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EFFECTS OF 17β-ESTRADIOL ON THE CYTOARCHITECTURE OF PYRAMIDAL CA1 NEURONS IN NORMOGLYCEMIC AND DIABETIC MALE SPONTANEOUSLY HYPERTENSIVE RATS

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Abstract—Previous work has shown a reduction of apical dendritic length and spine density in neurons from the CA1 hippocampus subfield of spontaneously hypertensive rats (SHRs). These abnormalities are prevented by treatment for 2 weeks with 17β-estradiol. In view of the fact that diabetes and hypertension are comorbid diseases, we have now studied the effect of Streptozotocin-induced diabetes on the dendritic tree and spines of CA1 hippocampus neurons, and also compared the regulation of these parameters by 17β-estradiol in diabetic and normoglycemic SHR. Twenty-week-old male SHR received iv 40-mg/kg Streptozotocin or vehicle and studied 1 month afterward. A group of normoglycemic and hyperglycemic SHR also received sc a single 17β-estradiol pellet or vehicle for 2 weeks. Hippocampus sections were impregnated with silver nitrate following a modified Golgi's method and the arbor of CA1 pyramidal neurons analyzed by Sholl's method. 17β-Estradiol treatment of normoglycemic SHR reversed the reduced length of apical dendrites, the low spine density and additionally decreased blood pressure (BP). Diabetic SHR showed increased length of apical and basal dendrites but reduced spine density compared to normoglycemic SHR. Diabetes also decreased BP of SHR. Treatment with 17β-estradiol of diabetic SHR enhanced dendritic length, increased dendritic spine density and further decreased BP. Thus, changes of cytoarchitecture of CA1 neurons due to 17β-estradiol treatment of normoglycemic SHR persisted after diabetes induction. A decrease of BP may also contribute to the central effects of 17β-estradiol in SHR diabetic rats.

Key words: hippocampus, 17β-estradiol, dendrites, spines, diabetes mellitus, spontaneously hypertensive rat.

INTRODUCTION

Increased hippocampus vulnerability is an important consequence of hypertensive encephalopathy (Oppenheimer and Fishberg, 1928). Chronic elevation of BP causes atrophic changes, microvascular thickening with ischemia, cytotoxic edema, demyelination, beta-amyloid deposits and tau pathology of the hippocampus. These changes are accompanied by cognitive decline and increased risk of dementia (Skoog et al., 1996; Petrovitch et al., 2000; Mulvany, 2002; Korf et al., 2004; Wiseman et al., 2004; Paglieri et al., 2008).

The spontaneously hypertensive rat (SHR) model of primary hypertension shows a pronounced hippocampus pathology characterized by astrogliosis, neuronal loss, demyelination, decreased growth factor expression, decreased neurogenesis and enhanced mRNA expression of the mineralocorticoid receptor and aromatase (Sabbatini et al., 1999, 2000; Tomassoni et al., 2004; Pietranera et al., 2006, 2010, 2011, 2012). Changes of learning and memory have made SHR models for dementia and the attention-deficit hyperactivity syndrome (Paglieri et al., 2008). Functionally, the hippocampus is highly dependent on the integrity of connections within the trisynaptic circuit (Lorente de No, 1934). This neuronal connectivity of the hippocampus is compromised in SHR, as shown by the abnormal dendritic morphology of pyramidal neurons compared to normotensive Wistar-Kyoto rats (Sánchez et al., 2011; Brocca et al., 2013).

Uncontrolled diabetes mellitus also damages the hippocampus. This is reflected as disturbed memory, impaired neurogenesis, changes of gene expression, altered signaling cascades, decreased energy metabolism and poor cell survival (Saravia et al., 2002, 2004; Reagan, 2005; Revsin et al., 2005; Stranahan et al., 2008; Thomas et al., 2013). Morphological abnormalities also appear in the hippocampus of diabetic animals. Magariños et al. (2000) using the Golgi method and electron microscopy, have observed that Streptozotocin-induced diabetes causes retraction of the presynaptic mossy fiber terminals contacting the CA3 apical dendrites, in addition to synaptic vesicle depletion. They suggest that diabetes is an endogenous stressor and accelerates the effect of exogenous stress. Nitta et al. (2002) have reported in the hippocampus of diabetic rats a pronounced synaptic dysfunction, revealed by a decreased number of basal dendrites and abnormal spine structure. Lastly, impaired insulin and insulin growth factor 1 (IGF1) in BB rats is associated with neuronal apoptosis

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Abbreviations: ANOVA, analysis of variance; BP, blood pressure; GPER, G-protein-coupled receptor 30; IGF1, insulin growth factor 1; RAS, renin–angiotensin–aldosterone system; SHR, spontaneously hypertensive rats.

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and increased Bax/Bcl-x ratio in the hippocampus (Li et al., 2002).

Analysis of different parameters has shown a multifactorial derangement of the diabetic hippocampus, in which the advanced glycation end products, changes of adrenal steroid secretion and their brain receptors, increased oxidative stress, excess production of proinflammatory cytokines, loss of cholinergic neurons, development of microvasculopathy and impaired brain glucose transport play important roles (Wang et al., 2009; Ye et al., 2011; Ceretta et al., 2012; Sherin et al., 2012; Jing et al., 2013; Rocco et al., 2013; Zhang et al., 2013). These findings strengthen the view that encephalopathy of diabetes mellitus (Rowlands and Bellush, 1989; Gispen and Biessels 2000; Artola et al., 2002; Biessels et al., 2002) increases hippocampus vulnerability, resembling hypertension-induced damage.

The combined effects of hypertension plus diabetes on peripheral and central organ damage have been the subject of several studies. In humans, high BP and diabetes mellitus are considered comorbid diseases reaching an epidemic status (Yang et al., 2011). Thus, patients with hypertension are at a two- to threefold higher risk of developing diabetes mellitus than normotensive patients and viceversa (Mancia, 2005). Hypertensive patients with diabetes mellitus are more prone to developing severe cerebrovascular disease and cognitive impairment (Lago et al., 2007). Working with diabetic SHR, Tomassoni et al. (2004) have shown a potentiation of damage to the cerebrovascular tree with increased brain pathology. Yang et al. (2011) and DeVisser et al. (2011) have studied the differential impact of diabetes and hypertension in gray and white matter regions of the brain of SHR with or without Streptozotocin-induced diabetes. They have determined that white matter abnormalities are more common in diabetic animals, whereas neuronal loss requires both pathologies.

