Neuroprotection and Sex Steroid Hormones: Evidence of Estradiol-Mediated Protection in Hypertensive Encephalopathy

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Abstract: Besides their effects on reproduction, estrogens exert neuroprotective effects for brain diseases. Thus, estrogens ameliorate the negative aspects of aging and age-associated diseases in the nervous system, including hypertension. Within the brain, the hippocampus is sensitive to the effects of hypertension, as exemplified in a genetic model, the spontaneously hypertensive rat (SHR). In the dentate gyrus of the hippocampus, SHR present decreased neurogenesis, astrogliosis, low expression of brain derived neurotrophic factor (BDNF), decreased number of neurons in the hilus and increased basal levels of the estrogen-synthesizing enzyme aromatase, with respect to the Wistar Kyoto (WKY) normotensive strain. In the hypothalamus, SHR show increased expression of the hypertensinogenic peptide arginine vasopressin (AVP) and its V1b receptor. From the therapeutic point of view, it was highly rewarding that estradiol treatment decreased blood pressure and attenuated brain abnormalities of SHR, rendering hypertension a suitable model to test estrogen neuroprotection. When estradiol treatment was given for 2 weeks, SHR normalized their faulty brain parameters. This was shown by the enhancement of neurogenesis in the dentate gyrus, according to increased bromodeoxyuridine incorporation and doublecortin labeling, decreased reactive astrogliosis, increased BDNF mRNA and protein expression in the dentate gyrus, increased neuronal number in the hilus of the dentate gyrus and a further hyperexpression of aromatase. The presence of estradiol receptors in hippocampus and hypothalamus suggests the possibility of direct effects of estradiol on brain cells. Successful neuroprotection produced by estradiol in hypertensive rats should encourage the treatment with non-feminizing estrogens and estrogen receptor modulators for age-associated diseases.

Keywords: Estradiol, hippocampus, hypertension, hypothalamus, neuroprotection.

1. MOLECULAR AND BIOCHEMICAL BASIS FOR ESTRADIOL NEUROPROTECTION

Originally, estrogens have been considered hormones engaged in the control of reproduction and sexual behavior. Over the years, multiple reports have demonstrated their powerful modulatory effects on brain function. In this aspect, pathological conditions involving the hippocampus, includeing trauma, aging, neurodegeneration, excitotoxicity, oxidative stress, hypoglycemia, amyloid-β peptide exposure and ischemia are prevented in part or totally by treatment with estrogens [1-6]. Estrogen effects could be mediated by interaction with intracellular receptors (ER) of the ERα or ERβ subtype although nonclassical, non-genomic mechanisms are increasingly recognized to intervene in estrogen effects on the brain [7, 8]. Recent studies have focused on the rapid activation of mitogen activated protein kinase (MAPK/ERK) pathway for the neuroprotective effects of estradiol mediated by the G-protein coupled receptor 30 (GPR30), although the issue is not definitively resolved [8]. Rapid steroid effects are also possible by the interaction of ERα with insulin-like growth factor receptor (IGF-IR) in the plasma membrane. This interaction activates the MAPK/ERK pathway and other components of IGF-IR mediated signaling such as phosphatidylinositol-3-kinase (PI3K), AKT and glycogen-synthase kinase 3β (GSK3β) [8]. Investigations on the mechanisms of estradiol neuroprotection have also included the stimulation of brain-derived neurotrophic factor (BDNF), increased phosphorylation of CREB (cyclic AMP-response element binding protein) and stimulation of several anti-apoptotic molecules including Bcl_2 [8]. Modulation of one or more of these pathways results, at the cellular level, in the control of adult neurogenesis, prevention of cell death, increase of neuronal survival and neurite outgrowth, prevention of glutamatergic excitotoxicity, stimulation of synaptogenesis, and antioxidant and anti-inflammatory effects owing to estradiol exposure *in vivo* or *in vitro* [5,8-13]. Therefore, the cellular machinery set in motion during estrogen protection is intrinsically complex and may show dissimilar modalities depending on the pre- existing pathology.

