramidal neurons of 8 months old SHR have been demonstrated [89], expanding the vulnerability hypothesis to the effects of aging and hypertension.

Further expression of the specific vulnerability of CA1 neurons include the reduced dendritic branching, dendritic arbor retraction, loss of dendrites and changes of spine density and morphology caused by inflammatory factors [90], hypobaric hypoxia [91], old age [92], global cerebral ischemia [93], neurodegeneration of Alzheimer' transgenic mice [94] and type I diabetes mellitus [95]. It has been also recognized that the strong dendritic remodeling and atrophy caused in the CA3 region by chronic stress and glucocorticoid treatment may also occur in the CA1 pyramidal cells [96–98]. Regarding physiological effects, Gould et al. [36] first noticed that CA1 but not CA3 or C4 pyramidal cell dendritic spine density fluctuate depending on the estrogenic stage of the sex cycle. These findings led to hypothesize that fluctuation of female steroids impact upon the CA1 pyramidal cells, whereas stress and high glucocorticoids levels show a more selective effect upon the CA3 region [36,96–98].

In view of its high degree of vulnerability, we studied if 17β-estradiol normalized the defects of dendritic arbor and spine density in the CA1 hippocampus subfield of SHR. Using a modified Golgi impregnation technique and rigorous morphological criteria to determine dendritic staining, a two dimensional reconstruction of the dendritic tree of the CA1 was carried out in 4 groups of rats: WKY with and without 17β-estradiol and SHR with and without 17β-estradiol [99]. Camera lucida drawings of the Golgi-impregnated dendritic trees of representative CA1 pyramidal neurons from steroid-untreated WKY and SHR, and WKY and SHR receiving 17β-estradiol are shown in Fig. 3. WKY rats without or with estradiol (Fig. 3a and b) showed a well developed dendritic tree in contrast to the atrophic profile of SHR (Fig. 3c and d). The low dendritic length of SHR was normalized by 17β-estradiol treatment (Fig. 3e and f). Quantitative measurements of the apical dendritic tree confirmed a significant decrease in SHR compared to the other three groups (Fig. 3B). We also observed a preferential effect of

