THE CENTRAL ROLE OF MINERALOCORTICOIDS IN THE DEVELOPMENT OF SALT-SENSITIVE HYPERTENSION

by

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CERTIFICATE OF APPROVAL

This is to certify that the M.S thesis of

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THE CENTRAL ROLE OF MINERALOCORTICOIDS IN THE DEVELOPMENT OF CHRONIC HYPERTENSION

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ABSTRACT

Patients with the disease hyperaldosteronism due to elevated levels of mineralocorticoids (MCs) such as aldosterone have chronically elevated blood pressure. In the deoxycorticosterone acetate (DOCA)-salt model of hypertension, DOCA can be used to induce hyperaldosteronism. Excess MCs in combination with a high salt diet induces hypervolemia and increases sympathetic activity. The result is increased blood pressure. However, the mechanism by which sympathetic activity is increased is not known. Increased dietary salt alone does not increase blood pressure, but DOCA alone can increase blood pressure. Nevertheless, the increase is much greater with the combination of DOCA and salt.[4,5] MCs appear to act directly or indirectly in regions of the brain that control sympathetic activity, such as the paraventricular nucleus (PVN) of the hypothalamus, median preoptic nucleus (MnPO) and the organum vascularis of the lamina terminalus (OVLT). Therefore, the purpose of this study was to test the hypothesis that a synergism exists between MCs acting on these brain regions and a high salt diet to increase sympathetic tone using the DOCA-salt model in the rat. To test this hypothesis, we determined if neuronal activation in sympathetic regions of the brain as identified by Fos immunohistochemistry was higher in rats treated with DOCA and salt compared to Sham, Sham-salt and DOCA. Preliminary results showed that there was

increased Fos activity in the paraventricular nucleus (PVN), lateral parabrachial nucleus (LPB), periaqueductal grey area (PAG), and the bed nucleus of the stria terminalis (BNST) in DOCA-salt rats when compared to DOCA or Sham-salt rats. These findings support the hypothesis that a synergism exists between MCs acting on the brain and a high salt diet to increase sympathetic activity in the DOCA-salt model. We conclude that MC, such as aldosterone, can play a central role in the development of chronic high blood pressure.

The Central Role of Mineralocorticoids in the Development of Salt-Sensitive Hypertension

INTRODUCTION

In the deoxycorticosterone acetate (DOCA)-salt model of hypertension, excess mineralocorticoids (MCs) in combination with a high salt diet induces hypervolemia and increased sympathetic activity, which are thought to increase blood pressure.[1-3] The hypervolemia is caused by MCs acting at the kidney to increase sodium and water retention. *However, the mechanisms by which DOCA-salt treatment causes increased sympathetic activity are unknown.* The study reported here was designed to test the hypothesis that excess MCs act in the brain to increase basal sympathetic activity, or tone, but only in combination with a high salt intake.

THE POTENTIAL ROLE OF CENTRAL MINERALOCORTICOIDS IN SYMPATHETIC TONE

Indirect evidence to support this hypothesis includes the fact that mineralocorticoid receptors (MR) are distributed throughout the brain in neurons and glia, and are abundant in the periventricular regions of the hypothalamus.[4,5] In a recent study, MR were identified in DOCA-salt rats in areas of the hypothalamus known to be involved with regulation of sympathetic tone, such as the median preoptic nucleus (MnPO) and the organum vascularis of the lamina terminalis (OVLT).[6] Another study demonstrated that intracerebroventricular (ICV) infusion of the MR antagonist, RU28318, decreased blood pressure in DOCA-salt rats, but there was no direct measure

of sympathetic activity.[7] Furthermore, recent studies of rats with congestive heart failure, another model of increased sympathetic activity, demonstrated that intracarotid (IC) or ICV infusion of the MR antagonist, spironolactone decreased blood pressure, paraventricular nucleus (PVN) firing rate, and renal sympathetic nerve activity (RSNA).[8,9] These data suggest that MCs may be acting centrally to support sympathetic activity, but this thesis has not been directly tested in DOCA-salt rats. Moreover, the combination of MCs and salt cause a greater increase in blood pressure than either MCs or salt alone.[4,5] Thus, it appears that the effect of MCs to increase sympathetic activity requires salt, but this has also not been investigated.