In addition to data gathered from living animals and cultured preparations, the estrogen protection hypothesis has been extended to various human neurodegenerative and psychiatric syndromes, such as stroke, cerebral ischemia,

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schizophrenia, Parkinson and Alzheimer`s disease, depression, aging-related disorders and mild cognitive impairment [14]. However, there is no general consensus on whether estrogen treatments in humans is devoid of undesirable effects and actually provide beneficial actions for neurological diseases.

The aim of this Minireview is to summarize our experience on estrogen neuroprotection in a genetic model of hypertensive encephalopathy, taking advantage that some abnormal parameters exhibited by the hypertensive brain are estrogen-sensitive. In other studies, we have presented proof that findings in hypertensive rats may be expanded to age-related diseases including aging itself and diabetes mellitus [15,16].

2. BRAIN ABNORMALITIES OF ESSENTIAL HYPERTENSION

Essential hypertension is a clinically and epidemiologically defined disease of a polygenic nature with a prevalence of 20-40% in humans. It constitutes a major risk factor for heart failure, renal disease and stroke. It is a severe disease, causing 5 million premature deaths each year worldwide (WHO World Health Report, 2002). The damaging effects of hypertension can be prevented by treatment with well known hypotensive drugs including inhibitors of the angiotensin-converting enzyme (ACE), diuretics, calcium channel blockers, β-blockers and angiotensin II receptor antagonists. However, this classical repertoire of anti-hypertensive drugs should also include steroids with brain protective activities, which may preclude the development of hypertensive encephalopathy, as discussed later in this Mini-Review.

The last expression, "hypertensive encephalopathy", stands for the brain damage caused by a persistent elevation of blood pressure. Uncontrolled hypertension is accompanied by a pronounced neuropathology. In hypertensive subjects, remodeling of the microvascular wall with vasoconstriction and ischemia plays a substantial role in neuronal damage [17,18]. Inside the brain, the hippocampus is highly vulnerable to the effects of hypertension, as demonstrated by the atrophy of the hippocampus and temporal lobe, increased cognitive decline and risk of Alzheimer´s disease found in hypertensive subjects [19,20]. Pathological hallmarks of Alzheimer`s disease such as neurofibrillary tangles, senile plaques and neuronal lesions have been described in the brain of hypertensive patients [21]. Therefore, a relationship exists between hypertension, cerebrovascular disease, decline of cognition and signs of hippocampal dysfunction [22].

A pronounced encephalopathy also characterizes experimental models of hypertension. The spontaneously hypertensive rat (SHR) is a genetic model of essential (or primary) hypertension, used to study cardiovascular disease. The SHR strain was developed by Okamoto in Japan in 1963, after breeding Wistar-Kyoto rats with high blood pressure. SHR is distinguished by changes typical of human hypertension, including cardiac hypertrophy, stiffness of the vascular tree, nephropathy and encephalopathy. In the brain, SHR show increased expression of the marker glial fibrillary acidic protein (GFAP), blood-brain barrier disruption, cytoskeletal breakdown, decreased forebrain white matter volume and abnormal neurogenesis [23-26]. Somehow, SHR presents an accelerated brain aging. Changes in learning and memory displayed by SHR made these animals models of dementia and other behavioral disturbances [22]. The question remains if hippocampal changes are consequences of a pre-existing hypertension or whether hypertension and brain pathology reflect a central defect in this strain [25-28]. Hormonal imbalances are frequent companions of hypertension. For example, adrenal steroids and their receptors may participate in the brain abnormalities of experimental hypertension [29-33], while aldosterone plays a role in 10-15% of patients with essential hypertension [18]. The known relationship between menopause and high blood pressure indicates that a decline of estrogens starts or impairs a preexisting hypertension. Since estrogens exert beneficial effects on the hippocampus [1,34], we decided to unveil if estrogen neuroprotective functions also apply to the hippocampaus of hypertensive animals.

The intervention of endocrine factors in the development of hypertension is more obvious after administration of the mineralocorticoid aldosterone or deoxycorticosterone acetate (DOCA) to rats drinking a concentrated salt solution [24]. Interestingly, hippocampal defects of DOCA-salt hypertensive rats have features in common with SHR, suggesting some etiopathogenic factors may be shared by genetic and mineralocorticoid-mediated hypertension. It therefore seems that the hippocampus in hypertension is under double hormonal control, in a negative fashion by excess production of adrenal steroids and in a beneficial way by sex steroid hormones.

