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Mineralocorticoid Treatment Upregulates the Hypothalamic Vasopressinergic System of Spontaneously Hypertensive Rats

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Key Words
Vasopressin · Vasopressin receptor · Fos · Paraventricular nucleus · Adrenal steroids · Hypertension · Preoptic nucleus · Organum vasculosum laminae terminalis

Abstract
Mineralocorticoid effects in the brain include the control of cardiovascular functions, induction of salt appetite, interaction with the vasoactive neuropeptides arginine vasopressin (AVP) and angiotensin II and development or aggravation of hypertension. In this regard, mineralocorticoids may play a pathogenic role in rats with a genetic form of hypertension (spontaneously hypertensive rats, SHR). Our objective was to compare the response of the hypothalamic vasopressinergic system to mineralocorticoid administration in SHR and control Wistar-Kyoto (WKY) rats. Sixteen-week-old male SHR showing a systolic blood pressure of 190 ± 5 mm Hg and normotensive WKY rats (130 ± 5 mm Hg) were treated subcutaneously with oil vehicle or a single 10-mg dose of deoxycorticosterone acetate (DOCA). After 2 h, rats were sacrificed and brains prepared for immunocytochemistry of Fos and vasopressin V1a receptor (V1aR) and for non-isotopic in situ hybridization of AVP mRNA. In the basal state, SHR demonstrated a higher number of AVP mRNA- and V1aR-immunopositive cells in the magnocellular division of the paraventricular hypothalamic nucleus (PVN) than WKY rats. After DOCA injection, SHR responded with a significant increase in both parameters with respect to vehicle-injected SHR. In WKY rats, DOCA was without effect on AVP mRNA although it increased the number of V1aR-positive cells. Changes in the number of Fos-positive nuclei were measured 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 DOCA treatment, a significant increment occurred in the OVLT but not in the PVN or MnPO of the SHR group only. These data suggest that the enhanced response of the vasopressinergic system to mineralocorticoids may contribute to the abnormal blood pressure of SHR.

Introduction

Mineralocorticoid actions in the brain include modulation of memory and learning processes, maintenance of ionic balance and membrane excitability, regulation of cardiovascular functions and fluid ingestion, interaction with neuropeptidergic and neurotransmitter systems and control of the hypothalamic-pituitary-adrenal axis [1–4].

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Part of mineralocorticoid effects are subsequent to hormone binding to intracellular receptors (type I or MR) expressed in anatomically-defined regions of the brain [1, 4–6]. However, non-genomic actions due to direct membrane effects of parent hormones [7] or their reduced metabolites have also been described, particularly with tetrahydro derivatives of the mineralocorticoid deoxycorticosterone (DOC) which binds to the GABA/benzodiazepine receptor [8].

When given to experimental animals, mineralocorticoids are important inducers of salt appetite and hypertension. In normal rats, van de Berg et al. [9] have shown that activation of central MR but not glucocorticoid receptors increases systolic blood pressure (BP) and that daily warming and stress imposes mineralocorticoid dependency of the pressor response. Adrenal steroids have also a role in spontaneously hypertensive rats (SHR), which present abnormalities in the BP response to hormones. For instance, it is known that adrenal-derived corticosterone is essential for the development of hypertension in young SHR, while alterations in corticosteroid-negative feedback occur in adult animals [10, 11]. Mineralocorticoids appear to participate in the maintenance of hypertension of SHR. In this regard, administration of the MR antagonist RU28318 to SHR induces a long-lasting reduction in BP, whereas infusion of aldosterone increases BP of adrenalectomized SHR but not Wistar-Kyoto (WKY) rats [12, 13]. Receptor studies have shown increased hippocampal and hypothalamic MR binding capacity in SHR compared to WKY, enhanced activation of MR in heart and kidney and increased heart MR mRNA content in the stroke-prone SHR [14–16]. Thus, different experimental paradigms suggest that disturbances of the response to stress, high sensitivity to mineralocorticoids and abnormalities of central and peripheral MR may contribute to development or maintenance of hypertension in the SHR strain.

Among other brain regions, functional alterations of the paraventricular hypothalamic nucleus (PVN) seem particularly relevant to our present issue. The PVN has been postulated as one of the sites responsible for the development of hypertension in SHR [17]. Lesions of the PVN delay development of hypertension, while pharmacological blockade of this nucleus in SHR reduces sympathetic vasomotor tone [18, 19]. The PVN synthesizes and secretes a variety of neuropeptides involved in stress responses and hypertension, among them arginine vasopressin (AVP) and corticotropin-releasing hormone (CRH) [20, 21]. The PVN of unstressed SHR is enriched in CRH mRNA, and following stress SHR but not WKY rats show activation of early genes and enhanced synthesis of CRH and CRH receptor mRNA [17, 22]. Both magno- and parvocellular CRH neurons are more numerous in human hypertensive patients [23], which together with findings in SHR suggest a hyperfunction of the CRH system in hypertension. The vasopressinergic system is also abnormal in SHR. These animals present a 3-fold higher expression of AVP mRNA in the PVN at 4 weeks of age still persisting at 10 weeks, increased plasma levels of AVP and attenuation of hypertension after pharmacological antagonism of the vasopressin V1a receptor (V1aR) [24, 25]. The V1aR is present in vasopressinergic magnocellular neurons and may autocontrol excitability and hormone release [26, 27], which may be important for a role of AVP in hypertension.