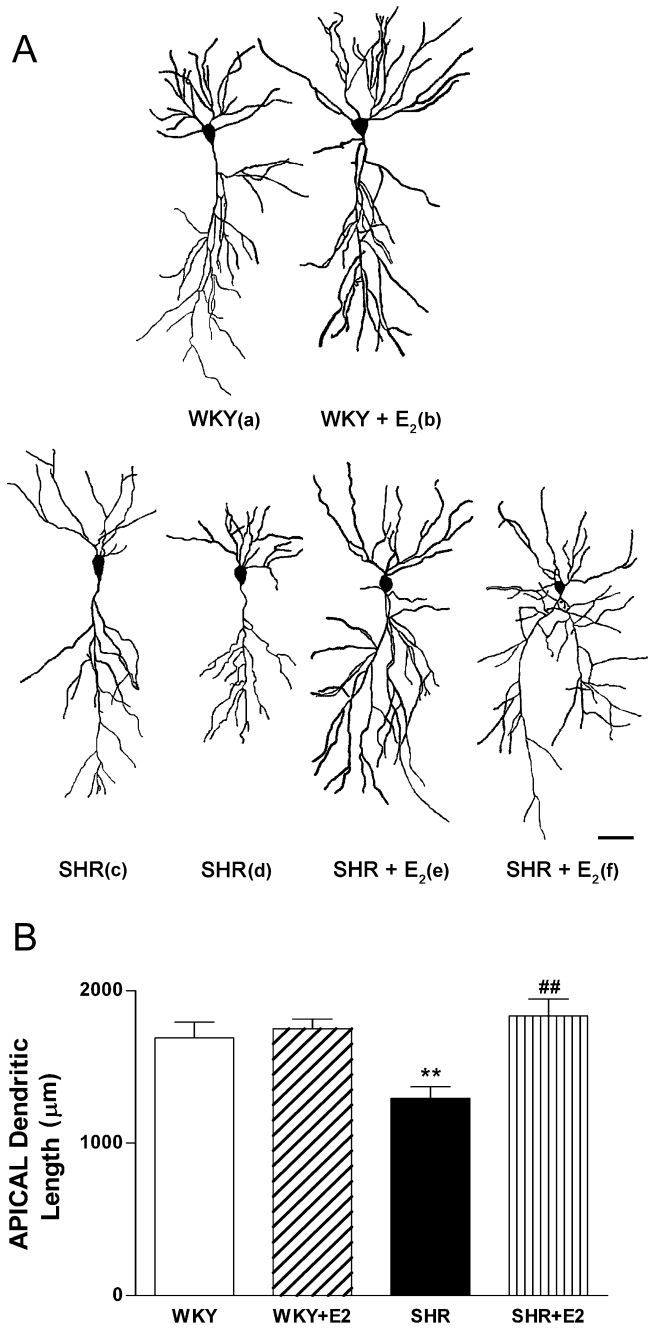

Fig. 3. (A) The upper images correspond to camera lucida drawings showing normal dendritic arbors of two neurons from WKY rats without (a) or with estradiol treatment (b). The lower drawings correspond to neurons from SHR showing dendritic atrophy (SHR c and SHR d) and neurons of estradiol-treated SHR (SHR e and SHR f) showing a more developed dendritic tree. Scale bar: 50 μm. (B) Length of CA1 apical dendrites (μm) in the four experimental groups. Length was significantly decreased in SHR vs. WKY ($^{**}p < 0.01$). Estradiol treatment significantly increased dendritic length in hypertensive rats (SHR vs. SHR + E_2: $^{\#\#}p < 0.01$) but not in WKY rats. Results correspond to $n = 15$ WKY and WKY plus estradiol-treated rats, $n = 17$ SHR rats and $n = 10$ SHR plus estradiol-treated rats.
Modified from Ref. [99] with permission.