3. ESTRADIOL CONTROL OF BLOOD PRESSURE AND HYPERTENSINOGENIC PEPTIDES IN A MODEL OF ESSENTIAL HYPERTENSION.

One possibility is that estrogen protection of the brain results secondary to changes of the cardiovascular and peripheral blood pressure systems, whereas another possibility is that estrogen action is exerted directly upon the brain. Thus, estrogens increase the vasodilators nitric oxide (NO) and atrial natriuretic peptide and interact with vascular endothelial growth factor (VEGF) but oppose angiotensin II-induced hypertension and catecholaminergic activity [35-38]. Estrogen action in the brain also reduces blood pressure and attenuates damage due to ischemia and stroke [4,6,39]. It is known that some estrogen effects in the brain may be genomically mediated, although antioxidant, membrane and synaptic effects are recognized to take part in estrogen neuroprotection [14,40]. One effect of estrogens related to hypertension could be due to binding to the ERβ subtype. ERβ is abundantly expressed in the hippocampus and the hypothalamic paraventricular nucleous (PVN) that synthesize arginine vasopressin (AVP) [7], a hypertensinogenic peptide under negative regulation by estradiol [41]. Along this line, it became important to show if estradiol control of blood pressure involves the down-regulation of the vasopressinergic system, implicating that the expression of the peptide as well as its receptors is the target of estrogen action.

To study the effects of estradiol on blood pressure, AVP expression as well as other parameters discussed in subsequent headings of this Minireview, we employed as a standard procedure 4 month old normotensive male Wistar-Kyoto rats and SHR. Mean resting blood pressure measured by a tail-cuff method, was 192 ± 10 mm Hg in SHR and 113 ± 5 mm Hg in WKY. For steroid treatment, rats received sc a single 12 mg pellet of estradiol-benzoate or an equivalent pellet of cholesterol in control rats. All animals (WKY and SHR) were used 2 weeks after pellet implantation to analyze the different parameters; at this time levels of estradiol varied between 1200 and 2000 pg/ml serum. Although, these may seem pharmacological in range, it should be reminded that hippocampal levels of estradiol are 6-fold higher than plasma levels (Hojo *et al*., 2004); therefore, the levels of estradiol needed for neuroprotection in the tissues may be actually much higher than plasma levels. After estradiol treatment was applied to SHR, it showed a hypotensive effect, decreasing mean blood pressure of by 40 mm Hg, although rats still remain slightly hypertensive (148 ± 8 mm Hg). Estradiol treatment also produced a slight but statistically significant decrease of the cardiomegaly shown by SHR [42]. The animal procedures followed the NIH Guide for the Care and Use of Laboratory Animals (Assurance Certificate N A5072-01 to Instituto de Biología y Medicina Experimental) and received approval of the Institute's Animal Care and Use Committee.

To study the regulation of AVP by blood pressure conditions and hormone treatments, we used an *in situ* hybridization method with an oligonucleotide probe specific for AVP mRNA. Data showed that in normotensive WKY rats, AVP mRNA was moderately abundant in posterior magnocellular cells of the PVN but its expression was much stronger in SHR, with new cells containing the AVP transcript now appearing in the medial portion of the magnocellular PVN of SHR [24]. After SHR received estradiol for 2 weeks, the AVP mRNA signal was considerably attenuated respect of steroid-naïve animals. These data was quantitatively confirmed by computerized image analysis [24]. In the basal state, SHR demonstrated a higher number of AVP mRNA and vasopressin type V1a receptors (V1aR) immunopositive cells in the magnocellular division of the paraventricular hypothalamic nucleus (PVN) than WKY rats. To test if SHR were hyperresponsive to mineralocorticoids, we injected once deoxycorticosterone (DOCA), a potent mineralocorticoid receptor (MR) agonist. SHR responded with a significant increase in AVP mRNA and V1aR with respect to vehicle-injected SHR. In WKY rats, DOCA showed no effect on AVP mRNA, although it increased the number of immunoreactive V1a-positive cells. That PVN neurons are hyperresponsive in hypertension was also suggested by measuring Fos, an early gene widely accepted as a marker of cell stimulation. Changes in the number of Fos-positive nuclei were obtained in the PVN, median preoptic nucleus (MnPO) and organum vasculosum of the lamina terminalis (OVLT), a circumventricular region showing anatomical connections with the PVN. In vehicle-injected rats, the PVN of SHR showed a higher number of Fos-positive nuclei than in WKY rats, whereas after a single injection of the mineralocorticoid DOCA, a significant increment occurred in the OVLT but not in the PVN or MnPO of the SHR group only. Therefore, these data suggest that SHR show an activation of anterior hypothalamic areas (OVLT) that in turn activate the PVN neurons. Thus, the enhanced response of the vasopressinergic system to mineralocorticoids may contribute to the abnormal blood pressure of SHR, suggesting that SHR may be a form of endocrine (mineralocorticoid-induced) hypertension of central origin. This chain of events speaks of a complex interaction between estrogens and AVP, which may take place at the PVN and also at anterior hypothalamic (OVLT) levels. Whereas, these regions stimulate the vasopressinergic system and blood pressure of SHR, down-regulation of AVP and blood pressure by estradiol may involve not only the PVN but also the OVLT. Another possible mechanism that could explain the differential modulation of the DOCA response in the control and hypertensive rats could be the expression of the mineralocorticoid receptor (MR). Preliminary experiments showed higher density of immunoreactive MR+ cells in the PVN of SHR respect of normotensive WKY rats (Pietranera *et al*., unpublished).