Estrogens are recognized protective factors for neurodegenerative diseases. In connection with these properties, treatment of SHR with 17β-estradiol normalizes dendritic arborization and spine number of the CA1 subfield (Brocca et al., 2013), and prevents development of abnormalities involving neurogenesis, growth factor expression, hilus neuronal number and astrocyte reactivity of the hippocampus (Pietranera et al., 2008, 2010, 2011). Therefore, 17β-estradiol protects the hippocampus from hypertensive encephalopathy. Likewise, some hippocampus parameters injured by diabetes are reversed by treatment with 17β-estradiol, which increases cell proliferation and doublecortin-positive neuroblasts in the dentate gyrus, and decreases astrogliosis of type I diabetic rodents (Saravia et al., 2004, 2006). In the cerebral cortex, 17β-estradiol reduces lipid peroxidation, and strengthens the antioxidant systems of diabetic-ovariectomized rats (Ulas and Cay, 2010), whereas chronic 17β-estradiol treatment reduces cortical and striatal infarct volume in male diabetic rats with middle cerebral artery occlusion (Toung et al., 2000).

Since previous studies have addressed the regulatory effects of 17β-estradiol in the hippocampus of hypertensive or diabetic models separately, we first aimed to compare the morphology of dendritic arbor and spine density in normoglycemic and hyperglycemic SHR with 1-month-long diabetes. Once this objective was accomplished, we investigated if 17β-estradiol modulated the hippocampus cytoarchitecture in a combined hypertensive + diabetic model. The results may shed light on the therapeutic value of sex steroid hormones in hypertensive encephalopathy comorbid with diabetes mellitus.

EXPERIMENTAL PROCEDURES

Animals

Male SHRs were obtained from the Institute of Biology and Experimental Medicine Animal facility. Animals were 20 weeks old at the beginning of the experiment. All rats were housed under controlled conditions of temperature (22 °C) and lighting conditions (lights on 07:00–19.00 h) with free access to food and water.

Mean BP was measured by an indirect tail-cuff method (Blood pressure system, Kent Scientific Corporation: Torrington, Connecticut, USA). For steroid treatment, a group of SHR were anesthetized using a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) given ip and implanted sc with a pellet containing 12 mg of 17β-estradiol benzoate (Sigma–Aldrich, St. Louis, MO, U.S.A.) dissolved in cholesterol during the last 2 weeks of the experiment. Another group of SHR was implanted with cholesterol pellets only. This 17β-estradiol treatment provides neuroprotection in different experimental conditions (Ferrini et al., 1999; Pietranera et al., 2008, 2010, 2011; Brocca et al., 2013). For diabetes induction, SHR received via the tail vein 40-mg/kg Streptozotocin (Sigma–Aldrich, St. Louis, MO, U.S.A.) dissolved in 0.5 M sodium citrate buffer. Two days after the injection glycosuria was determined using Keto-Diastix (Bayer Diagnostics, Buenos Aires, Argentina). Glycemia was determined at the time of killing using a one-touch ULTRA (Johnson and Johnson, Milpitas, CX, U.S.A.). Normoglycemic and hyperglycemia SHR were used 1 month after diabetes induction.

Animal experiments followed the NIH Guide for the Care and Use of Laboratory Animals and were approve by the Ethics Committee of the Institute of Biology and Experimental Medicine. Efforts were made to minimize animal suffering and to reduce the number of animals used in the different experiments.

Golgi staining for analysis of dendrite length and spine number in CA1 hippocampus neurons of normoglycemic and diabetic hypertensive rats

The procedures followed for perfusion of rats intracardially and fixation of brains for preparation for Golgi staining were already described (Brocca et al., 2013). We employed a variant of the Golgi procedure thoroughly described in previous publications (Beauquis et al., 2010; González-Burgos et al., 2012; Brocca et al., 2013). Neurons impregnated with silver nitrate were studied in the CA1 region of the dorsal hippocampus at

plates 27–33 of the brain atlas of Paxinos and Watson (1997).

Golgi impregnated pyramidal neurons selected for reconstruction and measurement of the dendrite length had to comply with a four-point morphological criterion, as already described in Brocca et al. (2013). The CA1 area was chosen because in comparison with other hippocampus regions, it shows higher estrogen sensitivity in female rats (Woolley et al., 1990; Inagaki et al., 2012). The dendritic length was studied by the Sholl method (Sholl, 1953). The number of intersections per shell in the Sholl analysis was also plotted against the distance from the center of neuronal soma. Drawings of the complete neuron and its neurites were analyzed with NIH software ImageJ running the Sholl Analysis Plugin v1.0. An average of four Golgi-impregnated neurons per brain was considered to meet the required criteria. The number of rats used for determination of dendritic length was 20 for SHR and 24 for diabetic SHR and the number of rats was 10 for SHR plus 17β-estradiol and 10 for SHR diabetic plus 17β-estradiol.

Golgi-impregnated dendritic spines was counted according to Woolley et al. (1990), following a previously described four-point criteria (Brocca et al., 2013). Spines of apical dendrites were counted in the *stratum radiatum*, whereas those pertaining to basal dendrites were counted in the *stratum oriens* of the hippocampus. Spines were counted in 15 segments per animal. Apical and basal spines were averaged per animal and results were expressed as the number of spines per μm dendritic length. Number of rats in each experimental group was the same as shown above for dendritic length.

Statistical analysis

Results were analyzed by a two-way analysis of variance (ANOVA) followed by the post hoc Bonferroni test. Statistical analyses were performed with Prism 4 GraphPad software (San Diego, CA, USA). A p value < 0.05 was considered significant.

