Neuro endocrinology

Neuroendocrinology 2001;74:396–406

Received: April 9, 2001
Accepted after revision: August 8, 2001

Changes in Fos Expression in Various Brain Regions during Deoxycorticosterone Acetate Treatment: Relation to Salt Appetite, Vasopressin mRNA and the Mineralocorticoid Receptor

Luciana Pietranera[a] Flavia E. Saravia[a] Bruce S. McEwen[c] Louis L. Lucas[c] Alan Kim Johnson[d] Alejandro F. De Nicola[a,b]

[a]Laboratory of Neuroendocrine Biochemistry, Instituto de Biologia y Medicina Experimental and Department of Human Biochemistry, Faculty of Medicine, University of Buenos Aires, [b]Instituto Universitario de Ciencias de la Salud, Fundación Barceló, Buenos Aires, Argentina; [c]Laboratory of Neuroendocrinology, The Rockefeller University, New York, N.Y., [d]Department of Psychology and Pharmacology and the Cardiovascular Center, University of Iowa, Iowa City, Iowa, USA

Key Words
Adrenal steroids · Fos · Adrenal steroid receptors · Vasopressin · Salt appetite · Supraoptic nucleus · Paraventricular nucleus · Amygdala · Organum vasculosum laminae terminalis

Abstract
Salt appetite, a conditioning factor for hypertension and cardiovascular diseases, is produced when high doses of mineralocorticoids are given to experimental animals. A commonly used procedure to identify neuronal activation is to determine the number of Fos-immunoreactive cells. In rats with established salt appetite after 8 days of deoxycorticosterone acetate (DOCA) treatment, Fos-positive cells were studied in seven brain areas. Significant increases in Fos activity were recorded in the paraventricular (PVN) and supraoptic (SON) nuclei, median preoptic nucleus (MnPO), organum vasculosum of the lamina terminalis (OVLT), preoptic area (POA), bed nucleus of the stria terminalis (BNST) and amygdala (AMYG). In most of these areas, increased Fos expression was also observed early (2 h) after a single DOCA injection, well before salt appetite develops. Using a mineralocorticoid receptor (MR) antibody, we studied whether Fos-active regions also expressed MR. MR-positive cells were found in the OVLT, MnPO, AMYG and BNST, but not in the POA, PVN and SON. In the PVN and SON, nevertheless, prolonged or single DOCA treatment increased expression of mRNA for arginine vasopressin (AVP). The present demonstration of Fos activation, in conjunction with differential expression of MR and stimulation of AVP mRNA, suggests that a neuroanatomical pathway comprising the AMYG, osmosensitive brain regions and magnocellular nuclei becomes activated during DOCA effects on salt appetite. It is recognized, however, that DOCA effects may also depend on mechanisms and brain structures other than those considered in the present investigation. Since some Fos-positive regions were devoid of MR, a comprehensive view of DOCA-induced salt appetite should consider nongenomic pathways of steroid action, including the role of reduced DOC metabolites binding to GABAergic membrane receptors.

Copyright © 2001 S. Karger AG, Basel

KARGER

Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2001 S. Karger AG, Basel
0028–3835/01/0746–0396$17.50/0

Accessible online at:
www.karger.com/journals/nen

Alejandro F. De Nicola
Laboratory of Neuroendocrine Biochemistry
Institute of Biology and Experimental Medicine, Obligado 2490
1428 Buenos Aires (Argentina)
Tel. +54 11 4783 2869, Fax +54 11 4786 2564, E-Mail denicola@proteus.dna.uba.ar

Downloaded by:
Univ. of California San Diego
132.239.1.231 - 6/27/2017 7:20:40 AM

Introduction

High salt ingestion is a conditioning factor for the genesis of hypertension and cardiovascular diseases. In experimental animals, the development and maintenance of salt appetite is hormonally regulated. Indeed, hypersecretion of endogenous adrenal steroids caused by ACTH administration or stress [1–3] or high doses of exogenous mineralocorticoids have been shown to stimulate drinking of concentrated NaCl solutions [4, 5]. The role played by the classical mineralocorticoid receptor (MR), the interaction of steroids with vasoactive peptides and enzymes and the neuroanatomical circuitry responsible for this behavior are the subject of continuing investigations in relation to mineralocorticoid-induced salt appetite [6–13].

The observation that deoxycorticosterone acetate (DOCA)-induced salt appetite goes into effect after a latency period of about 24–48 h suggested the involvement of a classical MR acting at the genome [4, 5, 9]. The same conclusion was derived from experiments showing that salt appetite was considerably reduced if animals also received an MR antagonist or MR antisense oligonucleotides [8, 11, 14]. MR-expressing cells in the amygdala (AMYG), lateral hypothalamus, circumventricular organs and the anteroventral third ventricular area (AV3V) were considered targets for hormonal induction of salt appetite [6, 15, 16]. However, an exclusive genomic effect may not be the final answer, because DOCA also acts as a precursor for tetrahydrodeoxycorticosterone (THDOC), a metabolite that binds to the GABAa/benzodiazepine receptor complex [12]. In this regard, administration of THDOC and the GABAa receptor agonist flunitrazepam resembled DOCA with regard to the induction of salt appetite in intact, sodium-replete rats [11]. Additionally, DOCA metabolites activate the mesolimbic peptidergic/dopaminergic structures associated with reward and goal-seeking behavior, introducing an additional anatomical locus as well as mechanistic possibilities for the mineralocorticoid effects on salt appetite [12, 13].

Neuroanatomical studies suggested a steroid-sensitive neural pathway for salt appetite control which included the AMYG, lateral hypothalamus and structures contained within the AV3V region [15, 16]. This pathway may differ from the angiotensinergic pathway originating in the subfomical organ (SFO) and descending to the organum vasculosum of the lamina terminalis (OVLT) [16]. Lesions placed in the steroid-sensitive route made animals resistant to DOCA induction of salt appetite [4, 15–17]. In addition to these structures, the paraventricular (PVN) and supraoptic (SON) nuclei also showed steroid sensitivity, since magnocellular cell synthesis and secretion of arginine vasopressin (AVP) is modulated by mineralocorticoids. Thus, DOCA treatment increased AVP mRNA in the PVN and SON, increased plasma AVP [9, 10] and stimulated peptide binding to V1 receptors in models of salt appetite and/or hypertension [18]. Therefore, the PVN and SON could become direct mineralocorticoid targets or functionally depend on afferent outputs from steroid-sensitive areas projecting to these nuclei.

A commonly used procedure to identify the cellular site of hormone action is to determine the number of Fos-positive cells by immunocytochemistry [19]. In response to osmotic changes caused by sodium depletion and hypertonic saline, or variations in the level of protein hormones, neuropeptides and steroids, specific sets of neurons become activated, thereby increasing nuclear Fos expression [20–28]. Several steroids induce Fos in the nervous system, among them glucocorticoids and mineralocorticoids [23, 26, 28, 29]. In one report, it was shown that systemic DOCA treatment increased Fos staining in the OVLT and to a lesser magnitude in the median preoptic nucleus (MnPO), but not elsewhere [28], whereas in another study, direct application of THDOC or aldosterone in the AMYG increased Fos immunostaining in the OVLT, bed nucleus of the stria terminals (BNST), MnPO, PVN, SON and SFO [26]. In view of the aforementioned evidence, several objectives were pursued to elucidate the participation of different cell groups in DOCA-induced salt appetite. First, to determine which brain regions are activated following DOCA treatment, Fos-immunopositive nuclei were determined in seven forebrain structures from rats before salt appetite was induced (2 h) and after salt intake was established (8 days) by systemic steroid exposure. Second, to confirm the involvement of hypothalamic neuropeptides, changes in AVP mRNA were monitored in the PVN from rats receiving DOCA for 2 h or 8 days. Third, to elucidate if steroid effects involve a genomic type of interaction, the presence of MR-positive cells was studied in brain areas showing increased Fos immunoreactivity in response to DOCA treatment.