Previous studies demonstrated increased AVP mRNA expression in the dorsal magnocellular division of the PVN in SHR that was further increased following short-term treatment with a mineralocorticoid in SHR but not in WKY rats [24,43]. Increased AVP synthesis may adversely affect the cardiovascular system and increase blood pressure [44]. Along this line, blockade with a vasopressin V1 receptor antagonist attenuates genetic and mineralocorticoid hypertension [45]. In addition, downregulation of the AVP system may facilitate neurogenesis, because inhibition of V1 vasopressin receptors increases neurogenesis in the dentate gyrus of chronically stressed mice [46]. Thus, estrogen reduction of AVP mRNA expression may impact on hypertension and sustain neurogenesis of SHR, and it should be considered an important target of estradiol neuroprotection. This may be a direct estrogen effect, because estradiol activation of ERβ in the PVN decreases AVP transcripts. These data indicate that expression of ER in the brain, especially the hypothalamus, mediates the protective effects of estrogen against angiotensin II-induced hypertension [38], while in contrast, depletion of estrogens increases hypertension of SHR [36,47]. Thus, experimental evidence support that estrogens protect from development of hypertension may be in part centrally mediated, and in certain way, antagonize the deleterious stimulation of the vasopressinergic system by mineralocorticoids.

4. ESTRADIOL MODULATION OF THE ASTRO-GLIAL CELL REACTION IN SPONTANEOUSLY HYPERTENSIVE RATS (SHR)

A common reaction of the nervous system to injury, neurodegeneration, ischemia, diabetes and toxins takes place in the astrocyte population, that migrate, alter their phenotype and show hypertrophic and proliferative changes [48]. Several molecular triggers of astrogliosis are known and have been recently reviewed [49]. Astrogliosis is commonly assessed by measuring the up-regulation of the cytoskeletal protein glial fibrillary acidic protein (GFAP) [50]. GFAP is strongly expressed by reactive astrocytes. Reactive astrocytes change their gene expression, release proinflammatory mediators that attract macrophages and

microglia and induce their local and distal proliferation. The gain of detrimental effects by reactive astrocytes is incompletely understood but might result from specific signaling cascades targeting microglia and other inflammatory cells [50]. Whereas, some beneficial functions have been recognized for reactive astrocytes [51], there are also supporting data showing that astrogliosis contributes to neurodegeneration [52].

In SHR, we examined the response of GFAP-immunoreactive astrocytes in three subregions of the dorsal hippocampus, namely, the *stratum radiatum* below the CA1 and CA3 subfields and the hilus of the dentate gyrus. In the experiment using the normotensive WKY strain, density of GFAP immunoreactive cells varied from 60 to 160 cells per unit area (mm^2) in the three hippocampal subfields. Instead, hypertensive rats show a 2-fold higher density of GFAP immunopositive cells in SHR than WKY for the CA1, CA3 and the dentate gyrus hippocampal regions. Also, the GFAP-labeled cells show a more reactive phenotype in SHR compared to WKY rats. Estradiol treatment for 2 weeks produced a significant downregulation of GFAP immunoreactive cell density in the SHR, whereas the steroid was inactive in normotensive animals.