THE ROLE OF THE HYPOTHALAMUS AND BRAIN STEM IN SYMPATHETIC TONE

Areas in the hypothalamus that are involved with sympathetic tone lie close to the third ventricle (for a review of hypothalamic anatomy see ref [11]). These include the MnPO, subfornical organ (SFO), and OVLT. The SFO and OVLT are termed circumventricular organs and lack a blood-brain barrier; therefore, their neurons are positioned with appropriate receptors or transporters to monitor substances in the blood. Also near the third ventricle is an important integrating center known as the PVN. The MnPO, SFO and OVLT all project to the PVN. The PVN has a parvocellular area and a magnocellular area; it is known that the parvocellular neurons project directly to brain stem regions, including the rostral ventrolateral medulla (RVLM). The RVLM provides most of the descending sympathetic motor drive to cardiovascular targets. Sympathetic premotor neurons in the PVN also synapse in the intermediolateral nucleus (IML) of the

spinal cord to control sympathetic activity of peripheral organs.[10] Thus, if both salt and DOCA activate sympathetic activity, increased activity in these regions would be expected.

FOS-IMMUNOREACTIVE NUCLEI AS MARKERS OF NEURONAL ACTIVATION

One procedure commonly used to identify neuronal activation is based on quantifying the number of Fos-immunoreactive cells. Fos is a transcription factor encoded by an immediate early gene.[Ω] Fos can be induced rapidly with acute stimulation, such as a shock, yet is transiently expressed and thus degraded quickly.[20] The Fos protein binds to AP-1 sites in promoter regions of genes coding for neurotransmitters, receptors, and other intracellular signaling molecules. Thus, Fos resides in the nucleus and is a marker of cell activation.[21] Fos expression is an all-or-none response and thus is very low in control or unstimulated rats, and the induction of c-Fos protein is typically detectable 1-4 hours after stimulation.[20] Moreover, previous studies suggest that constant stimulation will produce constant Fos expression.[14]

In one study of rats with DOCA-salt treatment, significant increases in Fos activity were identified in the PVN and OVLT, indicating neuronal activation.[12] However, the rats were given 4 large doses of DOCA by subcutaneous injection of 10mg/rat/day and sacrificed 24 hours after the last injection.[13] Bolus injections are stressful to an animal,[15] and because Fos expression is sensitive to external and internal

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[Ω] Immediate early genes comprise the cascade of gene expression responsible for initiating the process of stimulus-induced adaptive change, and were identified as transcription factors that were regulated in brain by excitatory synaptic activity (*Andreasson KI, Kaufmann WE 1990*)

stresses, it is possible that this dose and/or delivery method is inducing the observed neuronal activation. Nevertheless, because Fos was expressed in these chronically treated rats, it appears that the DOCA and salt treatment are providing a constant stimulation, which may produce a constant Fos expression. Moreover, while Fos expression was observed in PVN[15] region, whether neuronal activation in the RVLM results from DOCA-salt administration is unknown. Finally, whether brain regions activated by DOCA and salt alone are different from the combination has not been determined.

HYPOTHESIS AND SPECIFIC AIM

These studies therefore suggest this hypothesis: *In DOCA-salt rats, synergism between salt and MC in the brain activate the parvocellular PVN and RVLM and thereby increase sympathetic activity.* The hypothesis was tested by determining if Fos expression is greater in DOCA-treated animals drinking salt water (DOCA/salt) than in DOCA-treated animals drinking water (DOCA/H_2O) or in Sham-treated rats drinking salt water (Sham/salt). I predicted that virtually no Fos would be present in Sham/water (control) rats, that low levels of Fos would be present in Sham/salt or DOCA/water rats and that the highest levels would be present in DOCA/salt rats.