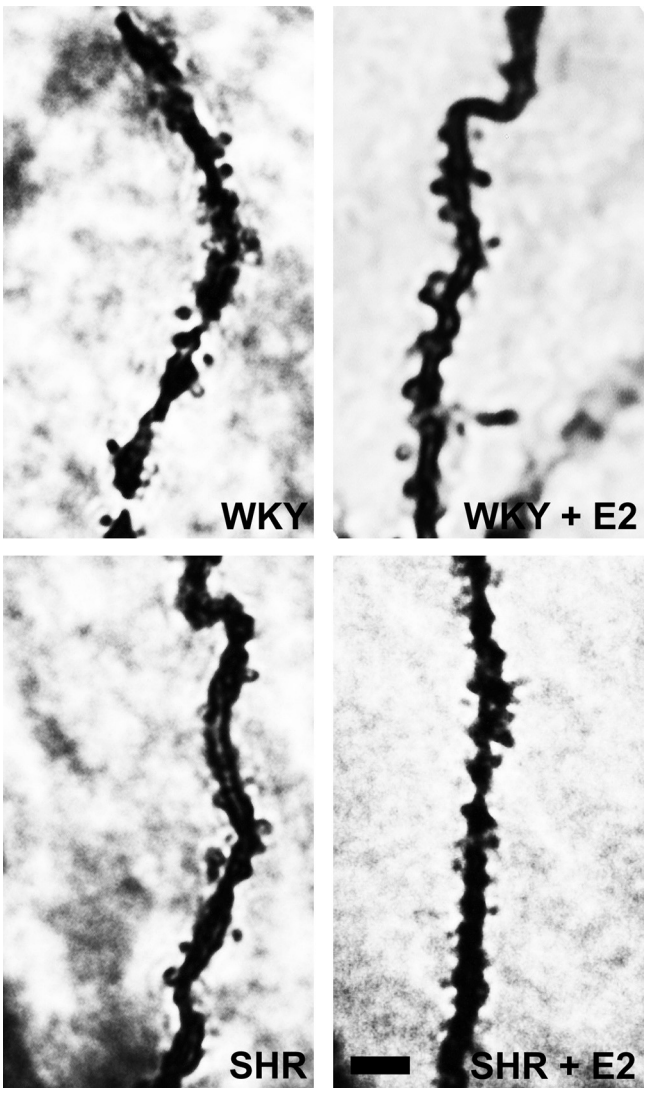

Fig. 4. Representative images of dendrites stained with the Golgi Technique. Spines protruding from second order dendrites were assessed for the apical dendrites located in the stratum radiatum below the CA1 hippocampus subfield. The microphotographs show abundant apical spines in WKY, WKY + E_2 and SHR + E_2 rats; in contrast fewer spines are observed in SHR. Scale bar: 5 μm.
Modified from Ref. [99] with permission.

17β-estradiol in dendrite length in the middle 120–200 μm range from the neuronal soma, whereas this effect was less marked or absent at shorter or longer distances, according to Sholl analysis. Quantitative analysis of the branching of the dendritic tree revealed fewer dendritic intersections in steroid untreated SHR than in the 17β-estradiol-treated hypertensive group. However, in contrast to results of apical dendrites, 17β-estradiol did not modify the basal dendritic length at any shell distance from the CA1 pyramidal cell soma.

Estrogen remodeling was not confined to dendrites, and also reached spine density. Golgi-stained spines protruding from second order dendrites were assessed separately for the apical and basal dendrites located in the hippocampus stratum radiatum and oriens, respectively, and results expressed as the number of spines per μm dendritic length. We found that SHR contained fewer apical dendritic spines than WKY, whereas 17β-estradiol treatment significantly increased spine density of apical and basal dendrites of SHR compared to steroid-untreated SHR, but it was without effect on WKY rats. The photomicrographs of Fig. 4 showed the conservation of spines in the SHR receiving 17β-estradiol treatment.

The neuritogenic effects of estrogens on the CA1 neurons were not unexpected. Neuroanatomically, CA1 pyramidal cells comprise the primary output cells of the hippocampus, whereas input originates from glutamatergic Schaffer collaterals of the CA3 region. These collaterals contact apical dendrites in the stratum radiatum and basal dendrites in the stratum oriens [100]. 17β-Estradiol increases the density of excitatory synapses in the CA1 region of

female rats, although male rats are much less sensitive to estrogen-induced synapse formation in the hippocampus [101]. The latter finding is in agreement with the our results in WKY male rats, which remained unresponsive to 17β-estradiol treatment. The estrogenic effect on the CA1 region has been attributed to the strong anti-oxidant, anti-glutamatergic, neurotrophic and mitochondrial protective activities of female hormones [26,28,79,102]. An important intermediate of estrogen neurotrophic effects is BDNF, which strongly stimulates the generation of dendritic spines [57,103]. It was already mentioned that a depletion of BDNF mRNA occurs in the dentate gyrus, but not the CA1 or CA3 areas of SHR., whereas estrogen treatment of SHR precludes BDNF mRNA depletion in the dentate gyrus, without affecting it in the CA1 area [66]. However, input to CA1 neurons is processed at several levels, encompassing the enthorrinal cortex, dentate gyrus, the mossy fibers, the CA3 neurons and the Schaffer collaterals. Therefore, up-regulation of BDNF by 17β-estradiol in the dentate gyrus may convey a trophic signal to the CA1 pyramidal neurons, explaining the improved response to 17β-estradiol treatment of SHR. In the next section, we summarize experiments showing that enhanced expression of the estrogen-synthesizing enzyme aromatase in SHR, could also take part in neuritogenic effects of 17β-estradiol.