Previous reports using deoxycorticosterone acetate (DOCA)-treated normal rats developing a salt appetite and hypertension have shown that AVP mRNA is rapidly upregulated in magnocellular cells of the PVN, receptor binding of AVP is increased and cells of osmosensitive regions become activated, according to the enhanced number of Fos-positive nuclei [6, 28–30]. Based on data obtained in DOCA-treated Sprague-Dawley rats, the present investigation compared the response of the vasopressinergic system to a single vehicle or DOCA administration of SHR and WKY rats. Two variables were measured in the PVN: the number of AVP mRNA-producing cells using in situ hybridization and V1aR-positive cells by means of immunocytochemistry. Additionally, Fos-positive nuclei were counted in the PVN and anterior hypothalamic regions as a measure of cell activation [31]. We thus intended to elucidate if the AVP system, being a central target of mineralocorticoids, is hyperreactive to the mineralocorticoid DOCA in this genetic model of hypertension.

Materials and Methods

Experimental Animals

Sixteen-week-old male WKY rats and SHR (purchased from the Lanari Institute, Faculty of Medicine, University of Buenos Aires) were housed under conditions of controlled humidity and temperature (22°C) with lights on from 07:00 h to 19:00 h. Animals were given food and water ad libitum. Systolic BP determined by an indirect tail-cuff method in conscious rats demonstrated all SHR were hypertensive (190 ± 5 mm Hg) whereas all WKY rats were normotensive (130 ± 5 mm Hg). A group of WKY rats and SHR was given a single subcutaneous injection of vegetable oil (0.2 ml) whereas another group received 10 mg/rat DOCA (Sigma, St. Louis, Mo., USA) dissolved in vehicle [6]. All groups (n = 4 per group) were sacrificed 2 h after injection. The protocol was approved by

the Animal Care and Use Committee of the Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina. For in situ hybridization or immunocytochemical procedures, rats were ether anesthetized and perfused intracardially with 0.9% NaCl followed by cold 4% paraformaldehyde in 0.1 *M* phosphate buffer pH 7.4. Brains were rapidly removed and incubated during 1 h at 4°C in the same fixative. After this step, brains were cryoprotected by overnight incubation in 15% sucrose in phosphate-buffered 0.9% NaCl (PBS), frozen on dry ice and kept at –80°C until use. Coronal sections (16 μm) were obtained in a cryostat and placed onto gelatine-coated glass slides.

In situ Hybridization (ISH) for AVP mRNA

Non-isotopic ISH was used to measure AVP mRNA levels, following EMBO (European Molecular Biology Organization) procedures [32], as previously used in our laboratory [6]. The 48 mer AVP probe 5′-GTA-GAC-CCG-GGG-CTT-GGC-AGA-ATC-CAC-GGA-CTC-TTG-TGT-CCC-AGC-CAG-3′ was complementary to the glycoprotein coding region of the AVP gene [33]. The probe was labeled with digoxigenin (Boehringer, Germany) at the 3′ end using the enzyme terminal transferase (Gibco, Gaithersburg, Md., USA). Slides were immersed in 4% paraformaldehyde for fixation during 20 min at 4°C and then washed with PBS. This double fixation procedure was used previously to study AVP mRNA and Fos protein expression [6]. Protocols for prehybridization and hybridization were previously described [6, 34]. The concentration of labeled probe was 10 n*M*. A second incubation (overnight) at 4°C was carried out with an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:5,000; Boehringer). Development of the reaction was carried out in the dark at room temperature by exposing the sections to the alkaline phosphatase substrates NTB (nitro-blue tetrazolium) and BCIP (bromo-chloride-indolphosphate) (Gibco) during 150 min. The specificity of the hybridization was determined by (1) competition of the labeled probe with a 20-fold excess unlabeled probe and (2) replacing the antidigoxigenin antibody by non-reactive serum. Under these conditions, the AVP signal was reduced to background levels.

V1a Receptor Immunoreactivity

Slides were immersed in 4% paraformaldehyde for fixation during 6 min at 4°C and then washed with PBS [6]. They were treated with 10% normal goat serum for 10 min at 37°C followed by incubation with the V1aR antibody at 1/750 dilution for 40 h. This antibody (anti-V1a rec, Alpha Diagnostics International, Inc., San Antonio, Tex., USA) was made against 19 amino acids at the extracellular N-terminal domain of the rat V1a receptor, and its specificity has been previously reported [35]. Sections were washed twice with PBS, incubated in goat anti-rabbit serum (1/200) for 2 h and processed following the ABC kit instructions (Vector Labs, 'Elite' ABC reagent). Development was carried out using 0.5 mg/ml diaminobenzidine containing 0.01% H_2O_2 and 0.25 g/ml nickel ammonium sulfate during 6 min at room temperature. Finally, slides were dehydrated with ethanol and xylene and mounted with Permount.

Fos Immunoreactivity

Slides were immersed in 4% paraformaldehyde for fixation during 6 min at 4°C and then washed with PBS [6]. They were treated for 15 min with 10% normal goat serum, followed by a primary antibody (Fos H-125 polyclonal rabbit antiserum, St. Cruz Biotechnology) at 1/1,000 dilution in PBS 0.15% Triton X-100 at room temperature overnight. Sections were washed twice with PBS, incubated in goat anti-rabbit serum (1/200) in PBS 0.15% Triton X-100 for 1 h and processed following the ABC kit instructions (Vector Labs, 'Elite' ABC reagent). Development was carried out using 1 mg/ml diaminobenzidine containing 0.01% H_2O_2 during 2 min at room temperature. Finally, slides were dehydrated with ethanol and xylene and mounted with Permount. Specificity and controls for the H-125 antibody have been reported previously [36].