The **specific aim** of this study was to determine if there is greater activity in sympathetic regions of the brain as a result of the combination of MCs and salt than in excess MCs or salt alone. To do this, Fos immunohistochemistry was performed on brains of DOCA/salt, DOCA/water, Sham/salt, and Sham/water rats.

METHODS

SURGICAL PROCEDURES

Under isoflurane anesthesia and using sterile conditions, male Sprague-Dawley rats (280-290g) underwent a unilateral nephrectomy and six received 200mg/kg DOCA in a silastic subcutaneous implant and five the silastic implant without DOCA.[16] The silastic pellet method was utilized because it provides a slow, continuous release that is less stressful to the rat than a bolus subcutaneous injection.[15] In addition, extra care was taken to eliminate stress to the animal, by frequent handling and petting, for the duration of the treatment. To measure blood pressure, a femoral arterial catheter was implanted and routed subcutaneously to the nape of the neck where it was exteriorized and secured. This catheter was connected to a pressure transducer and a Grass (7P1) polygraph to monitor increases in blood pressure. In another study, blood pressure was measured via a femoral arterial catheter using biotelemetry. This method involved the DOCA and Sham rats being placed in individual cages containing an RLA-3000 radiotelemetry receiver (Data Sciences, St. Paul, MN). Every 10 minutes, a 10-second sample of systolic, diastolic, and mean blood pressure and heart rate was taken, and the data were averaged to obtain a single data point for each parameter. This method provided a continuous measurement of blood pressure and heart rate throughout the day.

BLOOD AND BLOOD PRESSURE MEASUREMENTS

After seven days (Day 8), the rats' blood pressure was recorded for 10 minutes after they had acclimated to the polygraph area and appeared unstressed. A blood sample

was collected afterwards via the arterial catheter. Plasma sodium levels, plasma protein and hematocrit were determined. The rats were then maintained on either a 1% NaCl, 0.2% KCl solution or distilled water as their sole drinking fluid for 7 days. On Day 14, the animals' blood pressure and blood sample were analyzed as well. From the biotelemetry measurements, blood pressures were collected on Day 0-1, Day 3-4, and Day 7-8, after introduction of the high salt diet.

FOS IMMUNOREACTIVITY

On Day 15, the animals were rendered unconscious with excess CO_2 and perfused transcardially with saline followed by 2% paraformaldehyde/PBS. The brains were removed and stored in 2% paraformaldehyde/PBS overnight at 4°C. Brains were cryoprotected in 20% sucrose and stored overnight. Horizontal, 40 μM sections were then made in a cryostat; every third slice was collected and all were treated with 0.3% H_2O_2 for 15 minutes. Free floating sections were then blocked in 450:1 goat serum/10ml PBS/Triton for four hours. A primary antibody that only recognizes the c-Fos protein (Fos H-125, Santa Cruz Biotech, Inc., Santa Cruz) was added at 1/5,000 dilution in PBS/Triton/BSA at room temperature overnight in a shaker. Sections were washed three times with PBS, incubated with a secondary antibody (1:50 biotinylated anti-rabbit IgG/10ml PBS/Triton) for one hour, and then processed following the ABC kit instructions (Vector laboratories, 'Elite' ABC reagent). Development was carried out using 1mg/ml diaminobenzidine (DAB) containing 0.01% H_2O_2 during 3 minutes at room temperature. Finally, sections were mounted on gelatin-coated slides and left to dry overnight. Slides were then dehydrated with ethanol and xylene and coverslipped.