7. Rol of aromatase on 17β-estradiol effects in the hippocampus of SHR

Local estrogen synthesis by brain cells has been early shown by Naftolin et al. [104] and Balthazart et al. [105]. The estrogen-synthesizing enzyme, aromatase (*cyp19a1b*) has been found in the hippocampus of mice, rats, fish, birds, monkeys and humans [104–110]. The enzyme seems highly active in the hippocampus, since in mice the content of 17β-estradiol in this tissue is six-fold higher than in plasma [54]. Aromatase immunoreactivity has been localized in neuronal perikarya, dendrites, axonal processes and terminal boutons [104]. Reactive astrocytes following brain injury also become aromatase positive in several brain areas including the hippocampus [111–114].

The role of aromatase in the hippocampus involves synaptic development and plasticity and positive regulation of neurogenesis [115,116]. These last reports have shown that inhibition of aromatase activity with letrozole decreases cell proliferation and increases cell apoptosis in hippocampus. Estrogen biosynthesis by aromatase is tightly regulated, as reported for human glioma cell cultures, hippocampal dispersion cultures, and different brain regions from rat, mouse, quail, songbirds and fish [108,109,117–120]. Both androgens and estrogens show modulatory effects on aromatase, due to the presence of androgen and estrogen-response elements in the *cyp19a1b* gene [106,108,109,121–123]. Interestingly, tonic estrogen treatment of ovariectomized mice increases by 69% aromatase gene expression in the hippocampus, whereas cyclic estradiol administration has the opposite effect [124].

To investigate the role of aromatase in the hypertensive brain, we first determined aromatase mRNA expression by real time PCR in hippocampus from WKY rats and SHR [125]. The study showed higher basal level of aromatase mRNA in SHR respect of WKY rats (Fig. 5A vs. B). 17β-Estradiol treatment of SHR produced a further elevation of aromatase mRNA, surpassing the high basal levels of the same group implanted with cholesterol. In contrast, steroid treatment was inactive regarding aromatase mRNA levels of WKY rats. Secondly, immunocytochemistry was used to localize at the microanatomical level the basal distribution of aromatase protein in SHR and WKY rats and eventual changes in its expression levels. Aromatase immunoreactivity was localized in cell processes and cytoplasm of pyramidal neurons in the CA1, CA2, and CA3 subregions and it was highest in the CA1 pyramidal layer of the hypertensive group receiving 17β-estradiol. Whereas weak immunostaining was obtained in WKY (Fig. 5A), fibers and some neuronal bodies were stained in SHR (Fig. 5B). Strong immunostaining was also found in the hilar region of the dentate gyrus, especially in fiber collaterals, axonal varicosities and occasional cell bodies of the SHR group receiving 17β-estradiol (Fig. 5C). In addition, the length of aromatase immunoreactive fibers was higher in SHR vs. WKY rats, and it further increased in SHR but not in WKY rats, receiving steroid treatment. The immunoreactive fibers of the hilus might correspond to mossy fibers arising from granule cells of the dentate gyrus [126]. Double immunofluorescene staining demonstrated that GFAP+ positive astrocytes were devoid of aromatase immunostaining. Similarly, double-labeling for GFAP-aromatase in the CA1 and CA3 areas, discarded the presence of the enzyme in GFAP+ astrocytes.

It is likely that the increased basal levels of aromatase mRNA and protein expression in some regions of hippocampus and its stimulation by 17β-estradiol had profound influences for the encephalopathy of SHR [125]. The neuroprotective role of brain aromatase has been thoroughly reviewed [114,127]. In this trend, stimulation of aromatase may be linked to the preferential enhancement of BDNF, doublecortin-positive neuroblasts, neuronal density in the hilus and attenuation of astrogliosis found in SHR receiving 17β-estradiol [11,72]. Altogether, our data suggest that a combination of exogenously administered 17β-estradiol, plus estrogens locally synthesized by the enhanced aromatase, may provide critical steroid levels to alleviate the encephalopathy of SHR. A question remains, however, regarding the neuroanatomical site where systemic 17β-estradiol administration regulates hippocampal aromatase expression in SHR. Direct estrogen effects on the hippocampus are possible, because ERα and ERβ levels were preserved in hypertensive animals. Nevertheless, afferent pathways arising from estrogen-sensitive subcortical regions also regulate hippocampal plasticity [128]. This paracrine, indirect mechanism may be also involved in 17β-estradiol effects on aromatase expression. Further investigations are warranted to understand the role of estrogen-stimulated aromatase expression in the hippocampal circuit with behavior, learning, memory and neuroendocrine responses of SHR.