Quantitative Analysis

A computer-assisted image analysis system (Bioscan Optimas, Edmonton, Wash., USA) was used to determine the number of Fos and V1aR-immunoreactive cells and the number of cells expressing AVP mRNA. For all measures, counts were generated automatically by the software. The investigators who processed the cell counting were blinded to the procedure. Cell densities (number of cells/unit area) were determined in 6–9 anatomically matched sections of the PVN, the median preoptic nucleus (MnPO) or the organum vasculosum of the laminae terminales (OVLT) per rat. The anatomical limits of the PVN [17] corresponded to plates 17 (bregma –1.3)–18 (bregma –1.8) of the Paxinos and Watson atlas of the rat brain [37]. Limits of the OVLT and MnPO were those considered in a previous publication [6]. Data were expressed as the mean number of labeled cells ± SEM. Unpaired t-test or one-way analysis of variance followed by the Bonferroni post-hoc test were used to determine group differences.

However, we consider that ISH data may be misleading when comparing different experimental groups, since this method is at best semiquantitative. In order to test the reproducibility of the method, the number of cells expressing AVP mRNA from the same animal sections was measured in two different experiments. The number of AVP mRNA-expressing cells per hemi-PVN was 133 ± 12 (exp. A) and 129 ± 14 (exp. B), $t = 0.83$, two-tailed p value: 0.49 (non-significant). Thus, reproducibility with a high expression mRNA such as AVP seemed acceptable using our standard methodology. In the case of immunocytochemistry, consistency of the procedure was intended by staining sections from all experimental groups simultaneously and also including sections from different animals in the same slide. This procedure eliminated conflicts that may arise using different batches. To minimize inter-experimental variability, conditions of the procedures were kept rigorously throughout the assays.

Results

In WKY rats, cells expressing AVP mRNA were moderately abundant in the magnocellular posterior portion of the PVN (fig. 1, 2A). In this group of animals, DOCA administration did not change the numerical density or regional distribution of AVP mRNA-producing cells (fig. 2B). In untreated SHR, however, significantly more AVP mRNA-producing cells were present in the magnocellular PVN than in WKY ($p < 0.001$) (fig. 1, 2C). In the SHR group, DOCA treatment further increased the number of AVP mRNA-containing cells ($p < 0.05$ vs. untreat-

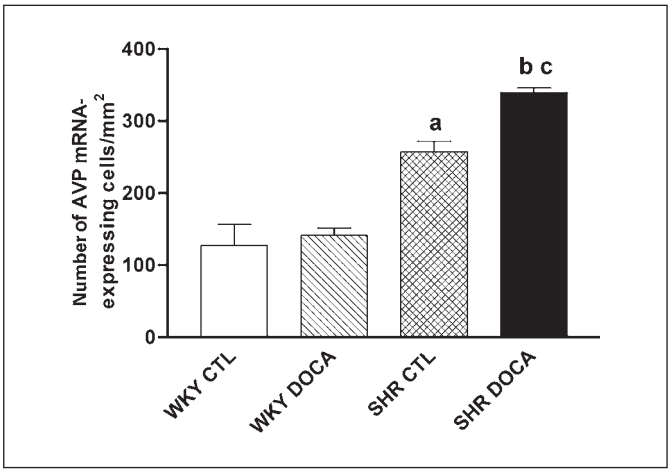

Fig. 1. Effect of DOCA treatment on the number of AVP mRNA-expressing cells in the paraventricular nucleus (PVN) of SHR and WKY rats. Data correspond to measurement of cell densities (number of positive cells/mm^2) in untreated WKY rats and SHR (WKY CTL, SHR CTL) or rats receiving 10 mg DOCA s.c. 2 h before sacrifice (WKY DOCA, SHR DOCA). Six to 9 anatomically matched sections were counted per rat; data were pooled per animal and animals (n = 4 per group) were used as independent variables. Statistical analysis demonstrated that without treatment SHR contained more AVP mRNA-expressing cells than WKY (a: $p < 0.001$) and the same result was obtained following DOCA treatment (b: SHR DOCA $p < 0.001$ vs. WKY DOCA). DOCA treatment further increased AVP mRNA labeled cells in SHR (c: $p < 0.05$ vs. SHR CTL) but was inactive in WKY rats (WKY CTL vs. WKY DOCA: ns).