RESULTS

BP and glycemia levels in SHR with or without Streptozotocin-induced diabetes mellitus. Effects of 17-estradiol treatment

One month after administration of Streptozotocin, SHR were markedly hyperglycemic, with mean blood glucose measuring above 500 mg/dl (Table 1). However, the hyperglycemia of diabetic SHR was slightly, but significantly reduced when diabetic SHR received the 17β-estradiol treatment for the last 2 weeks of the experiment (SHR + STZ vs. SHR + STZ + E_2, $p < 0.01$). Changes of blood glucose were not observed in 17β-estradiol-treated SHR. Although all SHR showed a sustained elevation of BP, induction of diabetes mellitus in this group produced a hypotensive effect (SHR vs. SHR + STZ, $p < 0.001$). By itself, 17β-estradiol slightly decreased BP of SHR by 20 mmHg ($p < 0.05$), whereas diabetes induction decreased the mean value of BP by 27 mmHg (SHR vs. SHR + STZ, $p < 0.001$). However, combination of 17β-estradiol plus diabetes markedly reduced the BP of SHR to 140 mmHg, the lowest values of all experimental groups (SHR vs. SHR + STZ + E_2, $p < 0.001$) (Table 1). These levels were still higher than those usually found in Wistar-Kyoto control rats, which approximated 110 mmHg (Brocca et al., 2013). Body weights of diabetic rats were lower than those of normoglycemic SHR, either in the presence or in the absence of 17β-estradiol (Table 1).

Dendritic length of normoglycemic and diabetic SHR with and without 17- estradiol treatment

The length of apical dendrites was analyzed in the CA1 region from the hippocampus of four groups of rats: SHR, SHR plus 17β-estradiol, diabetic SHR and diabetic SHR plus 17β-estradiol. A two-way ANOVA revealed significant changes between the mentioned groups ($F_{\text{treatment};1,43} = 17.87$). Post hoc analysis indicated that induction of diabetes mellitus enhanced by 28% the apical dendritic length of SHR compared to non-diabetic SHR (Fig. 1A; $p < 0.01$). Likewise, the post hoc test showed that 17β-estradiol treatment significantly increased apical dendritic length of hypertensive rats regardless of their glycemic condition (SHR vs. 17β-estradiol -treated SHR, $p < 0.05$; diabetic SHR vs 17β-estradiol-treated diabetic SHR: $p < 0.01$). The results also showed that mean dendritic length of the diabetic SHR + 17β-estradiol group was 60% longer compared to the SHR group, whereas dendrites of the SHR + 17β-estradiol group were 36% longer vs. the SHR group. Therefore, diabetes increased apical dendritic length of hypertensive rats and further enhanced the 17β-estradiol up-regulation of this

Table 1. Effects of 17β-estradiol (E2) treatment on glycemia, blood pressure and body weight in SHR without or with diabetes mellitus (DM)

Group	Glycemia (mg/dl)	Blood pressure (mm Hg)	Body weight (g)
SHR	116.7 ± 5.4	188.4 ± 2.8	328.0 ± 6.6
SHR + E2	122.3 ± 6.6	168.6 ± 3.5*	271.6 ± 4.4***
SHR + DM	565.2 ± 10.2***	161.1 ± 4.7***	235.5 ± 5.9***
SHR + DM + E_2	508.6 ± 15.8***##φ	140.9 ± 5.6***#φ	206.2 ± 6.8##φ

* $p < 0.05$.
*** $p < 0.001$ vs. SHR.
\# $p < 0.05$.
\#\# $p < 0.01$ vs. SHR + STZ.
φ $p < 0.001$ vs. SHR + E_2.

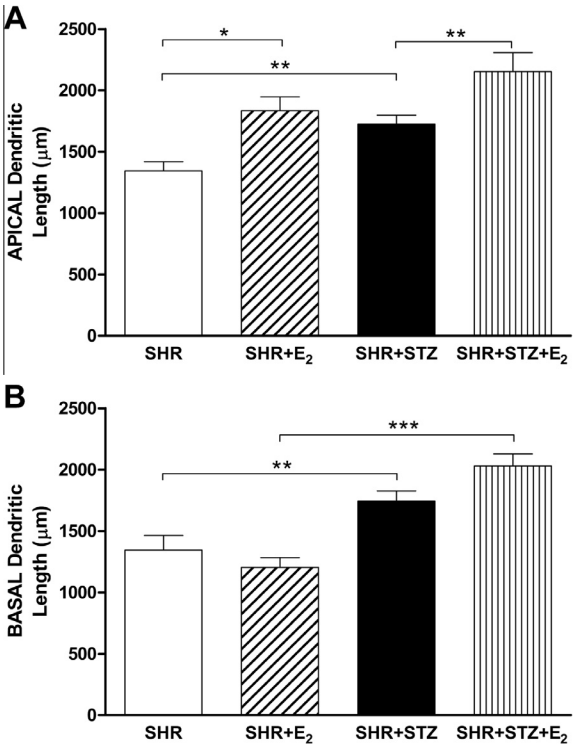

Fig. 1. A Mean length of CA1 apical dendrites (μm) measured in four groups of rats: SHR, SHR plus 17β-estradiol (E_2), diabetic SHR (SHR + STZ) and diabetic SHR receiving 17β-estradiol (SHR + STZ + E_2). Significant differences were found between the SHR and SHR + STZ groups ($^{**}p < 0.01$). 17β-estradiol treatment increased dendritic length of hypertensive rats (SHR vs. SHR + E_2: $^{*}p < 0.05$) and of the diabetic hypertensive group (SHR + STZ vs. SHR + STZ + E_2: $^{**}p < 0.01$). B. Mean length of basal dendrites (μm) in the CA1 hippocampus of the experimental groups detailed in Fig. 1A.. Diabetes induction of SHR significantly increased the length of basal dendrites vs. SHR ($^{**}p < 0.01$). Whereas 17β-estradiol effect was inactive on the basal dendrites of SHR, the modulatory action of 17β-estradiol was observed after diabetes induction (SHR + STZ + E_2 group vs.SHR + E_2: $^{***}p < 0.001$).

parameter. In this regard, it is important to note that under the effects of 17β-estradiol, length of apical dendrites of the diabetic SHR (2153 ± 156 μm) exceeded the length of dendrites from control WKY rats, the normotensive strain of SHR (1816 ± 88 μm).