LIGHT MICROSCOPY

A computer-assisted image analysis system consisting of an Olympus microscope BX40 and a Sony video camera connected to a Power PC was used to quantify Fos expression. Fos-positive cells were identified as having dark nuclear staining and manually counted. The areas studied included the MnPO, third ventricle (3V), PVN, periaqueductal grey area (PAG), lateral parabrachial nucleus (LPB), and the bed nucleus of the stria terminalis (BNST). I could not easily distinguish the OVLT, SFO and RVLM using horizontal slices, and therefore they were not studied. All areas were observed with 10X power to control for area size. To ensure that all the cells in the whole area were accounted for, boundaries and anatomical limits for these areas were based on the Paxinos and Watson rat brain atlas.[23] Fos-positive cells were quantified within the regions as number of positive cells/slice.

RESULTS

BLOOD PRESSURE RESPONSES TO DOCA/SALT

The blood pressure measurements taken by catheter were generally inconsistent because data in most rats was collected before the rats were completely at rest. Therefore, the data collected by telemetry were used. As shown in Figure 1, the DOCA rats showed an increase in blood pressure one day (Day 1) after beginning excess salt intake in drinking water (Day 0). An increase in blood pressure from 100mmHg to 125mmHg was also observed via catheter measurement in a DOCA rat, before and after 1 day of excess salt, respectfully. By Days 3-4 and Day 7-8, the rats continued to show an increase in blood pressure. Sham rats did not show the same increase in blood pressure after being given salt at Day 1, Day 3-4 or Day 7-8.

PLASMA PROTEIN, HEMATOCRIT AND BLOOD SODIUM LEVEL RESPONSES AS A RESULT OF DOCA AND SALT TREATMENT

On Day 8 and Day 14, a blood sample was taken to analyze plasma protein, sodium levels and hematocrit in all groups of rats. Table 1 contains values for these blood parameters. Plasma protein did not change over the 6-day water treatment in all groups. Hematocrit also did not change in Sham/water, Sham/salt, DOCA/water and DOCA/salt rats, but slightly increased in the DOCA/1 day salt rat. Sodium levels were constant in Sham/water, Sham/salt, and DOCA/water rats, but increased for DOCA/salt rats, as illustrated in Figure 2.

Table 1 *Effects of treatments on blood parameters*

Day 8	Sham/water n=2	Sham/salt n=3	DOCA/water n=2	DOCA/salt n=2	DOCA/ 1 day salt n=1
Plasma protein	6.2,6.2	5.5,6.0,6.0	6.2,6.1	5.0,6.0	5.5
Sodium	143,142.5	143.1,142.0, 141.1	143.2,144.1	145.0,144.9	150.1
Hematocrit	40.5,40.3	38.5,44.8,44.5	37.0,43.0	44.0,43.5	40.5

Day 14	Sham/water n=2	Sham/salt n=3	DOCA/water n=2	DOCA/salt n=2	DOCA/1 day salt n=1
Plasma protein	6.0,6.1	5.96,6.0	6.0,6.1	5.9,6.0	6
Sodium	144.1,141.9	141.2,140.3, 143.3	143.0,144.2	150.5,146.7	142.3
Hematocrit	42.5,395	40.5,44.9,44.0	42.5,43.0	45.5,42.0	47.5

* Values are from the same rats on Day 8 and Day 14

QUANTIFICATION OF FOS IMMUNOREACTIVE NUCLEI IN DOCA AND SHAM BRAIN SLICES

For this experiment, animals in Sham/water, Sham/salt, DOCA/water and DOCA/salt groups were sacrificed on Day 15, the day after their last blood pressure measurement and blood draw. This was done to eliminate any stress caused by the Day 14 measurements, which would induce Fos. The DOCA/1 day salt rat was sacrificed on Day 2 for the same reasons. Brain sections were used for locating Fos-immunoreactive (ir) nuclei. Few Fos-positive nuclei were present in the control Sham/water rats, suggesting a constitutive and basal level of expression. As shown in Fig 3 E-H and Table 2, Sham/salt rats expressed slightly increased numbers of Fos-positive nuclei above basal levels in most regions explored (i.e MnPO, PVN, LPB, and PAG).