Therefore, steroid-naïve SHR presented a marked increase in the density and phenotype of GFAP+ astrocytes, which strongly resembled the astrogliosis reported for trauma, neurodegeneration, aging and diabetes mellitus, conditions taken as hallmark of neuronal damage [53- 55]. The astrogliosis detected in the hippocampus of hypertensive animals may be secondary to neuronal suffering. The estradiol effect on GFAP immunopositive astrocytes in SHR was not unexpected, because this hormone down-regulates the reactive astrocytosis in the brain of old rats, young castrated rats, and animals with traumatic or excitotoxic lesions [56,57]. Downregulation of GFAP is explained by the presence of an estrogen-response element in the 5′ upstream region of the GFAP promoter [58]. However, since sex steroid receptors are more abundant in neurons than astrocytes, the possibility exists that astrocytes responded indirectly, via direct hormone effects on neurons [56]. Disregarding the mechanism, the reduced GFAP reactive phenotype of the astrocytes might prove beneficial for neurogenesis and other cellular events damaged in hypertension. In a GFAP $^{-/-}$ background, cell proliferation in the granular cell layer of the dentate gyrus is substantially increased, suggesting that genetic attenuation of reactive gliosis stimulates cell proliferation and neurogenesis [59,60]. Rozowsky *et al.,* [58] reported that estradiol - even at physiological levels - inhibits GFAP expression *in vivo* and *in vitro* in a transcriptionally mediated manner. Thus, reduction of astrogliosis becomes a valuable index of estradiol neuroprotection in the hippocampus of hypertensive animals.

5. ESTRADIOL STIMULATION OF HIPPOCAMPAL NEUROGENESIS AND NEUROTROPHIC FACTOR EXPRESSION IN SHR.

It is widely recognized that estrogens play an important role in the hippocampus, with modulation of learning, memory and neuroendocrine functions [3,13,61,62]. One way estrogens modulate these processes due to the enhancement of neurogenesis – the proliferation, migration and differentiation of new neurons - in the dentate gyrus of adult animals [9,63-65]. In this region, and in the subventricular zone, neurogenesis continues into adulthood [64]. The effect on neurogenesis seems mediated by estrogen receptors (ER), since both ERα and ERβ mRNA are expressed by ≈ 80% of proliferating cells of the dentate gyrus [66]. The positive control of neurogenesis is also a sign of estrogen neuroprotection under pathological circumstances.

To study cell proliferation, rats were injected with bromodeoxyuridine (BrdU), a thymidine analog that incorporates into the S phase of the cell cycle. Quantitative analysis employing the optical disector method, demonstrated BrdU-labeled cells were reduced by one-half in SHR respect of normotensive WKY rats. Estradiol stimulation varied according to blood pressure status. Cell proliferation was unchanged in normotensive WKY rats, whereas estradiol treatment for 2 weeks significantly increased it in the SHR group to levels of WKY rats. Enhanced neurogenesis has been linked to learning, memory and neuroendocrine functions [9,13,63,65,66]. Since newly-formed neurons may optimize interconnections between the dentate gyrus and the CA3 pyramidal subfield [65], the whole hippocampal function may be influenced by changes of neurogenesis due to estradiol treatment of hypertensive rats. The finding that estrogen stimulation of cell proliferation was exclusive of hypertensive rats and absent from WKY rats is intriguing. Alvarez-Buylla and co-workers [67] have postulated that stem cells located in the subgranular cells of the dentate gyrus divide, express GFAP and have the characteristics of astrocytes. A proportion of these astrocytes function as neuronal precursors in the hippocampus [67]. Therefore, one possibility would be that the pre-existing phenotype of astroglial precursors in the dentate gyrus makes SHR preferentially sensititive to estradiol-mediated changes of cell proliferation.