Materials and Methods

Salt Appetite Induction

Sixty male Sprague-Dawley rats weighing 200–250 g were housed under conditions of controlled humidity and temperature (22 °C), with lights on from 7.00 to 19.00 h. Animals were caged singly, with free access to tap water and 3% NaCl given in separate bottles (double-preference test). A previously used treatment protocol for salt

Downloaded by:
Univ. of California San Diego
132.239.1.231 - 6/27/2017 7:20:40 AM

appetite induction was used in the present study [8–10]. Thus, after a 3-day period during which basal ingestion of salt and water was monitored, a group of 5 rats was given subcutaneous injections of DOCA (Sigma, St. Louis, Mo., USA) in vegetable oil on alternate days (4 injections of 10 mg/rat/day). Control rats (n = 5) received vegetable oil vehicle only. Animals were sacrificed 1 day after the last DOCA or vehicle injection. For acute treatment studies, another group of 5 rats received a single subcutaneous injection of 10 mg/rat DOCA dissolved in vegetable oil, whereas 5 controls received vehicle only (n = 5); both groups were sacrificed 2 h after injection. Three different groups of rats were used to study Fos immunoreactivity, MR immunoreactivity and AVP mRNA expression, respectively. Salt intake was pooled for all three groups. The protocol was approved by the Animal Care and Use Committee of the Instituto de Biologia y Medicina Experimental, Buenos Aires, Argentina.

Fos Immunoreactivity

Animals anesthetized with ketamine (60 mg/kg i.p.) were perfused intracardially with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 *M* phosphate-buffered 0.9% NaCl (PBS; pH 7.4). Brains were stored in the same fixative for 6 h at 4°C. After fixation, brains were cryoprotected in 20% sucrose and 0.1 *M* phosphate buffer and stored overnight at 4°C. Sixty-micrometer coronal sections were made in a vibrotome; the resulting free-floating slices were treated with 10% normal goat serum, followed by immunostaining for Fos according to the procedure of Xu and Johnson [24]. A primary antibody (Fos H-125 polyclonal rabbit antiserum, Santa Cruz Biotechnology, USA) was added at 1/3,000 dilution in 0.15% Triton X-100 at room temperature overnight in a shaker. Sections were washed twice with PBS, incubated in goat anti-rabbit serum (1:200) in 0.15% Triton X-100 for 1 h and processed following the ABC kit instructions (Vector Laboratories, '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, sections were mounted on gelatin-coated slides, dehydrated with ethanol and xylene and mounted with Permount. Specificity and controls for the H-125 antibody have been reported previously [24].

MR Immunoreactivity

Previously used immunocytochemical methods were followed [10, 30]. Animals were perfused with 0.9% NaCl, followed by 4% paraformaldehyde prepared in 0.2% saturated picric acid solution in ethanol and 0.1 *M* phosphate buffer, pH 7.2. Brains were removed, fixed overnight in the same solution maintained at 4°C and then cut every 60 µm in a vibrotome. Free-floating sections were incubated in 0.3% H_2O_2 in PBS during 15 min, washed with PBS and blocked with 10% normal rabbit serum. Sections were then transferred to a 1/300 dilution of the anti-MR antibody MCR N-17 (Santa Cruz Biotechnology) and incubated overnight at 4°C in a shaker. Sections were washed in PBS, incubated with the second antibody (biotinylated anti-goat antibody) and processed following the ABC kit instructions (Vector Laboratories, 'Elite' ABC reagent). Chromogen was 0.05% diaminobenzidine prepared in 0.05% Tris and 0.015% H_2O_2 during 8 min. Finally, sections were mounted on gelatin-coated slides, dehydrated with ethanol and xylene and mounted with Permount [10, 30].

In situ Hybridization for AVP mRNA

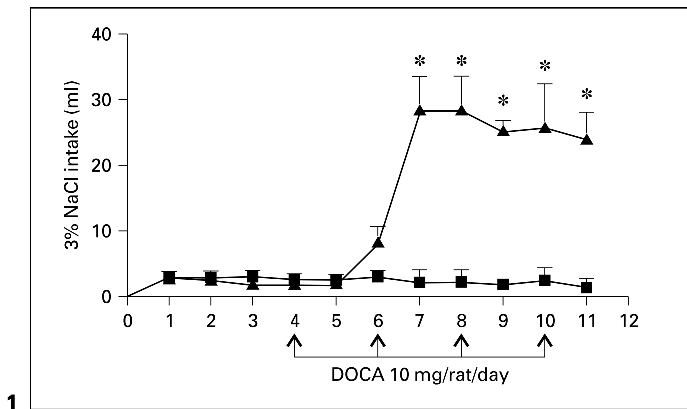
For in situ hybridization, rats were ether anesthetized and perfused intracardially with 0.9% NaCl followed by cold 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4. Brains were rapidly removed and incubated during 1 h at 4°C in the same fixative. After this step, brains were cryoprotected by overnight incubation in 15% sucrose in PBS, frozen on dry ice and kept at –80°C until use. Coronal sections (10 µm) were obtained in a cryostat and placed on gelatin-coated glass slides. Nonisotopic in situ hybridization was used to measure mRNA levels, following European Molecular Biology Organization procedures [31]. The 48-mer AVP probe 5′-GTAGACCCG-GGGCTTGGCAGAATCCACGGACTCTTGTGTCCCAGCCAG-3′ was complementary to the glycoprotein coding region of the AVP gene [32]. The probe was labeled with digoxigenin (Boehringer, Germany) at the 3′ end using the enzyme terminal transferase (GIBCO, Rockville, Md., USA) [33]. Slides were immersed in 4% paraformaldehyde for fixation during 6 min at 4°C and then washed with PBS. Prehybridization and hybridization were carried out as previously described [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 antidigoxigenin 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 nitroblue tetrazolium and 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 of unlabeled probe and (2) replacing the antidigoxigenin antibody by nonreactive serum. Under these conditions, the AVP signal was reduced to background levels.

Quantitative Analysis

A computer-assisted image analysis system (Bioscan Optimas, Edmonton, Wash., USA) was used to determine the number of Fos-immunoreactive cells. The areas studied included the AMYG (medial), preoptic area (POA) (medial), PVN (lateral magnocellular subdivision), SON, dorsal BNST (dBNST) (lateral division), ventral BNST (vBNST) (lateral division), dorsal MnPO (dMnPO), ventral MnPO (vMnPO) and OVLT. Anatomical limits for these areas were based on the Paxinos and Watson atlas [35]. Fos-immunoreactive nuclei (neuronal-like profiles showing brown nuclear staining) were counted in 6–9 sections per rat (5 animals per group) from the PVN, SON, AMYG, dBNST, vBNST, dMnPO, vMnPO, POA and OVLT. Data were expressed as the number of Fos-immunoreactive nuclei ± SEM per brain region analyzed. Photography was carried out with a Zeiss Axioplan optic microscope equipped with an automatic optic microscopy camera.