Fig 4 A-D shows Fos-ir nuclei from brains of DOCA/water animals. The numbers of Fos-positive cells are shown in Table 3. Levels were only slightly increased from basal levels in the explored regions, much like those of Sham/salt rats. In addition, while analyzing the sections, an unexpected region in each group of rats was noticed to express Fos-ir, the inferior olive (IO), which was later determined to be artifact.[22]

Fig 4 E-H shows photomicrographs of Fos-ir nuclei from brains of DOCA/salt rats. These levels were increased above basal levels in the explored regions. The increases in number of Fos-positive cells from the control Sham/water rat are shown in Table 4.

As shown in Fig 5 A-D, the DOCA rat with only 1 day of salt also showed highly increased levels of Fos expression in MnPO, LPB, BNST and PVN.

Figure 6 compares the numbers of Fos positive cells in the sympathetic regions of all groups of rats. The DOCA/salt rats appear to have higher levels of Fos activity than DOCA/water or Sham/salt rats in the PVN, LPB, PAG, and BNST.

Table 2 ***Numbers of Fos positive cells in sympathetic regions of the brain in Sham/water and Sham/salt rats***

	Sham/water n=2 (# Fos-positive cells/slice)	**Sham/salt n=3** (# Fos-positive cells/slice)
MnPO	2, 2	7,9,7
PVN	5,6	7,8,8
LPB	2,1	8,8,9
PAG	2,2	6,5,6

MnPO, median preoptic nucleus; PVN, paraventricular nucleus; LPB, lateral parabrachial nucleus; PAG, periaqueductal grey area

Table 3 ***Numbers of Fos positive cells in sympathetic regions of the brain in Sham/water and DOCA/water rats***

	Sham/water n=2 (# Fos-positive cells/slice)	**DOCA/water n=2** (# Fos-positive cells/slice)
PVN	5,6	7,8
PAG	2,2	8,10
LPB	2,1	10,7
IO	2,3	9,9

PVN, paraventricular nucleus; LPB, lateral parabrachial nucleus; PAG, periaqueductal grey area; IO, inferior olive

Table 4 ***Numbers of Fos positive cells in sympathetic regions of the brain in Sham/water and DOCA/salt rats***

	Sham/water n=2 (# Fos-positive cells/slice)	**DOCA/salt n=2** (# Fos-positive cells/slice)
PVN	5,6	36,32
LPB	2,1	21,18
PAG	2,2	34,30
IO	2,3	26,29
BNST	2,4	15,11

PVN, paraventricular nucleus; LPB, lateral parabrachial nucleus; PAG, periaqueductal grey area; IO, inferior olive; BNST, bed nucleus of the stria terminalis

DISCUSSION

INCREASED NEURONAL ACTIVATION IN SYMPATHETIC REGIONS OF THE BRAIN IN DOCA/SALT RATS

In this study, we tested the hypothesis that in DOCA/salt rats a synergism exists between salt and MCs in the brain to activate PVN and RVLM to increase sympathetic activity. The hypothesis predicted that the highest level of Fos-ir nuclei would be present in rats given DOCA and salt, while lower levels would be observed in Sham/salt and DOCA/water rats. Interestingly, Fos slightly increased with either DOCA or salt alone. However, in the animals with DOCA and 6 days of salt, there were far more Fos-positive nuclei in the PVN as well as other sympathetic regions, i.e MnPO, PAG, BNST, and LPB than DOCA/water or Sham/salt.

BLOOD PRESSURE RESPONSES IN DOCA RATS

The results show that the DOCA rats did show a rise in blood pressure after being given salt for a week, which suggests that in DOCA rats, salt is needed to increase blood pressure.

INCREASED FOS LEVELS IN SYMPATHETIC REGIONS OF THE BRAIN IN RESPONSE TO DOCA AND/OR SALT

The areas in the brain that we predicted to see activation of in response to DOCA and/or salt did indeed show Fos-ir nuclei in all groups of rats. These areas, MnPO, LPB and PVN, are autonomic and are integrating centers involved with sympathetic tone.