That estradiol stimulates neurogenesis in the subgranular zone and granular cell layer of SHR [28], was also substantiated by studying a further steps of neurogenesis. Using doublecortin (DCX) immunocytochemistry to label the population of neural progenitors (neuroblasts), conventional microscopy readily distinguished differences in DCX immunopositive cell morphology between WKY rats, steroid-naive SHR and hypertensive rats receiving estradiol treatment. Thus, abundant DCX immunoreactive cells were present in the subgranular cell layer of the dentate gyrus of WKY rats, with evidences of strongly stained cell bodies and cell processes. This profile contrasted with the scarce DCX immunoreactive cells of reduced intensity and fewer cell processes of SHR. The atrophic morphology of DCX positive cells of SHR changed following estradiol treatment, in which case aggregates of highly branched DCX-labeled cells of enhanced staining intensity appeared in the subgranule cell layer. Stereology procedures for cell quantitation determined a fifty percent reduction of DCX-stained cells in SHR compared to WKY, and a significant stimulatory effect on DCX-labeled cell number followed estradiol treatment. Thus, estradiol treatment of SHR

increased two steps of neurogenesis: cell proliferation, according to BrdU labeling, and the immature neuronal progenitors expressing the DCX marker.

Effects of estrogens on the hippocampus may be due to the interaction with neurotrophins such as brain-derived neurotrophic factor (BDNF), that could mediate some of the reported estrogen neuroprotective mechanisms [12]. The intermediary role of the growth factor is reinforced by the presence of an estrogen 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 estrogens [68,69]. Another interesting interaction between estrogens and BDNF is that both promote dentate gyrus neurogenesis. Similar to estrogens, evidences from *in vivo* and *in vitro* studies have shown that BDNF plays a key role in neurogenesis, by enhancement of cell proliferation, progenitor survival and terminal differentiation of newborn neurons [70,71]. Of great interest is that BDNF expression is down-regulated in hypertension and ischemia. This has been shown in a genetically hypertensive strain of Wistar rats, in which impaired learning and memory correlates with decreased BDNF expression in the dentate gyrus [72] and in SHR receiving carotid artery occlusion, in which BDNF mRNA and protein further decrease in the CA1 region and cortical areas [73]. Finally, a mutation in the BDNF specific receptor TrkB gene in the stroke-prone SHR [74], impairs neurotrophic function in this strain. Thus, cumulative evidences suggest that BDNF expression and hypertension are negatively interrelated.

In order to localize at the neuroanatomical level changes in the expression of BDNF mRNA, we employed a specific BDNF oligonucleotide probe hybridized to hippocampal sections from normotensive and hypertensive rats. A strong signal for BDNF mRNA was found on film autoradiograms of 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 SHR respect of the WKY rats. This reduction was more accentuated in the granule cell layer of the dentate gyrus, whereas the mRNA signal of the CA1 - CA4 hippocampal regions remained similar in control and hypertensive animals. Furthermore, estradiol administration to SHR restored the signal intensity of film autoradiograms to control levels, confirmed by proper statistical analysis. We also analyzed BDNF protein by a commercial ELISA method and found that BDNF protein content of whole hippocampus was reduced by half in estrogen-free SHR with respect to WKY rats. Similarly to changes obtained for BDNF mRNA, estradiol treatment restored BDNF protein levels of SHR to those of WKY

The neuroendocrine interactions taking place in the brain of SHR could prime the reduction of BDNF expression in the dentate gyrus. Besides hypertension, stress and high levels of glucocorticoids down-regulate BDNF mRNA and protein expression, which are the risk factors for the hippocampus [75]. Therefore, adrenal steroids could be involved in the reduction of BDNF in SHR, because activation of receptors for adrenal steroid represses transcriptional activity of the BDNF promoter site-specifically via interaction with other transcription factors [75]. In contrast to the negative regulation by adrenal steroids, female steroids show a stimulatory effect on BDNF. Modifications of hippocampal BDNF occur during the estrous cycle and estradiol replacement after ovariectomy increases the levels of BDNF mRNA and protein [12]. Estrogens induce BDNF transcription due to the localization of an estrogen-response element in the BDNF promoter [68]. However, in the granule cells of the dentate gyrus, extranuclear ERβ is the predominant receptor subtype, suggesting alternative mechanisms [76]. In our experiments, the stimulatory effect of estradiol in the hippocampus was obtained both at the BDNF mRNA as well as the protein level. We propose that estradiol 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 synthesized BDNF is stored into presynaptic terminals and later released to act postsynaptically [77]. In connection with this event, it has been recently shown that estrogen stimulates BDNF release from the dentate gyrus [69]. This complex interaction developing between estradiol / BDNF could result in trophic effects for the hippocampus, the dentate gyrus and stimulate neurogenesis. We speculate that BDNF restoration could mediate the neuroprotective effects of 17 β-estradiol on some faulty hippocampal parameters of SHR, especially neurogenesis.