8. Expectations for translational medicine

Translational medicine and its predecessor “from bench to bedside” intend to apply basic biomedical knowledge into clinical practice and bring new therapeutic options. Within this context, lessons learned from animal models might encourage the use of additional pharmacological interventions for hypertensive encephalopathy. Estrogens control reproductive and non-reproductive events in the brain, and have been successfully employed to prevent or attenuate neurochemical and brain functional abnormalities in models of diabetes mellitus, neurodegenerative diseases, aging, neuroinflammation, stroke and ischemia. Other applications of estrogen neuroprotection may include psychiatric diseases, mood disorders, and mild cognitive impairment.

Few reports have addressed the issue of estrogens being neuroprotectants to the brain of hypertensive animals. To fill this gap, our studies have shown many aspects of 17β-estradiol given chronically and continuously to SHR. First we have shown that estrogen treatment prevented the deficient expression of BDNF in the dentate gyrus of the hippocampus, a region committed to the birth of neurons in the adult brain. At the time that BDNF mRNA and protein expression was up-regulated, SHR increased cell proliferation and DXC+ neuroblasts in the dentate gyrus. After migration and

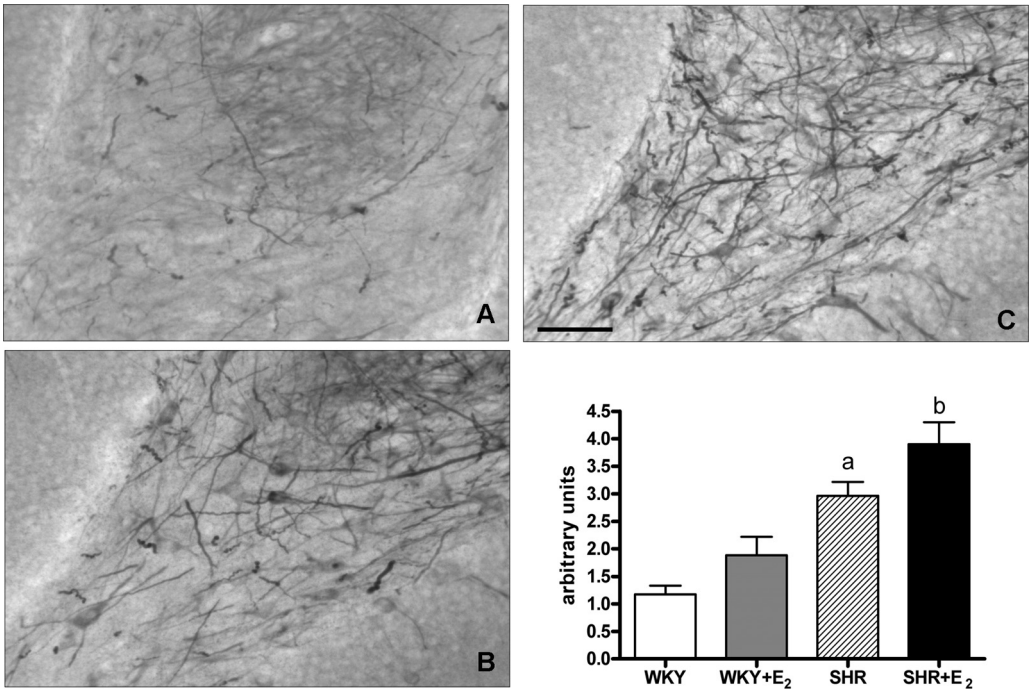

Fig. 5. *Photomicrographs*: Aromatase immunostaining in the hilus of the dentate gyrus of normotensive Wistar Kyoto rat (A), SHR (B) and SHR receiving 17β-estradiol treatment for 2 weeks (C). Immunostaining was more intense in fibers and cell processes in steroid-naïve SHR respect of WKY. 17β-Estradiol treatment further increased immunostaining for aromatase in cell processes and in sparse neuronal bodies of the dentate gyrus of SHR. Scale bar: 100 μm. *Graph*: Quantitative analysis of aromatase mRNA in whole hippocampus obtained by real time PCR of normotensive Wistar Kyoto (WKY) rats without or with 17β-estradiol treatment (WKY + E_2) and spontaneously hypertensive rats (SHR) without or with 17β-estradiol (SHR + E_2) treatment. Statistical analysis by ANOVA and post hoc test demonstrated higher aromtase mRNA in steroid-naïve SHR vs WKY or WKY + E2 ($^a p < 0.05$). Following 17β-estradiol treatment, SHR further increase aromatase gene expression ($^b p < 0.05$ vs all other groups). Results obtained from 10 to 12 animals per group.

Modified from Ref. [125] with permission.