Using a criterion applied to apical dendrites, the length of the basal dendritic arbor was determined in the four experimental groups (Fig. 1B). ANOVA demonstrated significant group differences ($F_{condition(1,48)} = 22.88$; $p < 0.001$). Post hoc comparison indicated that diabetes induction of SHR significantly increased basal dendritic length vs. the SHR group ($p < 0.01$). However, in contrast to the response of apical dendrites, 17β-estradiol did not increase basal dendritic length in normoglycemic or in diabetic SHR (p:NS). Still, the mean dendritic length of the SHR + diabetes + 17β-estradiol group was 50% longer than that of SHR, but only 16% longer than the SHR + diabetes group. Therefore, the results with basal dendrites suggested that diabetes induction enhanced the response to 17β-estradiol, because the diabetic SHR + 17β-estradiol group showed higher mean length values than the SHR + 17β-estradiol group ($p < 0.001$) (Fig. 1B). In consonance with apical dendrites, length of basal dendrites of the diabetic SHR + 17β-estradiol group (2030 ± 97 μm) also exceeded the level of control WKY rats (1534 ± 86 μm).

We next studied whether the effects of diabetes and 17β-estradiol applied to the whole apical dendritic tree of SHR or were confined to specific sectors. In order to elucidate this issue, the number of intersections per shell in the Sholl analysis was plotted against the distance from the center of soma (Fig. 2A). The analysis

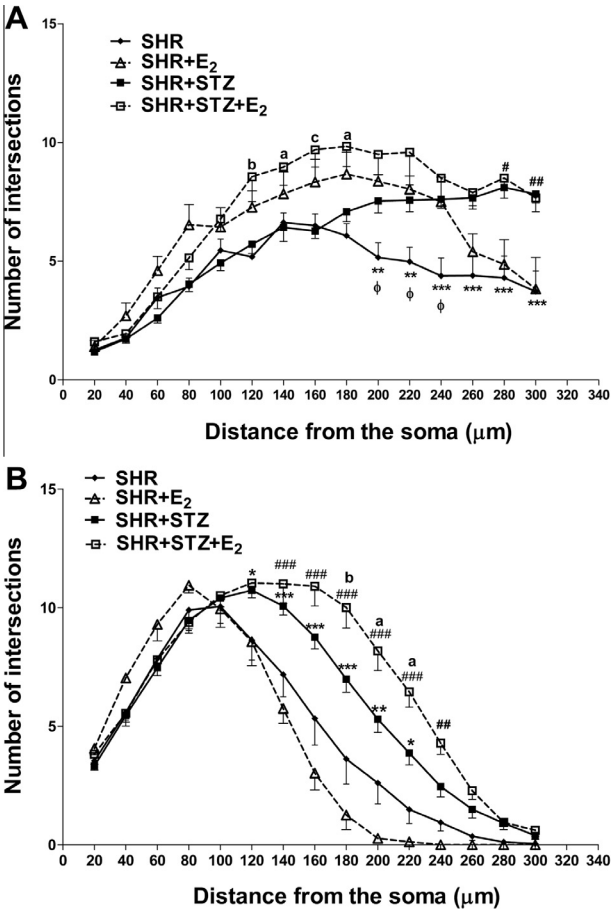

Fig. 2. A. Quantitative determination of the number of intersections in the Sholl analysis for apical dendrites. Significant differences were found for intersections in the circles ranging from 200–300 μm for SHR compared to the SHR + STZ group ($^{**}p < 0.01$, $^{***}p < 0.001$). Results also showed increased intersections in the SHR + E_2 and SHR + STZ-E_2 groups compared to their respective steroid-naïve groups at the 200–240 μm (SHR vs. SHR + E_2: $^{\varphi}p < 0.05$) and at 120–180 μm (SHR + STZ vs. SHR + STZ + E_2:: $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$), respectively. Comparison between the two 17β-estradiol-treated groups, (SHR + E_2 vs. SHR + STZ + E_2) revealed that in diabetic rats, 17β-estradiol increased branching at 280–300 μm ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$). B. The number of intersections plotted against radius of the Shell for basal dendrites showed a significant increase at the distance 120–220 μm from soma of the SHR + STZ group vs. SHR ($^{*}p < 0.05$, $^{**}p < 0.01$,$^{***}p < 0.001$). Treatment with the steroid of diabetic hypertensive rats produced a higher number of intersections at 180–220 μm (SHR + STZ vs. SHR + STZ + E_2: $^{a}p < 0.01$; $^{b}p < 0.001$). Diabetes induction also enhanced the response to 17β-estradiol at distances 140–240 μm (SHR + STZ + E2 vs. SHR + E2: $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$).

of branching of the apical dendritic tree reported significant differences in the ANOVA test with respect to distance from soma ($F_{(3,602)} = 11.76$, $p < 0.001$). The post hoc test showed that the diabetes effect on SHR was more concentrated in the long range distance dendrites (200–300 μm from soma; $p < 0.01$ or less vs. SHR), whereas the effects of 17β-estradiol treatment showed a wider distribution. In the latter case, significant differences were obtained in the 200–240-μm distance for 17β-estradiol treated SHR vs. SHR ($p < 0.05$), and 120–180-μm distance for 17β-estradiol-treated diabetic SHR vs. SHR ($p < 0.05$ or less).

The analysis applied to apical dendrites was also conducted to study the effects of diabetes and 17β-estradiol on the length of basal dendrites segregated into shell distances from soma (Fig. 2B). Branching of the basal dendritic tree in the four experimental groups reported significant differences in the ANOVA test with respect to distance from soma ($F_{\text{groups } (3,714)} = 10.17$, $p < 0.001$) As shown in Fig. 2B, plotting of the number of intersections vs. distance from soma center, showed significantly stronger effects of diabetes in the 120–220-μm distance from soma (diabetic SHR vs. SHR: $p < 0.05$ or less). Furthermore, in the basal dendrites of diabetic SHR, 17β-estradiol treatment significantly increased the number of intersections in the 180–220-μm distance ($p < 0.01$ or less). Therefore, the results indicated that the 17β-estradiol effect on basal dendrites, which was absent in normoglycemic SHR, reappeared when the SHR were rendered diabetic. Therefore, diabetes conditioned the response to 17β-estradiol on the basal dendrite branching, improving the 17β-estradiol effects on dendrites longer than 140 μm from the soma.