6. BASAL AND ESTRADIOL-STIMULATED AROMATASE EXPRESSION IN THE HIPPOCAMPUS OF SHR

Brain estrogen derives in part from ovarian secretion but it is also locally synthesized from cholesterol or androgen precursors by brain cells, as shown by the pioneering studies of Naftolin *et al.,* [78] and Balthazart *et al.,* [79]. The hippocampus of several species contains the enzymes necessary for estradiol biosynthesis, including the aromatization step converting C19 androgens into C18 estrogens [78-81]. The enzyme seems highly active in the hippocampus, since in mice the content of estradiol in this tissue is six-fold higher than in plasma [82]. Aromatase immunoreactivity is present in neuronal perikarya, dendrites, axonal processes and terminal boutons [78]. A role for neuronal aromatase activity in the hippocampus may involve synaptic development and plasticity [83]. In addition, reactive astrocytes strongly expressed aromatase following diverse forms of injury in several brain regions, including the hippocampus [84].

Brain estradiol biosynthesis is under regulatory control by a number of hormones and factors as reported for androgens and estrogens, which show modulatory effects on aromatase, explained by the presence of androgen and estrogen-responsive elements in the CYP19 aromatase gene [81,85]. Interestingly, tonic estrogen treatment of ovariectomized mice increases by 69% aromatase gene expression in the hippocampus, whereas cyclic estradiol administration has the opposite effect [86].

The goal of our studies was to identify possible differences between the hippocampus of SHR and the normotensive WKY rats in the basal and estradiol-stimulated

expression of aromatase mRNA and protein, and to localize the enzyme at the cellular level using double immunofluorescence histochemistry for aromatase and the astrocyte marker GFAP. Real time PCR of aromatase mRNA levels in whole hippocampus of WKY rats and SHR showed higher basal level of aromatase mRNA in SHR respect of WKY rats, whereas estradiol treatment during 2 weeks of SHR – but not WKY - produced a further elevation of aromatase mRNA, surpassing the high basal levels of the same group implanted with cholesterol. Microanatomical distribution of aromatase immunoreactivity localize it to cell processes, perikarya and occasional small varicosities of the pyramidal CA1, CA2, and CA3 subregions. In the CA1 area, the SHR group receiving estradiol showed the highest staining intensity compared to all other groups.

Weak aromatase immunostaining was also found in the granule cell layer of the dentate gyrus, but in contrast, strong aromatase immunolocalization was found in the hilar region of the dentate gyrus. A much stronger aromatase staining of fiber collaterals, axonal varicosities and occasional cell bodies was present in the hilar region of the dentate gyrus of SHR compared to WKY rats, which might correspond to mossy fibers arising from granule cells of the dentate gyrus. The length of immunoreactive processes was also significantly higher in steroid-naïve SHR respect of steroid-untreated WKY, which was significantly increased by estradiol but only in the hypertensive group. Finally, we observed that GFAP immunopositive astrocytes were devoid of aromatase immunoreactivity [87].

It is likely that the increased levels of aromatase and its stimulation by estradiol provide beneficial effects to the hippocampus of SHR. The neuroprotective role of brain aromatase has been already postulated [88], and as shown by Rune *et al.,* [83], it is involved in hippocampus synaptogenesis and synaptic plasticity. At this point, it seems desirable to interpret the positive and differential effects of serum- and locally produced estradiol on the hippocampus, considering that locally produced estrogens due to the high aromatase expression, did not modulate neurogenesis of steroid-naïve SHR. A question remains, however, respect of the neuroanatomical site where systemic estradiol administration regulates hippocampal aromatase expression. Direct estrogen effects on the hippocampus are well accepted. Nevertheless, additional evidence shows that afferent pathways arising from estrogen-sensitive subcortical regions regulate hippocampal plasticity [83]. This paracrine, indirect mechanism may be needed for full restoration of neurogenesis of SHR. Altogether, previous data in conjunction with the present investigation, suggest that a combination of exogenous estrogens and those locally synthesized by the enhanced aromatase expression may have an amplifying effect to alleviate the encephalopathy of SHR.