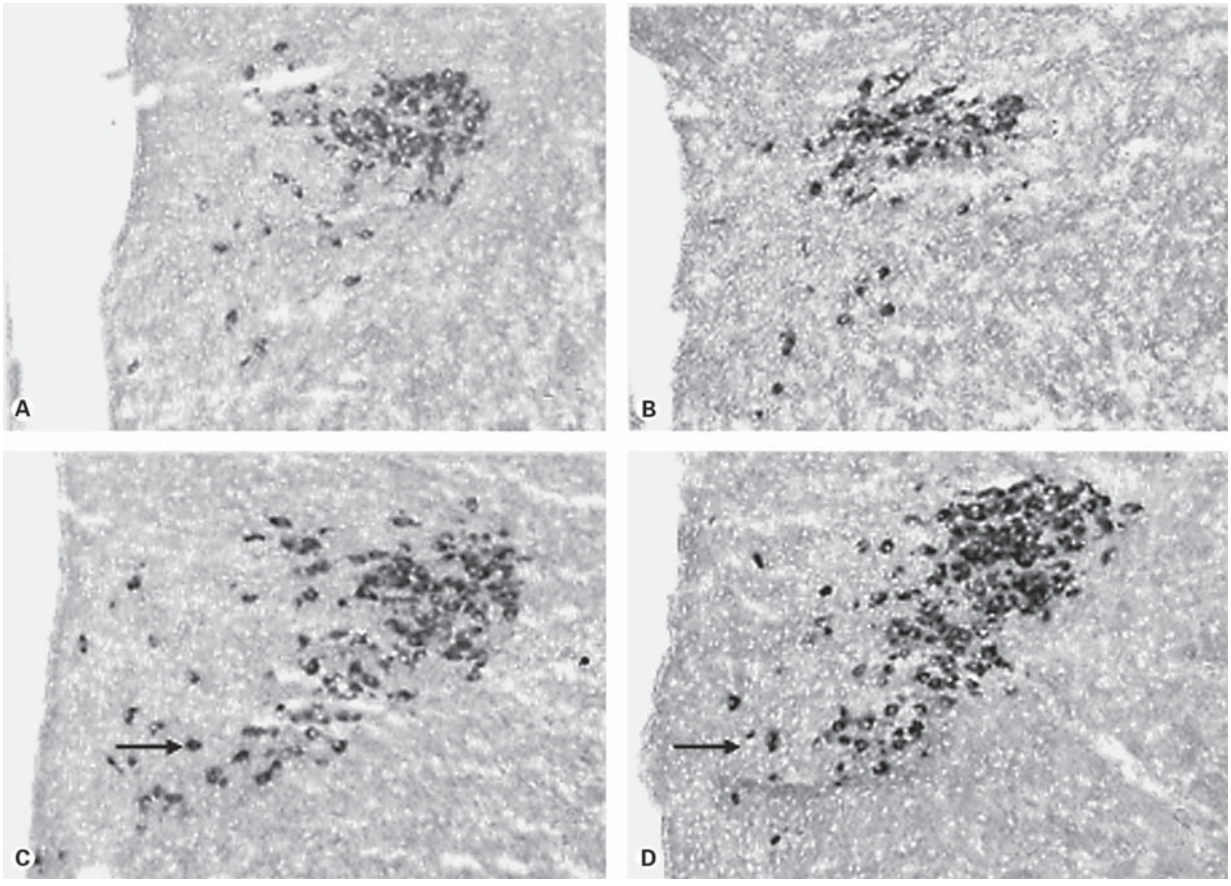

Fig. 2. In situ hybridization profile of AVP mRNA-expressing cells in a representative experiment. **A** WKY control; **B** WKY DOCA; **C** SHR control; **D** SHR DOCA. Clearly, AVP mRNA-expressing cells were more numerous in **C** than **A**. DOCA further increased labeled cells in SHR (**C** vs. **D**) but not in WKY rats (**A** vs. **B**). In addition, some magnocellular cells expressing AVP mRNA were allocated to the parvocellular division in both untreated and DOCA-treated SHR in comparison with their respective WKY group (arrows). 100×.

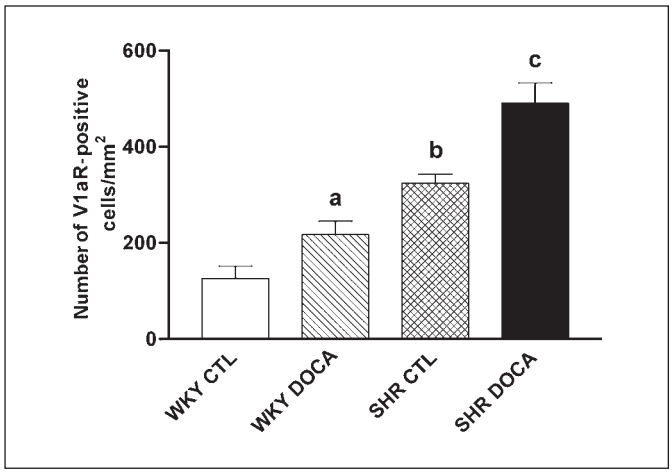

Fig. 3. Effect of DOCA treatment on the density (number of positive cells/mm^2) of V1aR-immunoreactive cells in the PVN of the groups of animals depicted in the legend to figure 1. Six to 9 anatomically matched sections were counted per rat; data were pooled per animal and animals (n = 4 per group) were used as independent variables. Statistical analysis demonstrated that DOCA treatment significantly increased V1aR-positive cells in WKY rats (a: $p < 0.05$ vs. WKY CTL). Both untreated SHR and DOCA-treated SHR showed more labeled cells than WKY (b: $p < 0.001$ vs. WKY CTL; c: $p < 0.01$ vs. WKY DOCA). ANOVA further demonstrated that the SHR DOCA group ranked the highest in terms of V1aR-positive cells.