Fig. 3 shows examples of camera lucida drawings of the four experimental groups. Golgi impregnation of hippocampus slices showed an atrophic profile of the dendritic arbor of normoglycemic SHR (A), compared with a stronger arborization of the basal and apical dendritic tree of diabetic SHR (C). 17β-Estradiol treatment of SHR produced a better developed dendritic arbor in a representative CA1 pyramidal neuron (B). The last drawing (D) corresponded to a diabetic rat receiving 17β-estradiol. In this case, a profuse dendritic arbor resulted from the combined effects of diabetes plus 17β-estradiol in the hypertensive rat.

Spine density of normoglycemic and diabetic SHR with and without 17-estradiol treatment

Spines protruding from apical dendrites were counted in the *stratum radiatum* and those projecting from basal dendrites were determined in the *stratum oriens* of CA1 pyramidal neurons. In the case of apical spines, quantitative evaluation by ANOVA demonstrated significant group differences ($F_{\text{treatment } (1,47)} = 60.37$; $p < 0.001$). Group comparison by the post hoc test (Fig. 4A), showed that the hypertensive + diabetes group contained a significantly lower spine number compared to normoglycemic SHR ($^{*}p < 0.05$). After 2 weeks of treatment with 17β-estradiol, apical dendrites from the CA1 region showed an increased spine number in both normoglycemic and diabetic SHR ($p < 0.001$ for both cases). Therefore, after 17β-estradiol treatment apical spine number was similar in both normoglycemic and diabetic SHR (p:NS). Fig. 4B shows the results of basal spines counted in dendrites of the *stratum oriens*. ANOVA demonstrated significant group differences ($F_{\text{treatment } (1,32)} = 25.84$; $p < 0.001$). Post hoc comparison demonstrated that similarly to apical spines, diabetic SHR contained a significantly lower number of basal dendritic spines than normoglycemic SHR ($p < 0.05$). The multiple comparison tests also showed that after 17β-estradiol treatment, spine number from basal dendrites was enhanced both in SHR ($p < 0.05$) and in diabetic SHR ($p < 0.001$).Therefore, 17β-estradiol supported spinogenesis of SHR regardless of their glycemic condition. Representative microscopic images of the apical spines are shown in Fig. 5. It is observed that hypertension and diabetes plus hypertension led to spine depletion (A, C). In contrast, enrichment of spines was observed after sex steroid treatment of normoglycemic SHR (B) and diabetic SHR (D).

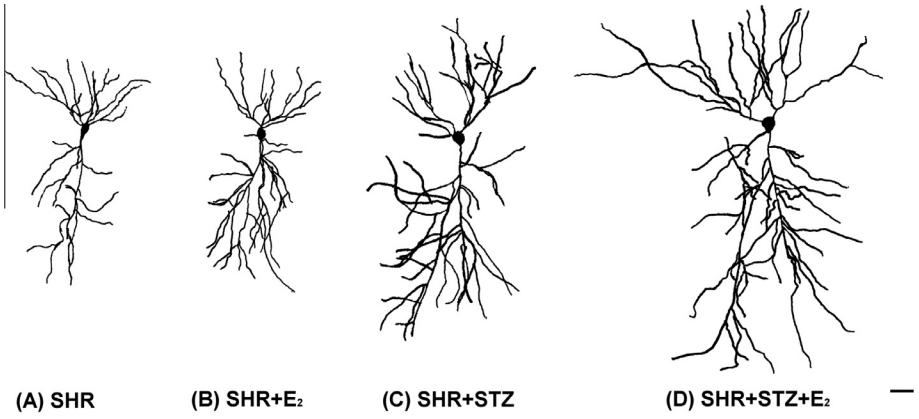

Fig. 3. Camera lucida drawings of CA1 pyramidal neurons of the four experimental groups as detailed in Fig. 1. Compared to untreated SHR (A, SHR), increased arborization of SHR occurred in animals receiving 17β-estradiol (B, SHR + E_2) and after diabetes induction (C, SHR + STZ). Dendritic sprouting looked stronger when diabetic SHR received 17β-estradiol (D, SHR + STZ + E_2). Scale bar = 50 μm.

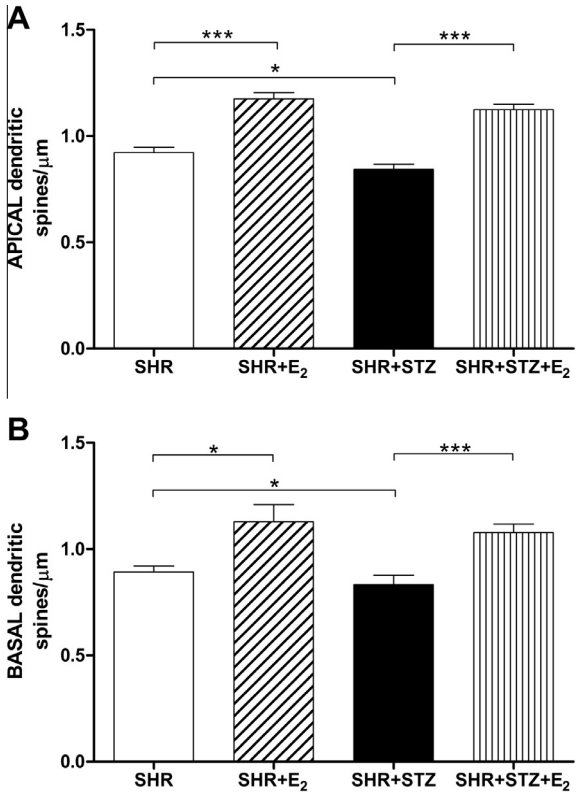

Fig. 4. Determination of spine density in the CA1 pyramidal neurons following the four-point morphological criterion detailed in the Experimental procedures section: Quantitative analysis evidenced a reduction of spine density both for the apical (A) and basal (B) dendrites in the SHR + STZ group ($^{*}p < 0.05$ vs. SHR). A significant surge of spine number was obtained after SHR received 17β-estradiol, either in the normoglycemic state (SHR + E_2: $^{***}p < 0.001$ for apical and $^{*}p < 0.05$ for basal dendrites vs. SHR) or in the hyperglycemic state (SHR + STZ + E_2: $^{***}p < 0.001$ for apical and basal dendrites vs. SHR + STZ).