7. CONCLUDING REMARKS ON ESTROGEN NEUROPROTECTION IN AGE –ASSOCIATED DISEASES

Neuroprotective effects are nowadays an expanding field in the mechanism of action of sex steroid hormones. Estrogens control many functions in neurons and glial cells in the brain, which bear important consequences for the treatment of aging and age-associated diseases such as diabetes mellitus and hypertension. Of special interest are those neuroprotective and beneficial that occur at the hippocampal and hypothalamic level, not necessarily related to reproduction. While the experiments here reported did not address the signaling mechanism(s) of estrogen action, they uncovered important biological consequences of estrogen effects in the brain in the course of hypertension.

Several lessons persist from the use of the SHR. Thus, our data demonstrated that estrogen treatment, albeit in pharmacological doses, is able to protect the hippocampus and hypothalamus, overcoming the undesirable effects of hypertension. In the hypothalamus, estradiol treatment of SHR alleviated the abnormal expression of the hypertensinogenic peptide AVP and its V1a receptor, suggesting that estradiol acts in the brain to decrease blood pressure. In the hippocampus, the effects of estrogens on neurogenesis and growth factor expression are highly relevant, since stimulation of endogenous progenitors to repair cellular damage would be important for learning and memory and neuroendocrine events taking place in the hippocampus. No less important are the down-regulation of the astroglial cell reaction, implying that they may a secondary response to an ongoing neurodegeneration which is prevented by estrogen therapy. Finally, effects on the expression of hippocampal aromatase at the mRNA and protein levels suggest an additional protective mechanism, in that the potential stimulation of locally synthesized estrogens

Table 1. Evidences for Estradiol Neuroprotection in the Brain of Spontaneously Hypertensive Rats

1.	Increased neuronal cell proliferation and differentiation in the dentate gyrus.
2.	Decreased astrogliosis in hippocampal subfields CA1, CA2 and CA3 and dentate gyrus.
3.	Increased expression of BDNF at the mRNA and protein levels in the dentate gyrus.
4.	Further increase of immunoreactive aromatase in the pyramidal CA1 hippocampal subfield and hilus of the dentate gyrus. Further increase of aromatase mRNA in whole hippocampus.
5.	Decreased expression of the mRNA for the hypertensinogenic peptide arginine vasopressin in the hypothalamus.
6.	Decreased blood pressure and cardiac hypertrophy.
7.	Similar changes were found after estradiol treatment in a model of mineralocorticoid-induced hypertension, the DOCA + salt-treated rat.
8.	Similarity of changes in both models suggests common mechanistic factors playing a role in estradiol neuroprotection of SHR and DOCA-treated rats.

may amplify mechanisms leading to alleviation of the hypertensive encephalopathy. The changes of brain parameters following estradiol treatment of SHR are summarized in Table **1**. In conclusion, animal models, such as SHR provide a unique preclinical background to design novel therapeutic strategies for age and age-associated diseases in humans.

There are concerns about the use of estrogens for the treatment of age-related pathologies, especially those involving cognitive impairment, menopausal symptoms and cardiovascular diseases. The Women Health Initiative (WHI) randomized clinical trial claimed that estrogen alone increases the risk of developing mild cognitive impairment [89], a process considered hippocampal-dependent. The WHI trial has been criticized on the grounds that the recruited women were obese and several years past menopause, and at an age when estrogen responsiveness diminishes. Other authors state that in humans there is a " window of opportunity " for hormone-replacement therapy, because it has been reported that estrogen therapy prevents the deleterious effects of brain aging if given at the perimenopause, whereas they are inactive or may even exacerbate neurodegeneration when given late in life [90]. However, undesirable effects may be circumvented by the use of non-feminizing estrogens, estrogen receptor modulators or the brain-active isomer 17α-estradiol. Future developments using new drugs and hormone derivatives are of great promise for neuroprotection in age-associated diseases.

CONFLICT OF INTERESTS

The author(s) confirm that this article content has no conflicts of interest.

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