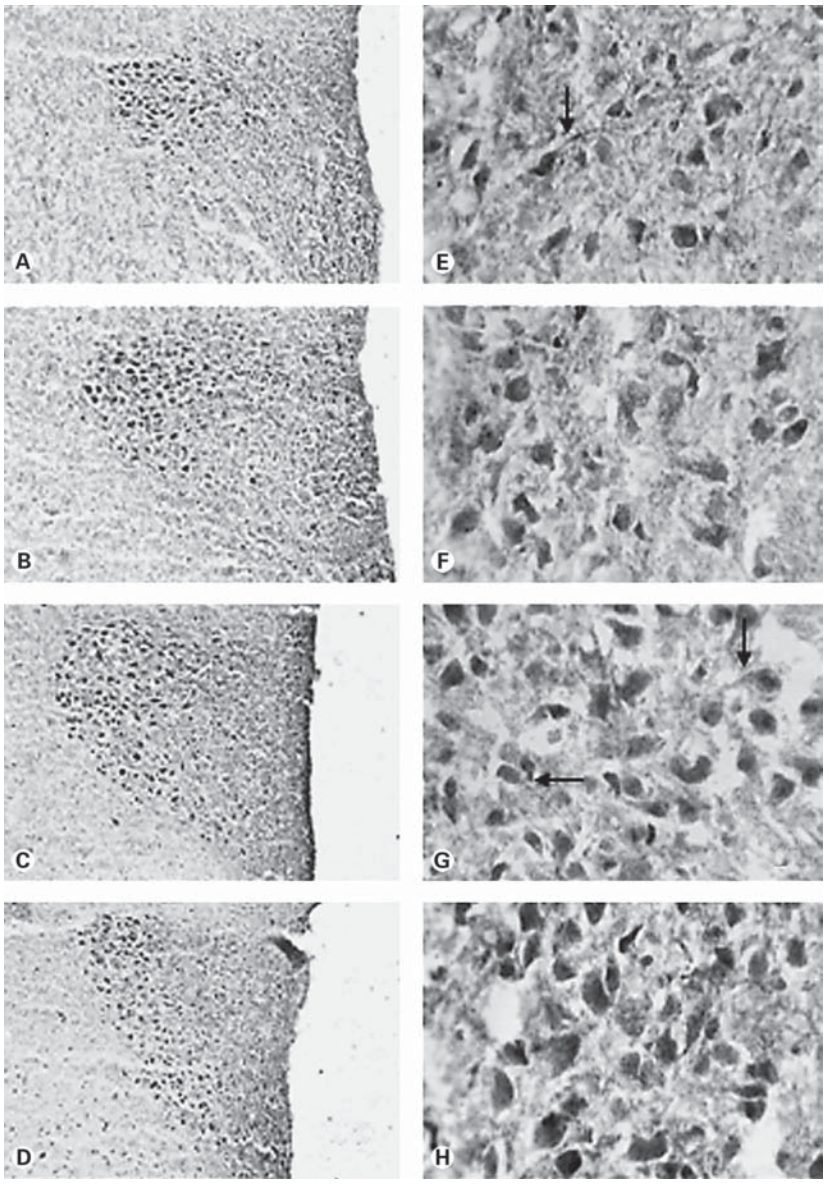

Fig. 4. Low (100×: **A**–**D**) and high (600×: **E**–**H**) magnification photomicrographs showing V1aR-immunopositive cells in WKY rats untreated (**A**, **E**) or receiving DOCA (**B**, **F**), and in SHR untreated (**C**, **G**) or receiving DOCA (**D**, **H**). Reactivity was present in cytoplasm and some cell processes (arrows). DOCA treatment elicited an increase in immunopositive cells in both WKY rats (**B**, **F** vs. **A**, **E**) and SHR (**D**, **H** vs. **C**, **G**), but the SHR plus DOCA group (**D**, **H**) showed the highest number of V1aR-immunoreactive cells in the PVN.

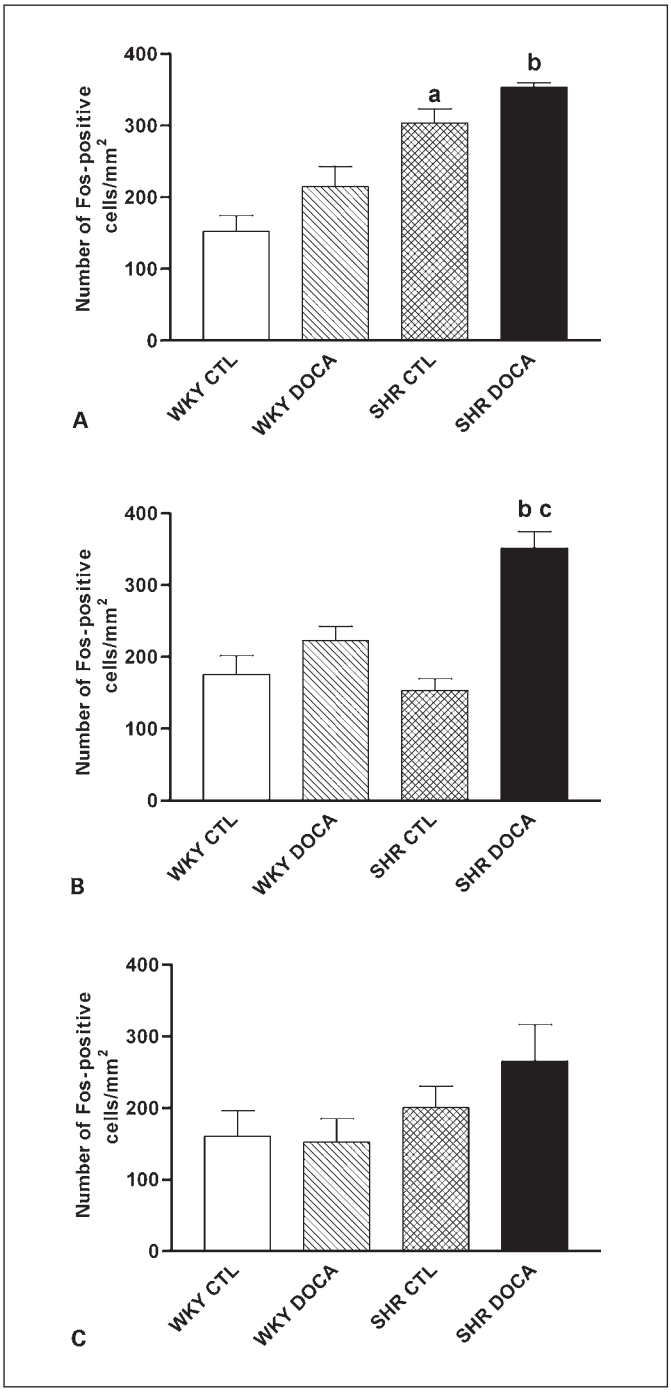

Fig. 5. Quantitative analysis of Fos-immunoreactive cells in the paraventricular nucleus (**A**), organum vasculosum of the lamina terminales (**B**), and median preoptic nucleus (**C**) of the groups of animals depicted in the legend to figure 1. Statistical analysis demonstrated higher number of Fos-positive nuclei in the PVN of SHR (a: $p < 0.01$ vs. WKY CTL, b: $p < 0.01$ vs. WKY DOCA). In the OVLT, the SHR DOCA group showed more Fos-immunoreactive nuclei than the other groups: b: $p < 0.01$ vs. WKY CTL and WKY DOCA; c: $p < 0.001$ vs. SHR CTL. No changes were obtained in the MnPO across the experimental groups.