We also assessed if 17β-estradiol treatment of diabetic SHR reverted to normal the density of dendritic spines. Comparison of this parameter showed that apical and basal spine density of the diabetic SHR + 17β-estradiol group was similar to that of control normotensive WKY rats (for apical spines: WKY 1.00 ± 0.01 per μm; SHR + diabetes + 17β-estradiol 1.12 ± 0.02: for basal spines: WKY 1.01 ± 0.01 μm, SHR + diabetes + 17β-estradiol 1.07 ± 0.02).

DISCUSSION

In the present investigation we studied the CA1 neuronal dendritic arbor and spine density of hypertensive rats with or without diabetes mellitus. First, we corroborated previous findings (Brocca et al., 2013) which demonstrated that 17β-estradiol treatment enhanced the length of the dendritic tree and spine density of the CA1 apical dendrites of SHR. In addition, we found that the positive modulation of 17β-estradiol on dendrites and spines of hypertensive rats remained in SHR rendered diabetic by Streptozotocin. Paradoxically, our data showed that diabetes potentiated the 17β-estradiol effect, because CA1 apical dendrites were longer in diabetic SHR receiving 17β-estradiol treatment, than in steroid-naïve SHR or diabetic SHR. This result suggests an amplifying effect of the pathological microenvironment of the hypertensive-diabetic rat on 17β-estradiol effects on hippocampus cytoarchitecture. Third, analysis by the Sholl method of the dendritic intersections showed that 17β-estradiol effects were more evident on long range distances from soma, whereas diabetes exerted additional effects on the middle range distances. At the clinical level, our results showed that diabetes induction and treatment with 17β-estradiol decreased BP and body weight of SHR. The estrogen effects may be due to decreases in food intake (Santollo et al., 2007), whereas the loss of body weight in diabetes can be ascribed to fat and protein catabolism. A question remains on whether reduction of BP of diabetic SHR originated in the changes of body weight. However, Susic et al. (1990) have shown several years ago that fasting SHR with weight loss equivalent to that of diabetic SHR did not modify the high BP levels of these animals.

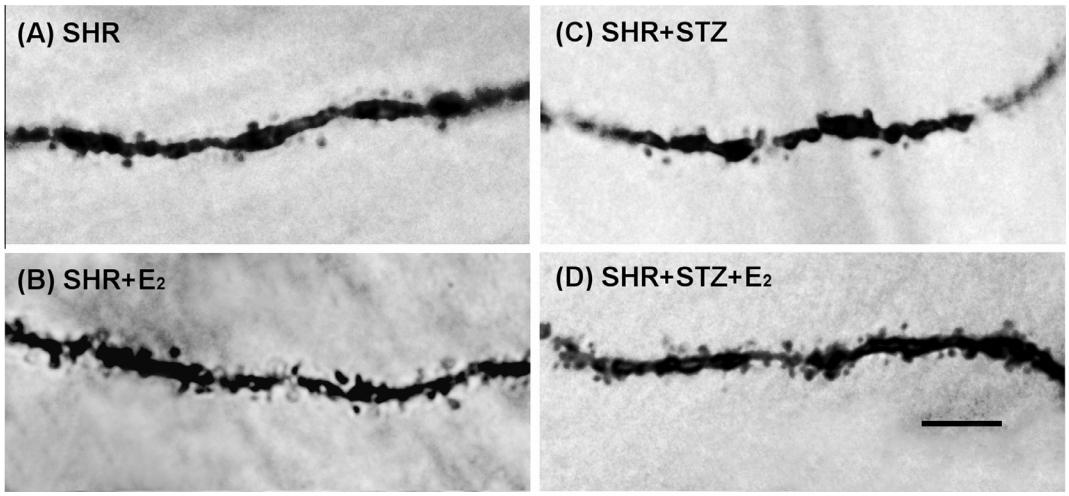

Fig. 5. Representative microphotographs of dendrites stained by the Golgi silver impregnation method. The images revealed low and comparable number of apical dendritic spines in SHR (A) and SHR + STZ (C) groups. The images in (B) and (D) showed that 17β-estradiol increased spinogenesis of SHR (B) and of SHR with induced diabetes mellitus (D: SHR + STZ + E_2). Scale bar = 5 μm.

Another finding from our work refers to the microanatomical site(s) of steroid action, because in contrast to apical dendrites, basal dendritic length was not regulated by 17β-estradiol given to normoglycemic SHR. This was already shown in previous work (Brocca et al., 2013) and replicated here. However, the effects of 17β-estradiol became apparent in the presence of diabetes, as length of basal dendrites of the diabetic + 17β-estradiol-treated SHR group was the longest compared to the other groups. Thus, diabetes induction modified in several ways the steroid response, and in addition, diabetes per se increased the length of the apical and the basal dendrites. It seems important to remark that following 17β-estradiol treatment of diabetic rats dendritic length was actually normalized and even enhanced over the length shown by WKY rats, the normotensive control of SHR. Similarly, spine density of both apical as well as basal spines returned to levels shown by control normotensive WKY rats.