differentiation, newborn cells extend their axons (mossy fibers) to contact the CA3 pyramidal region, and from there the Schaffer collaterals reach the CA1 pyramidal cell layer. It was in the latter region that 17β.estradiol increased length of dendrites and spinogenesis, both of which were defective in the hippocampus from steroid unprotected SHR. Therefore, the whole hippocampus circuitry could be modified by estrogens in the pathological brain of hypertensive rats. The mechanisms of estrogen action in SHR remain open for study. However, a clue comes from the observations that ERα, ERβ and levels of circulating 17β-estradiol did not differ between estrogenized and vehicle-treated SHR. However, SHR showed an unusual up-regulation of aromatase protein and mRNA expression in hippocampus, supporting the hypothesis that accumulation of endogenous plus exogenous estrogens could be mandatory for effective neuroprotection. Table 1 summarizes the findings of our work with SHR.

Finally, it should be mentioned that 17β-estradiol is not free from secondary effects in the uterus, vagina and breast in females, and cannot be used in males. Therapeutic options bypassing unwanted effects could be the non-feminizing estrogen 17α-estradiol, or natural compounds showing preferential affinity for ERβ, an isoform more commonly associated with protection from neurodegenerative diseases [129].

Table 1
Summary of 17β-estradiol treatment of spontaneously hypertensive rats (SHR).

1.	Implantation of a 12 mg 17β-estradiol-benzoate pellet for 2 weeks increased ~30-fold circulating steroid levels of SHR.
2.	This treatment regime significantly reduced blood pressure levels and the expression of the hypertensinogenic peptide arginine vasopressin in hypothalamus of SHR.
3.	17β-Estradiol treatment did not affect the expression of Erα or Erβ mRNA in hippocampus.
4.	Cell proliferation and neuronal progenitors were significantly stimulated in the subventricular zone of the dentate gyrus of estrogen-treated SHR.
5.	Concomitantly, there was an up-regulation of BDNF mRNA and protein in the dentate gyrus of hippocampus of SHR.
6.	17β-Estradiol also increased aromatase immunoreactive fibers in dentate gyrus and aromatase mRNA in whole hippocampus
7.	17β-Estradiol positively modulated the apical dendritic arbor and spine density in apical dendrites of CA1 hippocampus subfield.
8.	Neuroprotection may require the combined effects of exogenous 17β-estradiol plus the stimulation of endogenous estrogen synthesis in hippocampus of SHR.

Acknowledgements

This work was supported by grants from FONCYT (PICT 2012 # 0009), CONICET (PIP # 11220120100016) and the University of Buenos Aires (Ubacyt 20020100100089). These findings sources had no role in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

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