AVP mRNA determined by in situ hybridization was quantified within the lateral (mainly magnocellular) division of the PVN [32] using computerized densitometric analysis previously validated for hypothalamic neuropeptides (Optimas Bioscan program) [9, 10, 30]. Digitized images of tissue sections (6–9 sections per rat, 5 rats in each group) were displayed on the video screen under identical lighting conditions and the program was made to calculate the average optical density of the pixels contained within the boundaries of the outlined area by using a 256-unit gray level scale. The densitometric reading of a comparable area outside the PVN but devoid of AVP mRNA was considered as background and subtracted from the staining readings of the magnocellular cells. Results were expressed as the total immunoreactivity area ($\mu m^2 \times 10^3$) and immunoreactivity intensity of cells expressing AVP mRNA.

Downloaded by:
Univ. of California San Diego
132.239.1.231 - 6/27/2017 7:20:40 AM



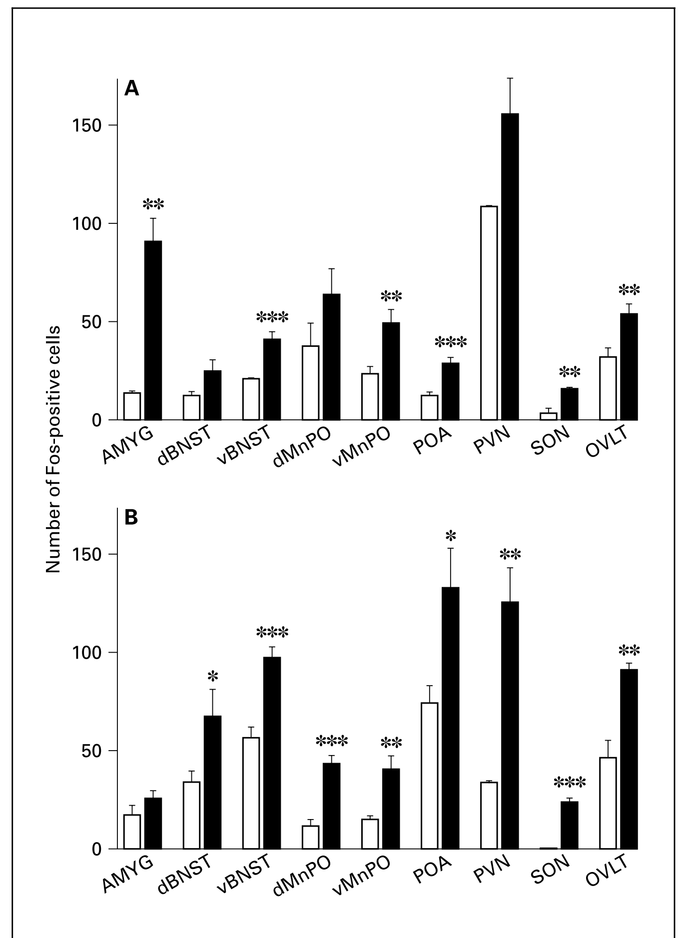


Fig. 1. Intake of 3% NaCl (ml/day) during mineralocorticoid treatment. After a control period of 3 days, DOCA (10 mg/rat/day; ▲) dissolved in vegetable oil vehicle was administered to a group of rats on alternate days (4, 6, 8 and 10; arrows). Control animals received the vehicle only (■). Animals were used 2 h after a single DOCA injection on day 4, or on day 11, 24 h after the last DOCA injection on day 10. All rats had free access to bottles containing 3% NaCl solution or tap water. * Significantly different from control rats ($p < 0.005$).

Fig. 2. Quantitative analysis of Fos-immunoreactive cell nuclei in brain regions from control (white columns) and DOCA-treated (black columns) rats. **A** Number of Fos-positive nuclei in rats receiving vehicle or a single injection of 10 mg/rat/day of DOCA, with animals sacrificed 2 h later. **B** Number of Fos-positive nuclei in rats receiving vehicle or 4 injections of DOCA (10 mg/rat/day) given on alternate days, with animals sacrificed on the 9th day, i.e. 24 h after the last DOCA injection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control rats (Student's t test).

Results

Salt Appetite Induction

Salt appetite was determined for the Fos immunoreactivity, MR immunoreactivity and AVP mRNA groups. Since animals receiving natriorexigenic doses of DOCA drank comparable amounts of salt solution, data from the different experiments were pooled for statistical purposes. Figure 1 shows, in agreement with previous studies, that salt drinking behavior following the first DOCA administration started after a delay of 24–48 h and continued at a rate of approximately 30 ml/day for the rest of the experiment. In contrast, salt appetite did not develop in rats receiving a single DOCA injection and used 2 h afterwards (fig. 1).

Fos Immunoreactivity

For this experiment, animals in the vehicle- and DOCA-treated group were killed on the 9th day (4 injections) or 2 h after a single injection, and brain sections were used for counting Fos-immunoreactive nuclei. Fos-positive nuclei were already present in control animals, suggesting a constitutive and basal level of expression. As shown in figure 2B, 4 injections of DOCA significantly increased the number of Fos-immunoreactive nuclei above basal levels in most regions explored, i.e. the dBNST, vBNST, dMnPO, vMnPO, POA, PVN, SON and OVLT. In the medial AMYG, a slight increase was noticed which did not reach significance. However, changes were not uniform in magnitude, as the greatest differences with vehicle were noted in the SON (26.5-fold), PVN (3.65-fold) and dMnPO (3.63-fold). Typical photomicrographs of Fos expression in the PVN (fig. 3A, B), MnPO (fig. 3C, D) and OVLT (fig. 3E, F) in control rats (fig. 3A, C, E) and rats receiving 4 DOCA injections (fig. 3B, D, F) are shown in figure 3.

Figure 2A shows Fos-immunoreactive nuclei from animals killed 2 h after a single vehicle or DOCA injection.

Downloaded by:
Univ. of California San Diego
132.239.1.231 - 6/27/2017 7:20:40 AM