There may be several factors involved in this paradoxical dendritic sprouting observed after induction of diabetes in SHR. Thus, as diabetic SHR showed decreased number of spines in both apical and basal dendrites, dendritic elongation may be a mechanism compensatory for the fewer spines of CA1 hippocampus neurons. Dissociation between dendrite length and spine density has been reported in other circumstances. In rats learning a spatial navigation task, Kolb et al. (2008) reported increased dendritic arborization but decreased spine density in pyramidal occipital cortex neurons. In Tg2576 mice with overexpression of the Swedish mutation of the human amyloid precursor protein (APP), there is increased dendritic elongation in pyramidal cortical neurons, coexisting with a reduction in spine density (Rocher et al., 2008.). In addition, increased dendritic arborization and sprouting with loss of spinophilin-labeled dendritic spines in the CA1 fields of hippocampus have been shown in Alzheimer's disease brains (Spires and Hyman, 2004; Akram et al., 2008). Along this line, it has been proposed that Alzheimer's disease represents a form of type III diabetes because the molecular and biochemical features overlap with both type 1 and type 2 diabetes mellitus (de la Monte and Wands, 2008). Changes of learning and memory have made SHR models for dementia (Paglieri et al., 2008), a hallmark of Alzheimer's disease. Therefore, the diabetic-SHR rat shares some features with Alzheimer's disease.

Another factor modulating the synaptic reorganization of CA1 neurons in diabetic SHR may rely on changes of BP. Diabetic SHR show decreased BP compared to normoglycemic SHR, although levels of Wistar-Kyoto rats (the normotensive background strain for SHR) were not reached. Diabetic SHR could be sodium depleted due to their marked polyuria with sodium excretion, a factor decreasing BP. Besides, several reports have confirmed a deficiency of the peripheral renin–angiotensin–aldosterone system (RAS) in experimental diabetes (Christlieb, 1976; Price et al., 1999; Giacchetti et al., 2005). Thus, decreased RAS and hyponatremia could explain the reduced BP of the SHR-diabetic cohort, with an impact on the length of the dendritic arbor. With these results on hand, we hypothesized that dendritic arborization may be related in an unknown way to BP status. This supposition is presented in Fig. 6, in which a plot of BP levels against dendritic length, demonstrated a significant negative correlation ($p < 0.01$). Thus, in SHR high BP was associated with low dendritic length and viceversa.

The present report supports the vulnerability of the hippocampus the damaging effects of diabetes mellitus and hypertension. Several groups have described gross morphological abnormalities, neuronal loss, atrophy of the dendritic tree, microvasculopathy, reduced neurogenesis, demyelination, astrogliosis, and cognitive decline in these diseases (Sabbatini et al., 1999, 2000; Magariños et al., 2000; Mulvany, 2002; Nitta et al., 2002; Saravia et al., 2004; Tomassoni et al., 2004; Wiseman et al., 2004; Paglieri et al., 2008; Pietranera et al., 2008, 2010; Stranahan et al., 2008; Thomas et al., 2013) The derangement of hippocampus arborization found in the present and former work in hypertension and /or diabetes should lead to dysfunctional hippocampus connectivity. The well-known hippocampus trisynaptic circuit ends up in the CA1 pyramidal cells, which receive most inputs from the CA3 region via the Schaffer collaterals, according to the classical neuroanatomical studies of Lorente de No (1934). However, hippocampus arborization shows a great degree of plasticity. For example, more than a decade ago, Gould et al. (1990) have shown that the CA1 region is specifically modulated by estrogens, which increase dendritic spine formation and synaptic density through the mediation of estrogen receptors.

Therefore, it seemed crucial to examine how hippocampus vulnerability could be rescued by estrogens in a model of diabetes–hypertension comorbidity. Whereas we have previously shown the stimulatory effect of 17β-estradiol on apical dendritic length and spine density of CA1 hippocampus in SHR (Brocca et al., 2013), the response to 17β-estradiol in a combined diabetes plus hypertension model have not been previously reported. Therefore, an important objective was to establish if similarities or differences existed in 17β-estradiol neuroprotection between SHR and SHR-diabetic animals. This approach was taken because

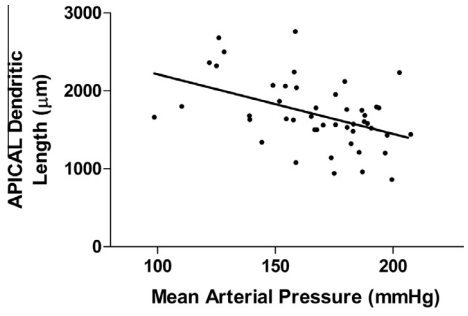

Fig. 6. Correlative study of dendritic arborization and blood pressure levels. The figure shows the combined plot of apical dendritic length of all groups of hypertensive rats vs. the mean arterial pressure measured by a tail-cuff method. A negative correlation was obtained with a Pearson r value of 0.453, *R* squared of 0.2055 and a two-tailed *p* value of 0.01. Number of XY pairs = 23.

estrogens are hippocampus neuroprotectants in a variety of pathological situations (Goodman et al., 1996; Behl, 2002; McEwen, 2002; McCullough and Hurn, 2003; Wise, 2006; Azcoitia et al., 2011), which included hypertension (Pietranera et al., 2004, 2006, 2008, 2010, 2011) and diabetes mellitus (Saravia et al., 2004; Saravia et al., 2006). 17β-estradiol was chosen as neuroprotectant because of its known modulation of dendritic length and spine density in the adult hippocampus *in vivo* (Gould et al., 1990; Christensen et al., 2011; Velázquez-Zamora et al., 2012; Luine and Frankfurt, 2013) and in hippocampus neurons *in vitro* (Prange-kiel et al., 2013).

However, the just-mentioned literature reports on 17β-estradiol effects have been investigated in female animals but not in male animals, both *in vivo* and *in vitro*. We used male SHR based on several circumstances. First, there are profound differences in BP between male and female SHR, with males showing significantly higher BP than females (Reckelhoff et al., 2000; Maris et al., 2005; Moulana et al., 2014). In addition, pronounced sexual dimorphisms and susceptibility to diabetes have been reported, with higher mortality in female rats (Vital et al., 2006). Besides, differences in the profile of neurosteroids between diabetic male and female rats have been reported (Pesaresi et al., 2010). In this context, the use of male SHR seems justified because of their higher BP and lower mortality during hyperglycemia compared to female animals. Third, the use of males would be consistent with our previous reports showing that several hippocampus parameters are estrogen-responsive in male SHR but not in male normotensive WKY rats (Pietranera et al., 2008, 2010, 2011; Brocca et al., 2013). Nevertheless, in view of increasing evidence that differences between male and female animals occur in the hippocampus after 17β-estradiol, the present results on the effects of 17β-estradiol in diabetic SHR should be circumscribed only to males.