ed SHR) (fig. 1, 2D). A further distinction between rat strains was the appearance in SHR of new labeled cells in the medial portion of the magnocellular PVN (fig. 2C, D). Counting of AVP mRNA-expressing cells in the medial PVN demonstrated that the SHR (101 ± 4) and SHR + DOCA (118 ± 4) groups showed a higher cell density than the WKY (36 ± 5) and WKY + DOCA (28 ± 5) groups. These findings suggested that strain differences exist regarding the distribution of AVP mRNA-containing cells. Furthermore, the stimulatory effect of mineralocorticoid treatment in the SHR group was restricted to dorsal magnocellular cells of the PVN.

In addition to the PVN, we also determined the AVP mRNA cell density in the supraoptic nucleus (SON), since the SON is an important site of AVP synthesis and secretion [20, 21]. In this case, untreated SHR showed a higher AVP mRNA cell number than WKY rats (140 ± 10 vs. 77 ± 7 cells per nucleus, $p < 0.01$). DOCA treatment had no effect on this parameter in WKY (73 ± 6) or the SHR groups (113 ± 4). Thus, DOCA effects on AVP mRNA seemed exclusive of the PVN.

In the next series of experiments, the V1aR was quantitated in the PVN of the four groups of rats (fig. 3, 4). In all groups, immunostaining was detected in cytoplasm and some cell processes (fig. 4, note arrows in E and G). Similar to findings with AVP mRNA, the hypertensive group receiving vehicle showed a significant increase in V1aR-expressing cells compared to untreated WKY ($p < 0.001$) (fig. 3, 4A, C). Quantitative analysis demonstrated that DOCA treatment increased the number of cells immunostained for V1aR in both rat strains. Thus, after DOCA treatment, V1aR-immunostained cells were more abundant in WKY rats vs. untreated WKY ($p < 0.05$) (fig. 4A, B, E, F), and a similar increase (~1.6-fold) was elicited by DOCA in the SHR group compared to untreated SHR ($p < 0.01$) (fig. 4C, D, G, H). However, ANOVA followed by post-hoc test demonstrated that the highest number of V1aR-immunostained cells was measured in the SHR + DOCA group (fig. 3, 4D, H; $p < 0.01$ vs. all other groups).

The number of Fos-positive immunostained cell nuclei was determined in the PVN, OVLT and MnPO of the four groups of experimental animals. As already shown [6], Fos-positive cells were present in control animals, suggesting that anesthesia and vehicle injections were sufficient to elicit a Fos response in animals killed 2 h afterwards. When the number of Fos-positive nuclei was counted per unit area in WKY rats, DOCA treatment did not produce changes in any of the three areas with respect to steroid-naïve animals of the same strain