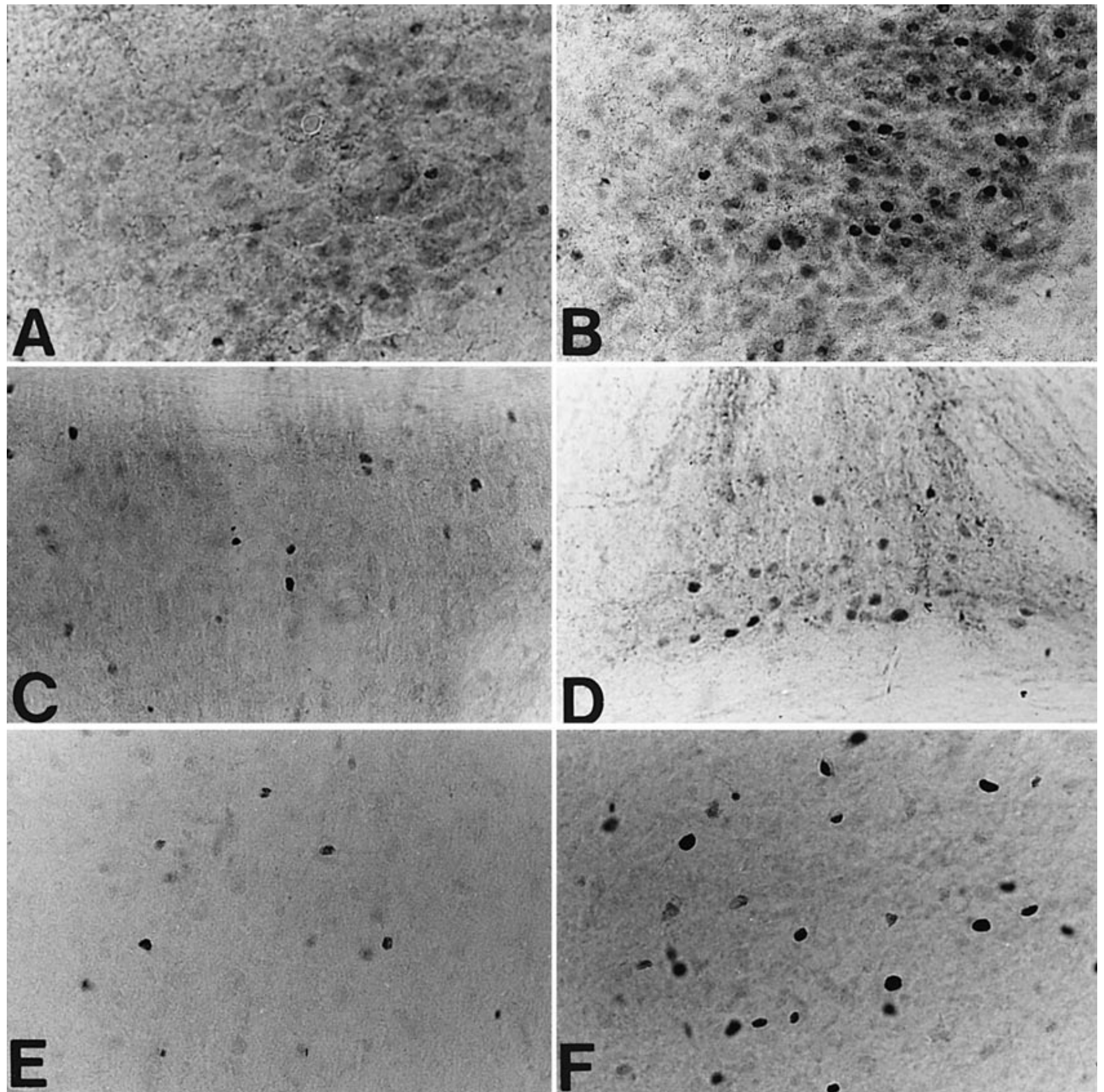


Fig. 3. Representative photomicrographs showing Fos-immunoreactive cell nuclei in a control rat (**A**, **C**, **E**) and a rat receiving 4 DOCA injections (**B**, **D**, **F**). **A**, **B** PVN. **C**, **D** MnPO. **E**, **F** OVLT. ×400.

This time period was too short for the development of salt-seeking behavior (fig. 1). As in the prolonged DOCA treatment group, most brain areas from animals receiving a single DOCA injection showed increased number of Fos-immunopositive nuclei (fig. 2). However, significant increments were recorded for the AMYG, vBNST, vMnPO, POA, SON and OVLT, while a nonsignificant increase was registered in the PVN, dBNST and dMnPO ($p > 0.05$). The most remarkable changes due to the single DOCA treatment occurred in the AMYG (6.7-fold) and SON (4.2-fold). Figure 4 shows representative photomicrographs of Fos-positive nuclei in the PVN (fig. 4A, B), MnPO (fig. 4C, D) and OVLT (fig. 4E, F) from control rats (fig. 4A, C, E) and rats receiving a single DOCA injection (fig. 4B, D, F).

MR Immunoreactivity

The ability of the MCR N-17 antibody to recognize the rat brain receptor was first tested in the dorsal hippocampus, due to its high content of MR [6, 36–40]. As shown in figure 5A, the hippocampus from male rats displayed MR immunoreactivity which was predominantly nuclear in

Downloaded by:
Univ. of California San Diego
132.239.1.231 - 6/27/2017 7:20:40 AM

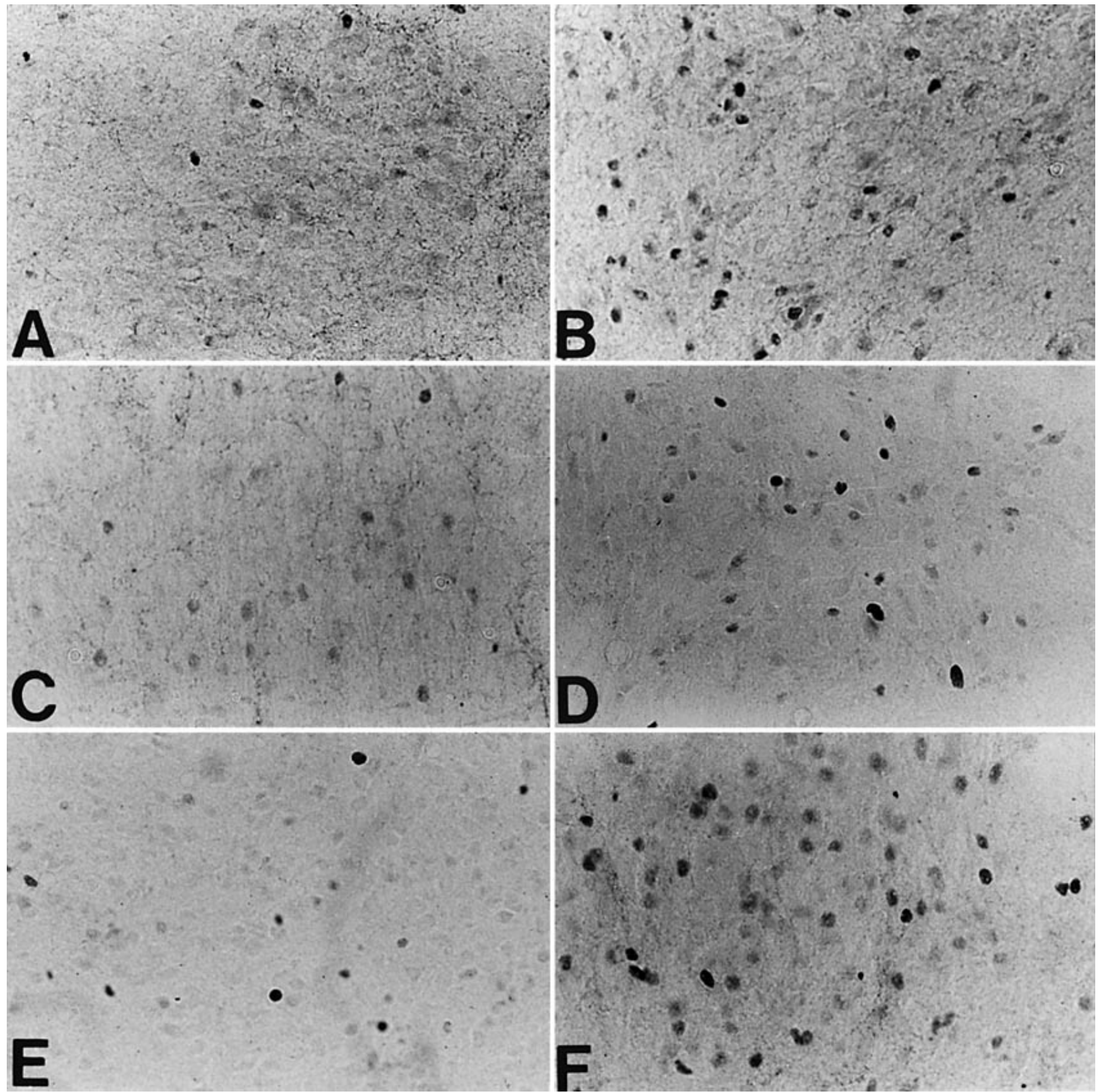


Fig. 4. Representative photomicrographs showing Fos-immunoreactive cell nuclei in a control rat (**A**, **C**, **E**) and a rat receiving a single DOCA injection (**B**, **D**, **F**). **A**, **B** PVN. **C**, **D** MnPO. **E**, **F** OVLT. ×400.

some cells of the pyramidal cell layer. However, and in agreement with a previous report [40], MR staining was absent in the PVN (fig. 5B) and SON (results not shown).