When 17β-estradiol was given to diabetic SHR, some differences emerged with steroid-treated normoglycemic SHR. In the case of the apical dendritic length, diabetes not only increased the apical dendritic length of hypertensive rats but also potentiated the 17β-estradiol up-regulation of this parameter. In addition, 17β-estradiol effects were distributed widely compared to a more restricted effect of diabetes. Regarding the basal dendritic length a direct 17β-estradiol was lacking, although the steroid effect appeared in the long range distance dendrites in the presence of diabetes. Thus, it seems that diabetes allowed the response of the basal dendrite branching to 17β-estradiol. Finally, with respect to dendritic spines, 17β-estradiol supported spinogenesis of both the SHR and SHR + diabetes groups. An interesting question then arises: why did 17β-estradiol effects on dendritic and spine morphology persist when brain pathology was probably greater due to simultaneous presence of hypertension and diabetes?

In this regard, the mechanism(s) available for the regulation of dendritogenesis and spinogenesis by estrogens should be recalled. The neurochemical basis for estrogen protection to CA1 neurons relies on its strong anti-oxidant, anti-glutamatergic, neurotrophic, anti-apoptotic and mitochondrial protective activities (Behl, 2002; McCullough and Hurn, 2003; Brinton, 2008; Azcoitia et al., 2011). These estrogen effects can be genomically mediated by the estrogen receptors alpha and beta (ER and ER β) or by membrane-initiated events. Among the latter, increasing evidence point to the G-protein-coupled receptor 30 (GPER) playing an important role in neuritogenesis (Ruiz-Palmero et al., 2013). GPER also regulates vasomotor tone, delays development of hypertension and plays a protective function in the cardiovascular system of SHR (Meyer et al., 2011; De Francesco et al., 2013). Therefore, differential activation of the last type of estrogen receptor, the decrease of BP due to estrogens (Belo et al.,2004) and the hypotensive effect of diabetes may influence the structure CA1 neurons of SHR.

Literature reports have presented evidence for 17β-estradiol neuroprotection in several pathological environments, i.e., diabetes, hypertension, aging, neuroinflammation, ischemia, injury and neurodegeneration (Saravia et al.,2004; Brocca et al.,2013; Chen et al., 2007; Pietranera et al., 2010; Arevalo et al., 2011; Mackenzie et al., 2012; Johann and Beyer, 2013; Schreihofer and Ma, 2013). Therefore, we would like to speculate that diabetes sensitizes the brain (Klein and Waxman, 2003) to estrogen effects at the cell membrane or nucleus, resulting in changes of synaptic connectivity of the CA hippocampus neurons. Among the factors involved in this sensitization, locally synthesized estrogens due to hippocampus aromatase may play a role. The work of Vierk et al. (2012) has shown that the regulation of synaptic plasticity in the hippocampus in response to sexual steroids may depend on aromatase. The activity of this enzyme specifically affects synaptic plasticity in females but not in males (Vierk et al., 2012). Interestingly, male SHR showed increased aromatase immunoreactivity and mRNA expression in the hippocampus (Pietranera et al., 2011), both of which are further enhanced following 17β-estradiol treatment. Preliminary results suggest that similar effects are taking place in the hippocampus of diabetic SHR (Brocca et al., unpublished). Thus, we suggest that the synaptic arbour of male SHR with and without diabetes mellitus respond to 17β-estradiol in a female-like manner. A matter of future endeavor would be to analyze the molecular mediators of estrogen effects in comorbid diseases, with the hope of finding a novel therapeutic approach for diabetic-hypertensive encephalopathy.

From the behavioral point of view, the SHR has been investigated as a model for the attention/hyperactivity syndrome and vascular dementia (Paglieri et al., 2008). Behavioral performance is also altered in diabetes mellitus and in streptozotocin-treated SHR (Tomassoni et al., 2004; Stranahan et al., 2008; Thomas et al., 2013). Thus, cognitive dysfunction, changes in learning and memory and hyperactivity of SHR may be the functional correlate for the abnormalities of neuronal cytoarchitecture found in the present investigation. Regarding the response to sex steroids, it is known that in normal animals exogenous or brain-derived 17β-estradiol has a strong impact on

learning, memory, hippocampus-dependent behaviors and long-term potentiation (González-Burgos et al., 2012; Inagaki et al., 2012; Vierk et al., 2012; Luine and Frankfurt, 2013). It has been postulated that these changes obey to increases in spine density, synaptic plasticity and neurophysiology of the CA1 neurons (Hojo et al.,2008). Therefore, further experiments are needed to elucidate if changes of hippocampus dendrites and spines due to estrogen treatment lead to improvement of cognitive processes in SHR with and without diabetes mellitus.

CONCLUSIONS

The results of the present study support previous reports demonstrating 17β-estradiol modulation of the cytoarchitecture of CA1 hippocampal neurons. The novelty of our study resides in the use of a hypertensive-diabetic rat mode to test 17β-estradiol effects in the hippocampus. Separately, these diseases are known to damage hippocampus structure and function. Normoglycemic SHR showed atrophic dendrites and low spine counting, both abnormalities being reversed by 17β-estradiol treatment. Concomitantly, the steroid decreased BP of SHR. Diabetic SHR showed elongated dendrites and spine depletion, findings observed by others in Alzheimer patients and some animal models of the disease. Induction of diabetes also decreased BP of SHR. In diabetic SHR, 17β-estradiol treatment further increased dendrite length and restored spine number. Correlative analysis demonstrated that dendritic length was negatively associated to BP levels. We suggest that changes of neuronal processes in SHR with or without diabetes are plastic events sensitive to steroid treatment and possibly to BP status. Therefore, the present data revealed new targets of 17β-estradiol effects in the brain of hypertensive diabetic rats.

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