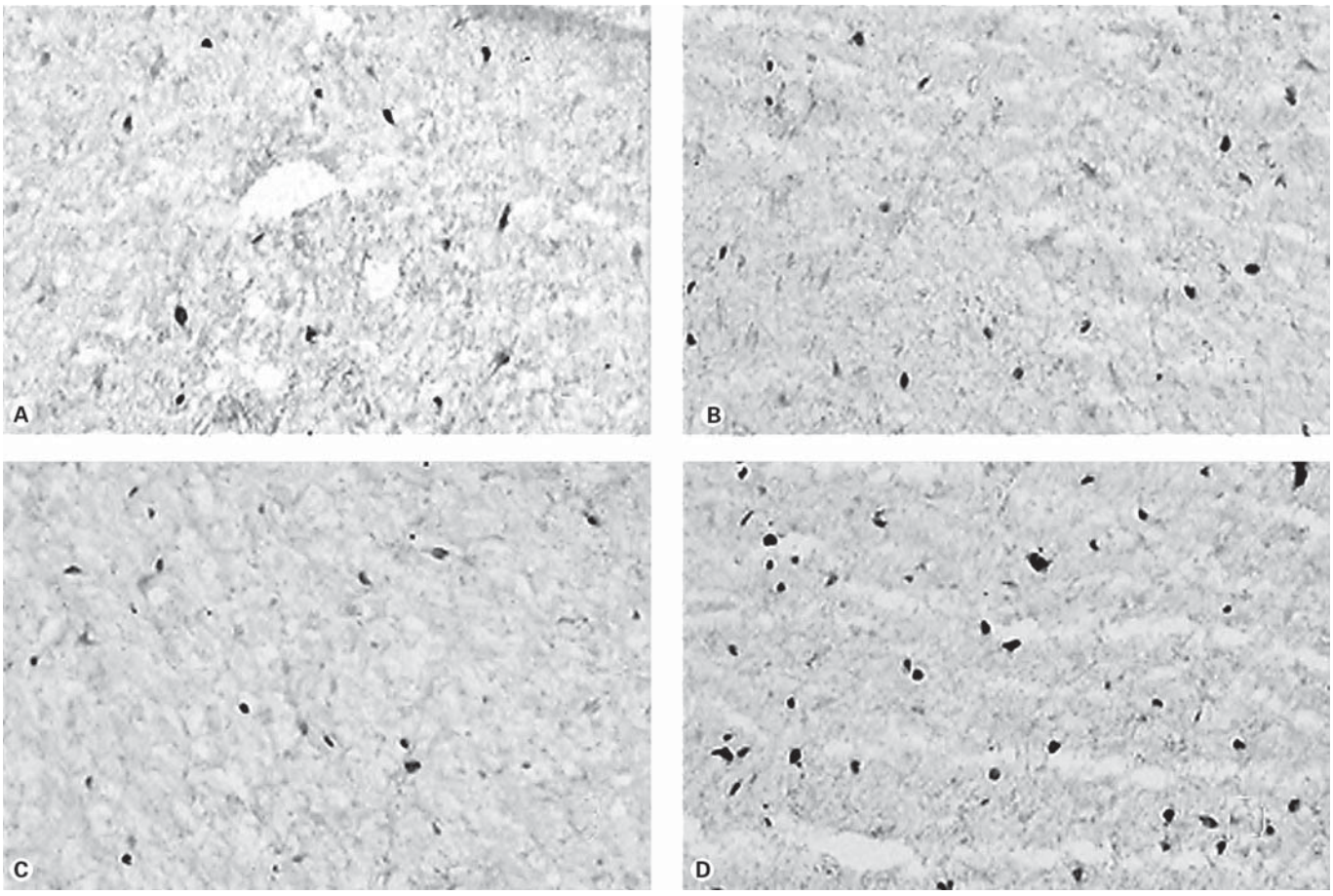

Fig. 6. Representative photomicrographs of Fos-immunoreactive nuclei in the OVLT of WKY (**A**), WKY plus DOCA (**B**), SHR (**C**) and SHR plus DOCA (**D**). Density of Fos-positive nuclei was higher in the SHR + DOCA rat than in rats corresponding to the other groups. 100×.

(fig. 5). In contrast, significantly more Fos-positive nuclei were present in the PVN but not in the other two areas of untreated SHR (fig. 5). Mineralocorticoid exposure of SHR was without effect in the PVN, but resulted in a robust increase of Fos-immunopositive cell nuclei in the OVLT (~2.3-fold, $p < 0.01$ vs. WKY + DOCA, $p < 0.001$ vs. untreated SHR, respectively) (fig. 6).

Discussion

The present study demonstrated constitutive differences of the vasopressinergic system of the hypothalamus as well as the hyperresponse of this system following short-term mineralocorticoid treatment in SHR compared to age-matched WKY rats. Changes occurring in the PVN and OVLT of the SHR strain included (a) increased number of AVP mRNA-producing cells in the dorsal magnocellular division of the PVN and in the SON of untreated SHR; (b) a significant increment in AVP mRNA-producing cells following DOCA treatment in the PVN but not in the SON; (c) the appearance of the AVP mRNA signal in the medial division of the PVN, the density of which was similar in the basal state and after DOCA treatment; (d) increased number of V1aR in the magnocellular division of the PVN of untreated SHR which showed a further increment after DOCA treatment, and (e) a significantly higher number of Fos-positive nuclei in the PVN of untreated SHR, which further increased in the OVLT but not in the PVN following mineralocorticoid treatment.

It is possible that the hyperresponse of the AVP-producing cells in SHR may have consequences for the cardiovascular system. In the present experiments, BP was

not determined because 2 h after DOCA administration may be too short for a significant effect on this parameter. On the contrary, DOCA effects on BP may take weeks to develop in SHR [39] and in normotensive rats drinking water, increases in BP and plasma AVP take longer than 7 days following DOCA injections on alternate days [28]. However, it is worthwhile pointing out that the roles of AVP in adult SHR have been debated, with some reports favoring its etiopathogenic role in hypertension and others denying it [reviewed in 21]. Nevertheless, it has been conclusively shown that adult SHR present higher expression of AVP mRNA in the PVN and increased plasma levels of AVP throughout the course of hypertension [24]. In a preliminary study, we found that immunoreactive AVP content of the posterior pituitary was significantly reduced by 30% in untreated SHR compared to WKY rats ($p < 0.01$), whereas DOCA treatment produced a further 10% reduction in the SHR group only [unpubl. data]. This suggests that a continuous release of AVP from the posterior pituitary occurs in SHR. However, since treatment with the AVP V1 receptor antagonist OPC-21268 during the developmental phase of hypertension, attenuated but did not completely block the high BP [25], it was concluded that AVP is probably one among a number of factors involved in genetic hypertension. In this regard, the function of the brain renin-angiotensin II (Ang II) system has been strongly implicated in SHR, since expression of the angiotensinogen mRNA, angiotensin II receptors and cell sensitivity to the peptide are all amplified in the subfornical organ, the anterior hypothalamic and anteroventricular third ventricular areas of SHR [38, 40, 41]. However, since Ang II binding is a potent releaser of AVP from the PVN [21, 42], newly-generated AVP could also contribute to Ang II effects on BP.

In this context, the finding that the AVP system is hyperresponsive to mineralocorticoids in SHR becomes important in the light of existing evidence that these hormones play a pathogenetic function in the maintenance of hypertension. Thus, a variety of experimental approaches including the injection of a MR antagonist, infusion of aldosterone or measurement of agonist binding to MR, support the participation of mineralocorticoids and MR in this form of hypertension [12–16]. It is likely that after DOCA hydrolysis, free DOC binds to MR in brain regions which control BP and cardiovascular functions. The MR-expressing cells are found in the hippocampus where MR and GR are regulated differentially by corticosterone [43], amygdala, lateral hypothalamus, the circumventricular organs, the anteroventral third ventricular area (AV3V) but not the PVN [2, 6, 44–46]. This anatomical location suggests that if stimulation of AVP synthesis requires the activation of a MR-dependent mechanism, it must take place in regions outside the PVN. Similarly, DOCA effects on development of salt appetite and hypertension involving magnocellular AVP, probably take place in a mineralocorticoid-sensitive circuit outside the PVN [6, 47].