MR-expressing cells of variable intensity were also found in other brain regions. In the MnPO, MR staining was mainly localized in perikaryon and long cell processes and occasionally in the nucleus (fig. 5C, D). Weak, although specific staining was obtained in the OVLT, another structure pertaining to the lamina terminalis (fig. 5F). Cells with MR-positive nuclear staining were also observed in the BNST (fig. 5E). Thus, a receptor-mediated, genomic effect of DOCA seemed feasible in MR-positive areas, whereas lack of detectable MR in the PVN and SON made nongenomic or indirect effects of the mineralocorticoid more likely possibilities.

AVP mRNA Expression

In animals receiving DOCA or vehicle for 2 h or 8 days, AVP mRNA levels were detected by nonisotopic in situ hybridization in the lateral PVN (mainly magnocellular) and SON. Measured by computerized image analysis, the total area of AVP mRNA expression in the PVN was

Downloaded by:
Univ. of California San Diego
132.239.1.231 - 6/27/2017 7:20:40 AM

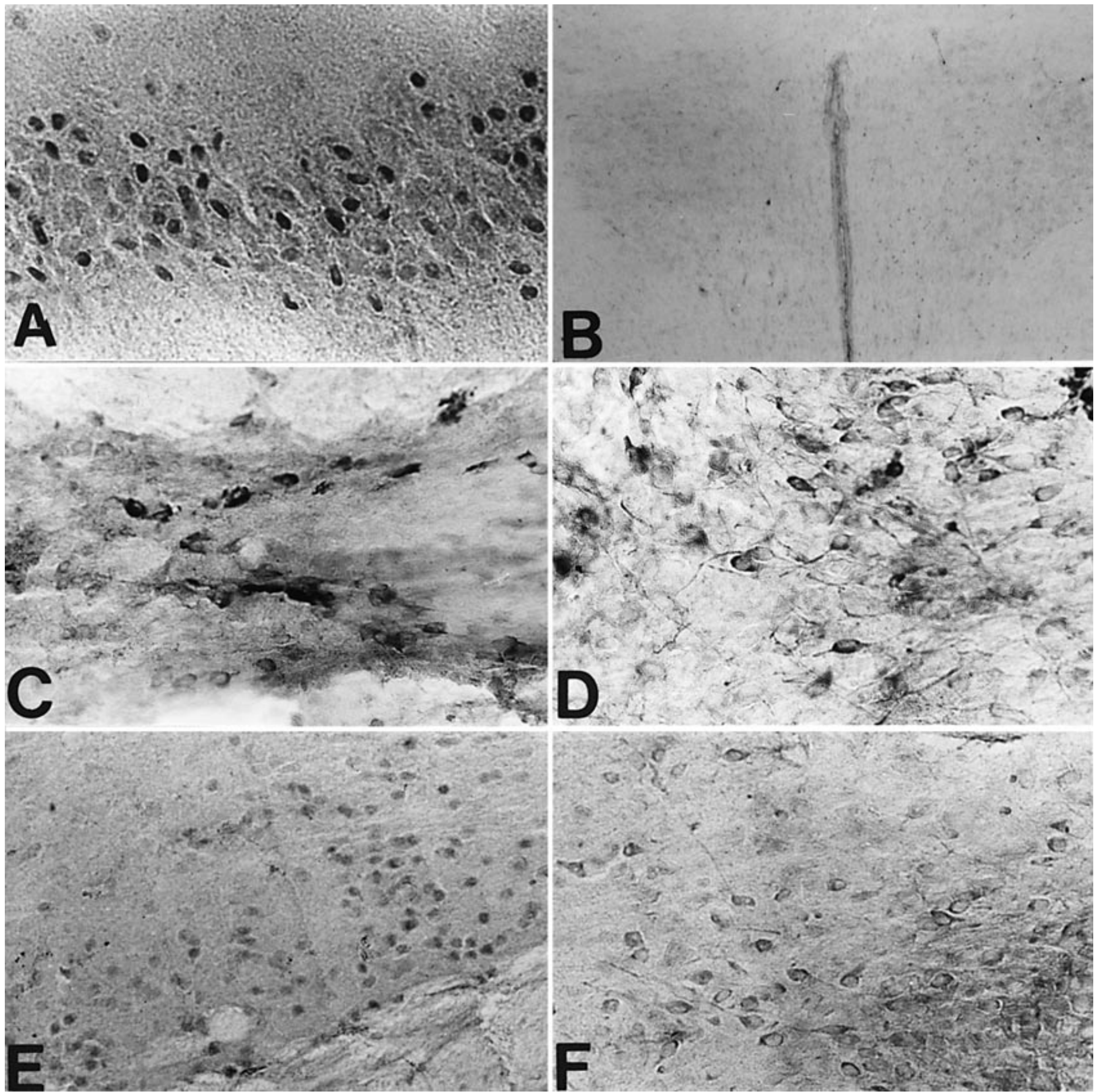


Fig. 5. MR immunoreactivity in the hippocampus (**A**), MnPO (**C**, **D**), BNST (**E**) and OVLT (**F**). The photomicrographs also show that the PVN (**B**) lacked detectable MR. MR showed a nuclear localization in the hippocampus (**A**) and BNST (**E**), whereas cytoplasmic staining prevailed in the MnPO (**C**, **D**) and OVLT (**F**). **A**, **C**–**F** × 400. **B** × 100.

2.3 times higher in rats receiving 4 DOCA injections than in controls ($p < 0.05$), while it was 3 times higher than controls in the SON ($p < 0.05$) (fig. 6C). In contrast to data in animals given 4 DOCA injections and showing a salt appetite, the AVP mRNA area in rats given a single DOCA injection did not differ significantly from the area of control animals (fig. 6A). However, cells expressing AVP mRNA in the 2-hour DOCA group showed increased staining intensity compared to those of control rats both in the PVN and SON (fig. 6B), whereas the intensity was unchanged in these nuclei after 4 DOCA injections (fig. 6D). Therefore, while a single DOCA injection increased the content of AVP mRNA, prolonged DOCA treatment possibly recruited additional cells expressing normal amounts of neuropeptide mRNA.

Downloaded by:
Univ. of California San Diego
132.239.1.231 - 6/27/2017 7:20:40 AM

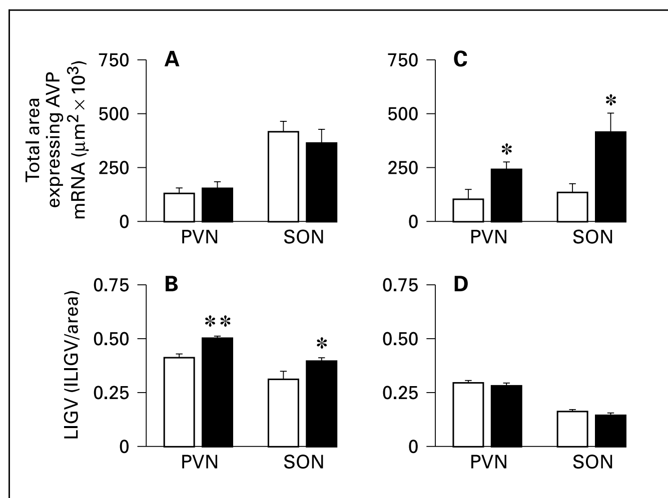


Fig. 6. Effects of DOCA treatment on AVP mRNA. Data correspond to measurement of the total area expressing AVP mRNA (in $\mu m^2 \times 10^3$) (**A**, **C**) and the intensity of the reaction (LIGV = ILIGV/area) (**B**, **D**) in hypothalamic PVN and SON from control rats (white columns) and DOCA-treated (black columns) rats receiving 1 (**A**, **B**) or 4 injections (**C**, **D**) of the mineralocorticoid. LIGV: mean log inverse gray value; ILIGV: integrated log inverse gray value. * $p < 0.05$, ** $p < 0.02$ versus controls (Student's t test).

Discussion

DOCA Stimulation of Fos Immunoreactivity

In the present study, we first determined the number of Fos-immunoreactive nuclei in some regions of the brain of rats receiving two different time courses of mineralocorticoid treatment: a prolonged course which induced a salt appetite and a short treatment which did not change this behavior. Under both conditions, Fos immunoreactivity was activated to some extent by mineralocorticoid treatment. Furthermore, Fos activation occurred not only in regions containing immunoreactive MR, but also in others devoid of detectable MR. Thus, the possibility exists that brain regions showing both Fos induction and MR immunoreactivity may be genomically responding to systemic DOCA treatment. However, early gene induction in otherwise MR-negative cells could indicate a membrane-mediated effect of the steroid or that activation in these areas was a secondary event following steroid action in MR-expressing cells located elsewhere.

Under our experimental conditions, rats with established salt appetite presented a significantly increased number of Fos-immunoreactive nuclei in the dBNST, vBNST, dMnPO, vMnPO, POA, OVLT, PVN and SON, while a single DOCA injection promoted significant Fos induction in the AMYG, vBNST, vMnPO, POA, SON and OVLT. Thus, neurons became activated in 'osmosensitive' structures of the lamina terminalis (OVLT, MnPO), hypothalamic regions (PVN, SON, POA) and two components of the so-called 'extended amygdala', i.e. the medial AMYG and BNST.

Our results also show that rats given a single DOCA injection displayed significant Fos induction in the AMYG, vBNST, vMnPO, POA, SON and OVLT. In contrast, the experimental group with established salt appetite demonstrated significant increases in Fos immunoreactivity in all of the sampled regions with the exception of the AMYG. Thus, compared with the nonappetitive group, in the salt-drinking group, neurons became activated in 'osmosensitive' structures of the lamina terminalis (MnPO), hypothalamic regions (PVN) and components of the so-called 'extended amygdala', i.e. the BNST, and deactivated in the AMYG. Therefore, the results of Fos immunoreactivity suggest that the expression of salt appetite involves a coordinative effect upon many brain regions, as suggested by Johnson et al. [41]. An additional point noted in our experiments was a substantial difference between control groups after the single and the 4 injections of vehicle, as exemplified for the PVN and POA (fig. 2). Although this effect did not interfere with the DOCA effect, changes in basal Fos levels could be due to injection stress [42] acting differently in regions with more active roles in the stress response.

Previous studies have used Fos to monitor changes in neural activation in DOCA-treated animals. Interestingly, divergent results were obtained with animal models of salt appetite induction and suppression due to different doses of DOCA. In rats with salt appetite due to subcutaneous DOCA pellet implantation, strong Fos staining was seen in the OVLT but to a much lesser extent in the MnPO [23]. In another study [26], as early as 1 h after implants of THDOC or aldosterone into the AMYG, salt appetite was induced and Fos immunoreactivity was enhanced in the OVLT, BNST, MnPO, SFO, PVN and SON. In contrast, low doses of DOCA able to suppress salt appetite caused by adrenalectomy reduced the number of Fos-positive nuclei in the OVLT and SFO [28]. Thus, experimental treatments designed to induce or suppress salt appetite go in parallel with changes in Fos. These results indicate the value of Fos as a neuroanatomical marker to elucidate the participation of neural regions sensitive to variations in hormonal input. Collectively, data obtained using changes in Fos immunoreactivity, lesion studies and steroid implants reinforce the hypothesis that one pathway involved in steroid control of salt appetite connects the

Downloaded by:
Univ. of California San Diego
132.239.1.231 - 6/27/2017 7:20:40 AM

AMYG, the amygdalofugal tract, BNST, AV3V structures and hypothalamic nuclei (PVN, SON) [11, 16, 41]. The question remains, however, whether DOCA causes the Fos response per se or whether the function of osmotic stress evoked by DOCA is the reason for this response. Although DOCA-treated rats exhibit normal plasma sodium levels [10], the possibility that osmotic changes in specific regions induce the Fos response cannot be discarded. Such questions still remain to be answered.

However, an intriguing fact is that before salt appetite begins, several regions showed positive Fos immunoreactivity in rats receiving a single DOCA injection. This observation questions the specificity of the DOCA/Fos response in relation to the time of development of salt appetite. It is possible that in addition to neuronal activation indicated by the Fos response, steroid effects upon other brain structures such as those pertaining to the basal ganglia dopaminergic systems [11, 13] may be needed for the full expression of salt-craving behavior. Future experiments are planned to study Fos activity in these areas from control and DOCA-treated rats.

Localization of MR Immunoreactivity

Salt appetite after systemic administration of DOCA usually develops after 24–48 h of hormone exposure. According to Sakai et al. [11], this delay is due to genomic effects under conditions of systemic steroid administration, a possibility supported by experiments showing that administration of MR antagonists and MR antisense therapy blocks DOCA-induced salt appetite [8, 11, 14]. To understand the relationship between Fos induction and genomic steroid effects, MR-immunoreactive cells were studied in areas of stimulated Fos expression. Extrahippocampal MR-immunoreactive cells and/or fibers were present in the OVLT, BNST and MnPO. Here, it is likely that salt appetite induction depended on the hormone interaction with an intracellular receptor, with subsequent modulation of the transcriptional machinery. Mineralocorticoid-selective receptors and binding sites were previously studied using a variety of techniques. A DOC-binding protein responsible for salt appetite and hypertension was described in the hypothalamus by Lassman and Mulrow [43]. Hypothalamic, amygdaloid nuclei and septal binding of labeled aldosterone was also observed during in vitro incubations with the spirolactone derivative [^{3}H]-ZK 91587 [39]. Yongue and Roy [44] found the highest cell nuclear concentration of endogenous aldosterone in the hypothalamus, while the AMYG contained about half of the hypothalamic levels. Localization studies using immunohistochemistry and in situ hybridization also disclosed that the MnPO, POA, SFO and medial AMYG expressed MR protein and mRNA [38, 40]. Moreover, after intravenous administration of [^{3}H]-aldosterone to adrenalectomized animals, considerable uptake occurred in MR-selective sites from circumventricular structures and the corticomedial AMYG, while the POA and other hypothalamic areas contained lower but still appreciable levels [6]. Thus, analysis of MR complements Fos expression studies to delineate the mineralocorticoid-sensitive neural circuit subserving sodium appetite.

However, some evidence also favors a role for the glucocorticoid receptor (GR), since GR occupation occurred in brain regions in animals given high doses of DOCA [8]. Also, treatment with the GR antagonist RU 486 did not inhibit but rather potentiated the DOCA effects on salt appetite [8], an effect possibly due to a weak agonist activity of this compound [45]. In the same way, coadministration of DOCA and dexamethasone facilitated mineralocorticoid-induced sodium intake [45, 46]. Thus, although DOCA effects on GR alone cannot explain mineralocorticoid-induced salt appetite, synergistic modulation of MR and GR may underlie this phenomenon.