Furthermore, while this investigation concentrated on AVP, it should be mentioned that oxytocin – which is also synthesized in the PVN – has an important role in salt regulation and water balance. In a preliminary experiment, we found that steroid-naïve SHR showed a slightly higher density of oxytocin mRNA-expressing cells compared to untreated WKY (86 ± 11 vs. 68 ± 14 cells per PVN), but differences were not significantly different. However, the oxytocin response to mineralocorticoids may require addition of salt to the drinking water. Previous data in control Sprague-Dawley rats demonstrated that oxytocin mRNA responded to DOCA treatment in animals drinking 3% NaCl, but not in those drinking water [28]. Thus, further studies are needed to define the response of oxytocin to acute mineralocorticoid treatment in hypertensive rats.

Fos immunoreactivity has been considered a landmark of cellular activation which helps to localize the regional site of hormone action [31]. In the present situation, a single DOCA injection stimulated the number of Fos-positive nuclei in the OVLT but not the PVN or MnPO of SHR, pointing to the OVLT as a mineralocorticoid target which may coordinate DOCA effects on AVP synthesis in the PVN [6]. This assumption is supported by the presence of MR and retention of injected mineralocorticoid in the OVLT [2, 6]. In this respect, the OVLT belong to a group of structures with efferent neuronal connections to the PVN influencing the secretion of AVP [48, 49]. Instead, changes in basal Fos levels in the PVN of SHR could be due to this region playing a more active role in the stress response of SHR [17, 22, 23]. Collectively, data obtained using changes of Fos immunoreactivity, reinforce the hypothesis that one pathway involved in DOCA effects may involve extrahypothalamic areas. However, this assumption considered that DOCA-effects in SHR involved a genomic mechanism involving the classical intracellular steroid receptor. This may be a partial answer, because DOC also acts as a precursor for tetrahydrodeoxycorticosterone (THDOC), an allosteric modulator of the GABAa receptor in mesolimbic regions [8]. Thus, DOC metabolism creates additional interpretations to our present data, in view of the

fact that GABAergic responses are different in the brain of SHR and WKY rats [50].

Another interesting aspect of the AVP system in SHR was the presence of additional AVP mRNA-producing cells in the parvocellular division of the PVN. These cells were already present in the PVN of steroid naïve animals, and also in the DOCA-treated SHR group. Traditionally, magnocellular-derived AVP was thought to respond to osmotic stimuli, traveling within axons which project to the neurohypophysis. AVP is released from these terminals and acts on the periphery [reviewed in 21]. However, morphological and neurochemical evidence suggests that magnocellular AVP also gains access to the pituitary portal circulation and influences ACTH secretion from the anterior lobe [20]. Magnocellular AVP is released after stress [51], although its cellular origin is unknown. Although further investigations are needed to understand the contribution of different populations of magnocellular-derived AVP to stress levels of circulating AVP in SHR [24], the magnocellular cells ascribed to the medial division of the PVN are an intriguing possibility.

Another abnormality of SHR was the increased number of V1aR-immunoreactive cells in the magnocellular division of the PVN from rats not receiving DOCA. After DOCA treatment, V1aR-immunostained cells were more abundant in both WKY rats and the SHR group, which is in agreement with a former study using binding techniques [30]. However, group comparison indicated that in the SHR group, DOCA most prominently increased the number of V1aR-containing cells. The V1aR is part of a transmembrane receptor family which is localized in the forebrain, circumventricular organs, cerebral blood vessels, choroids plexus, the PVN and SON [26]. Evidence exists that V1aR colocalize with AVP in magnocellular neurons, suggesting that AVP may act directly on these neurons to exert an autoregulatory control [26, 27]. Working through this mechanism, DOCA could cause supplementary release of AVP from a hyperresponsive PVN of the SHR.

In connection with the hyperresponsive PVN of the SHR, it is known that the hippocampus maintains a high excitatory tone over an inhibitory gabaergic tone impinging upon the PVN [1]. Theoretically, a faulty hippocampus may result in increased neuropeptide biosynthesis in the PVN. Indeed, the occurrence of neuronal loss, astrocyte reactivity and changes of neurogenesis in the hippocampus of SHR has been reported [52, 53]. However, total hippocampectomy of normal rats precipitated a marked increase in CRH and AVP mRNA limited to the parvocellular division [54]. Thus, increased AVP mRNA and V1aR in the magnocellular division cannot be fully ascribed to a faulty hippocampus, but more likely reflects the hyperreactivity of the PVN or stimulation of other regions (i.e. OVLT) by mineralocorticoid treatment.

Overall, this investigation opens the possibility that the abnormal mineralocorticoid/AVP interaction of SHR may have functional consequences. While hypertension in this rat strain may involve Ang II, monoamines and the neuropeptides CRF, oxytocin and proopiomelanocortin-derived peptides [21, 38, 40, 41], it may originate in part from the abnormal response of the vasopressinergic system to mineralocorticoids. As already discussed, mineralocorticoid hormones are increased in blood and their cognate receptors are hyperactivated in the brain and other tissues of SHR. The stimulated levels of AVP may affect BP after binding to V1aR in the PVN, a process which further stimulates AVP release. Furthermore, excess magnocellular AVP may be directed to vasopressinergic pathways projecting to centers of the brainstem and spinal cord involved in BP control [19, 21, 55], initiating and sustaining the hypertension.

However, it is worth mentioning that whereas the WKY inbred strain is usually employed as a control of the SHR strain, WKY rats are also described as hypoactive and show reduced responsiveness to a number of stressors [56]. Thus, one interpretation of the mRNA expression pattern is that SHR looks more like other normal strains whereas the WKY are more aberrant, in spite that the hypertensive rats derive from WKY animals. Thus, further studies are needed to look for additional factors which may generate the high sensitivity to DOCA and perhaps AVP in SHR.

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