DOCA Effects on AVP mRNA Levels

In the third part of the present study, we measured changes in AVP mRNA in magnocellular cells. In a previous publication, De Kloet et al. [7] postulated that the mineralocorticoid-selective MR located in the AV3V region acts in coordination with locally secreted neuropeptides to control fluid homeostasis. Among the intermediate vasoactive neuropeptides, AVP synthesized by the magnocellular cells of the PVN and SON seemed a likely candidate. Here, we showed that AVP mRNA staining intensity was increased in the PVN during short DOCA treatment, whereas the prolonged DOCA administration eliciting a salt appetite increased the total area of AVP mRNA expression. These data suggest that increased AVP mRNA synthesis per cell was the primary response of magnocellular cells to acute stimulation with DOCA, whereas additional AVP-producing cells were recruited upon prolonged DOCA treatment. In the latter case, although the level of AVP mRNA per cell was unaffected, increased numbers of neuropeptide-producing cells would assure a continuous replenishment of AVP in DOCA-treated rats. This assumption is supported by former publications from our laboratory, in which the changes in AVP mRNA and peptide in the PVN and SON were coupled with an increased AVP content in blood [9, 10]. AVP released from pituitary stores into the peripheral circula-

Downloaded by:
Univ. of California San Diego
132.239.1.231 - 6/27/2017 7:20:40 AM

tion after DOCA treatment contributes to the development of hypertension through its antidiuretic and vasoconstrictor effects [47, 48]. While it is unknown if peripheral AVP contributes to salt drinking behavior, the latter could be due to centrally derived AVP. Thus, besides releasing AVP into the bloodstream and the portal system, the PVN sends vasopressinergic projections to several brain areas, including the AMYG, circumventricular organs and the third ventricle and tissues around the PVN [49, 50]. Whatever the mechanism, a role of AVP in salt appetite is suggested by attenuation of salt drinking behavior when DOCA-treated rats receive peripheral infusions of a V1/V2 AVP receptor antagonist [51]. However, the exact mechanism(s) underlying the stimulatory effects of DOCA on AVP mRNA is as yet unclear. It is conceivable that DOCA may be unable to directly promote AVP gene expression due to the lack of MR and GR in magnocellular neurons of the PVN or SON. More likely is that steroid treatment may regulate synthesis of this neuropeptide by direct action on MR-containing cells present in the AMYG, BNST or structures of the lamina terminalis or elsewhere in the brain [6, 40].

Conclusions

The present demonstration of Fos activation in osmosensitive regions of the brain, in conjunction with the role of MR and hypothalamic AVP mRNA, focused on the anatomical locus and cellular pathways activated during DOCA effects on salt appetite. It is recognized, however, that DOCA effects on salt appetite may also depend on mechanisms and brain structures other than those analyzed in the present investigation, since DOC is extensively reduced in response to the GABAa receptor agonist THDOC [52], which does not bind to classical intracellular steroid receptors. A more comprehensive view of DOCA-induced salt appetite should thus consider the multiplicity of genomic and nongenomic pathways of steroid action in the brain.

Acknowledgments

This work was supported by grants from the National Research Council of Argentina (CONICET, PIP 4103), University of Buenos Aires (MT13, MT048), FONCYT (PICT 97 05-00438), Fundación Barceló (Buenos Aires) and by an International Agreement between NSF/CONICET.

References

1 Denton DA, Blair-West JR, McBurnie MI, Miller JAP, Weisinger RS, Williams RM: Effect of adrenocorticotrophic hormone on sodium appetite in mice. Am J Physiol 1999;277: R1033–R1040.
2 Weisinger RAS, Denton DA, McKinley MJ, Nelson JF: ACTH induced sodium appetite in the rat. Pharmacol Biochem Behav 1978;8: 339–342.
3 Weisinger RAS, Coghlan JP, Denton DA, Fan JSK, Hatzikostas S, McKinley MJ, Nelson JF, Scoggins BA: ACTH-elicited sodium appetite in sheep. Am J Physiol 1980;239:E45–E50.
4 Wolf G: Effect of deoxycorticosterone on sodium appetite of intact and adrenalectomized rats. Am J Physiol 1965;6:1281–1285.
5 Fregly MJ, Waters IW: Effects of mineralocorticoids on spontaneous sodium chloride appetite in adrenalectomized rats. Physiol Behav 1966;1:65–74.
6 McEwen BS, Lambdin LT, Rainbow TC, De Nicola AF: Aldosterone effects on salt appetite in adrenalectomized rats. Neuroendocrinology 1986;43:38–43.
7 De Kloet ER, Sutanto W, Van den Bergh D, Carey MP, van Haarst AD, Hornsby CD, Meijer OC, Rots NY, Oitzl M: Brain mineralocorticoid receptor diversity: Functional implications. J Steroid Biochem Mol Biol 1993;47: 183–190.
8 Vallee SM, Grillo C, Gonzalez S, Cosen-Binker L, de Kloet ER, McEwen BS, De Nicola AF: Further studies in deoxycorticosterone acetate treated rats: Brain content of mineralocorticoid and glucocorticoid receptors and effect of steroid antagonists on salt intake. Neuroendocrinology 1995;61:117–124.
9 Grillo C, Saravia F, Ferrini M, Piroli G, Roig P, Garcia S, de Kloet ER, De Nicola AF: Increased expression of magnocellular vasopressin mRNA in rats with deoxycorticosterone-acetate induced salt appetite. Neuroendocrinology 1998;68:105–115.
10 Saravia F, Grillo C, Ferrini M, Roig P, Lima A, de Kloet ER, De Nicola AF: Changes of hypothalamic and plasma vasopressin in rats with deoxycorticosterone-acetate induced salt appetite. J Steroid Biochem Mol Biol 1999;70:47–57.
11 Sakai RR, McEwen BS, Fluharty SJ, Na LY: The amygdala: Site of genomic and nongenomic arousal of aldosterone-induced salt intake. Kidney Int 2000;57:1337–1345.
12 Lucas LR, Pompei P, McEwen BS: Effects of deoxycorticosterone acetate and diazepam on neuropeptidergic neurons in rat striatum. Neuroreport 1997;8:811–816.
13 Lucas LR, Pompei P, McEwen BS: Salt appetite in salt-replete rats: Involvement of mesolimbic structures in deoxycorticosterone-induced salt craving behavior. Neuroendocrinology 2000;71:386–395.
14 Sakai RR, Ma LY, Zhang DM, McEwen BS, Fluharty SJ: Intracerebral administration of mineralocorticoid antisense oligonucleotides attenuates adrenal steroid-induced salt appetite in rats. Neuroendocrinology 1996;64:425–429.
15 Brody MJ, Johnson AK: Role of the anteroventral third ventricular region on fluid and electrolyte balance, arterial pressure regulation and hypertension. Front Neuroendocrinol 1980;6: 249–292.
16 Nitabach MN, Schulkin J, Epstein AN: The medial amygdala is part of a mineralocorticoid-sensitive circuit controlling NaCl intake in the rat. Behav Brain Res 1989;35:127–134.
17 Zhang DM, Epstein AN, Schulkin J: Medial region of the amygdala: Involvement in adrenal-steroid-induced salt appetite. Brain Res 1993;600:20–26.
18 Swords BH, Wyss JM, Berecek KH: Central vasopressin receptors are upregulated by deoxycorticosterone acetate. Brain Res 1991;559: 10–16.
19 Kovács K: c-Fos as a transcription factor: A stressful (re)view from a functional map. Neurochem Int 1998;33:287–297.

Downloaded by:
Univ. of California San Diego
132.239.1.231 - 6/27/2017 7:20:40 AM

20 Sharp FR, Sagar SM, Hicks K, Lowenstein D, Hisanaga K: c-Fos mRNA, Fos, and Fos-related antigen induction by hypertonic saline and stress. J Neurosci 1991;11:2321–2331.
21 Vivas L, Pastuskovas CV, Tonelli L: Sodium depletion induces Fos immunoreactivity in circumventricular organs of the lamina terminalis. Brain Res 1995;679:34–41.
22 Rowland NE, Fregly MJ, Han L, Smith G: Expression of Fos in rat brain in relation to sodium appetite: Furosemide and cerebroventricular renin. Brain Res 1996;728:90–96.
23 Lane JM, Herbert J, Fitzsimons JT: Increased sodium appetite stimulates c-fos expression in the organum vasculosum of the lamina terminalis. Neuroscience 1997;78:1167–1176.
24 Xu Z, Johnson AK: Central renin injections: Effects on drinking and expression of immediate early genes. Brain Res 1998;782:24–35.
25 Bruijnzeel AW, Stam R, Compaan JC, Croiset G, Akkermans LMA, Olivier B, Wiegant VM: Long-term sensitization of Fos-responsivity in the rat central nervous system after a single stressful experience. Brain Res 1999;819:15–22.
26 Ma LY, McEwen BS, Fluharty S, Sakai RR: Brain c-fos expression after acute adrenal steroid implants in the amygdala of the rat (abstract 856.3). 29th Ann Meeting Soc Neurosci, Miami Beach 1999.
27 Morien A, Garrard L, Rowland NE: Expression of Fos immunoreactivity in rat brain during dehydration: Effect of duration and timing of water deprivation. Brain Res 1999;816:1–7.
28 Weisinger RS, Burns P, Colvill LM, Davern P, Giles ME, Oldfield BJ, McKinley MJ: Fos immunoreactivity in the lamina terminalis of adrenalectomized rats and effects of angiotensin II type I receptor blockade or deoxycorticosterone. Neuroscience 2000;98:167–180.
29 Briski KP, DiPasquale BM, Gillen E: Induction of immediate-early gene expression in preoptic and hypothalamic neurons by the glucocorticoid receptor agonist, dexamethasone. Brain Res 1997;768:185–196.
30 Ferrini M, Piroli G, Frontera M, Falbo A, Lima A, De Nicola AF: Estrogens normalize the hypothalamic-pituitary axis response to stress and increase glucocorticoid receptor immunoreactivity in hippocampus of aging male rats. Neuroendocrinology 1999;69:129–137.
31 Landry M, Roche D: In situ hybridization: Methods and Application to the Multiple Labelings; EMBO Course On Basic Methods in Molecular Neuroanatomy. Université Pierre & Marie Curie CNRS, 1994.
32 Ivell R, Richter D: Structure and comparison of the oxytocin and vasopressin genes from rat. Proc Natl Acad Sci USA 1984;81:2006–2010.
33 Landry M, Roche D, Calas A: Short-term effects of centrally administered galanin on the hyperosmotically stimulated expression of vasopressin in the rat hypothalamus. An in situ hybridization and immunohistochemistry study. Neuroendocrinology 1995;61:393–404.
34 Saravia FE, Gonzalez SL, Roig P, Alves V, Homo-Delarche F, De Nicola AF: Diabetes increases the expression of hypothalamic neuropeptides in a spontaneous model of type I diabetes, the nonobese diabetic (NOD) mouse. Cell Mol Neurobiol 2001;21:15–28.
35 Paxinos G, Watson C: The Rat Brain in Stereotaxic Coordinates. Sydney, Academic Press, 1982.
36 McEwen BS, Weiss JM, Schwartz LS: Selective retention of corticosterone by limbic structures in rat brain. Nature 1968;220:911–912.
37 Birmingham MK, Sar M, Stumpf WE: Localization of aldosterone and corticosterone in the central nervous system, assessed by quantitative autoradiography. Neurochem Res 1984;9:333–350.
38 Ahima R, Krozowski Z, Harlan R: Type I corticosteroid receptor-like immunoreactivity in the rat CNS: Distribution and regulation by corticosteroids. J Comp Neurol 1991;313:522–538.
39 Grillo CA, Vallee S, McEwen BS, De Nicola AF: Properties and distribution of binding sites for the mineralocorticoid receptor antagonist [3H]-ZK 91587 in brain. J Steroid Biochem 1990;35:11–15.
40 Herman JP: Regulation of adrenocorticosteroid receptor mRNA expression in the central nervous system. Cell Mol Neurobiol 1993;13:349–372.
41 Johnson AK, de Olmos J, Pastuskovas CV, Zardetto-Smith AM, Vivas L: The extended amygdala and salt appetite. Ann NY Acad Sci 1999;877:258–280.
42 Bozas E, Triton N, Phillipidis H, Stylianopoulou F: At least three neurotransmitter systems mediate a stress-induced increase in c-fos mRNA in different rat brain areas. Cell Mol Neurobiol 1997;17:157–169.
43 Lassman MN, Mulrow PJ: Deficiency of deoxycorticosterone-binding protein in the hypothalamus of rats resistant to deoxycorticosterone-induced hypertension. Endocrinology 1974;94:1541–1546.
44 Yongue BG, Roy EJ: Endogenous aldosterone and corticosterone in brain cell nuclei of adrenal-intact rats: Regional distribution and effects of physiological variations in serum steroids. Brain Res 1987;436:49–61.
45 Shelat SG, King JL, Flanagan-Cato LM, Fluharty SJ: Mineralocorticoids and glucocorticoids cooperatively increase salt intake and angiotensin II receptor binding in rat brain. Neuroendocrinology 1999;69:339–351.
46 Ma LY, McEwen BS, Sakai RR, Schulkin J: Glucocorticoids facilitate mineralocorticoid-induced sodium intake in the rat. Horm Behav 1993;27:240–250.
47 Berecek KH, Murray RD, Gross F, Brody MJ: Vasopressin and vascular reactivity in the development of DOCA hypertension in rats with hereditary diabetes insipidus. Hypertension 1982;4:3–12.
48 Zicha J, Kunes J, Lebl M, Pohlova I, Slaninova J, Jelinek J: Antidiuretic and pressor actions of vasopressin in age-dependent DOCA-salt hypertension. Am J Physiol 1989;256:R138–R145.
49 Saito T, Yajima T, Watanabe T: Involvement of AVP in the development and maintenance of hypertension in rats; in Yoshida S, Share L, Yagi K (eds): Antidiuretic Hormone. Baltimore, University Park Press, 1981, pp 215–221.
50 Ota K, Kimura T, Inoue M, Funyu T, Shoji M, Sato K, Ohta M, Yamamoto T, Abe K: Effects of V1- and V2-vasopressin (AVP) antagonist on the pressor, AVP and atrial natriuretic peptide responses to a hypertonic saline infusion in conscious anephric rats. Eur J Endocrinol 1995;133:127–132.
51 De Nicola AF, Grillo CA, Saravia FE: Mineralocorticoid-induced changes in neuropeptide gene expression. 20th Eur Winter Conf Brain Res, Villars sur Ollon 2000, p 59.
52 Kraulis I, Foldes G, Traikov H, Dubrovsky B, Birmingham MK: Distribution, metabolism and biological activity of deoxycorticosterone in the central nervous system. Brain Res 1975;88:1–14.

Downloaded by:
Univ. of California San Diego
132.239.1.231 - 6/27/2017 7:20:40 AM