DOCA/water and Sham/salt rats did show slightly elevated Fos expression and DOCA/salt rats even more in the above areas, as is illustrated in Figure 6, suggesting that MCs and salt synergize to fully activate sympathetic regions in the brain.

An interesting area, the IO, was unexpectedly expressing increased Fos levels in DOCA/water as well as DOCA/salt rats. This region in the brainstem actually lies more medial and posterior than the RVLM, though they are very close to each other. The IO is not an autonomic region of interest; it detects peripheral nervous input from the viscera and relays the information to the cerebellum. It was determined later that this region is actually artifact and is known to constitutively express Fos-positive cells.[22] Another interesting area activated in all groups of rats is the PAG. The PAG is an autonomic region in the brain; it lies near the fourth ventricle and sends indirect output to autonomic preganglionic neurons. It also helps to redirect blood from viscera to muscles during "fight or flight". This also lies near the region where the locus coeruleus (A6 cell group) resides, which is the main noradrenaline center output center in the brain. Thus, our results suggest that the PAG may be activated as a response to DOCA and salt synergism in the brain to increase sympathetic outflow. BNST activation was only observed in DOCA/salt rats and the DOCA/1 day salt rat and is also another indirect autonomic center in the brain. Increased Fos-positive cells have also recently been observed in this region in response to DOCA.[17]

There is the possibility that the increased Fos expression seen in these sympathetic regions was due to the hypertension, and not the DOCA/salt combination. However, it was found that hypertension alone increased Fos levels only in the LPB and BNST, but not in hypothalamic regions.[19] Therefore, this suggests that increased Fos

expression observed in hypothalamic regions in this study appear to be due to the MCs and salt, whereas the LPB and BNST activation may be a result of increased blood pressure.

FOS LEVELS AND THE BLOOD PRESSURE RESPONSE IN THE DOCA/1 DAY OF SALT RAT

The DOCA/1 day salt rat was used later on in the study as we became interested in determining exactly when blood pressure starts to rise in DOCA/salt rats. The DOCA/1 day salt rat did show increased blood pressure from 100mmHg to 125mmHg after only 1 day of salt. Compared to DOCA/salt and DOCA/water rats, the DOCA/1 day salt rat had similar areas of activation in the brain (PVN, LPB and PAG) as indicated by increased Fos activity.

SOURCES OF ERROR

There are several sources of error that may have altered our results. The time of day that the blood pressure measurements were done with the polygraph tended to be in the late afternoon, and it was brought to my attention that the best time to take blood pressure measurements in the rat is in the morning when they are least active and their pressure is less likely to fluctuate. The biotelemetry method provided more accurate blood pressure readings for the groups of rats and therefore these data were used in place of the polygraph measurements. Also, c-Fos is an early expression gene, and since we are looking at activation of regions that have been stimulated for over a 2-week period, it

may well be that some of the Fos signal may have dissipated over that time, even though it has been shown that Fos can be constitutively activated with constant stimulation.[12]

CONCLUSIONS

MCs and salt do appear to *synergize* to fully activate sympathetic regions in the brain.

FUTURE DIRECTIONS

It would be best to use coronal sections of the brain to detect the autonomic regions. Since I could not detect Fos in OVLT, SFO or RVLM in the horizontal slices I could not test the hypothesis that MCs and salt synergize in the brain to increase sympathetic outflow via RVLM. With coronal slices, the OVLT, SFO and RVLM as well as the PVN are easily distinguishable and both regions could be detected for activation by Fos-immunohistochemistry. Also, another Fos protein, Fos B, is activated only with longer stimulation periods. Thus, it would be interesting to examine the sympathetic regions as well as other brain regions using Fos B immunohistochemistry with the 2-week long term DOCA/salt treatment.

CLINICAL RELEVANCE

The findings of this study will be significant because hyperaldosteronemia, a clinical condition that DOCA-salt hypertension models, causes chronic elevated blood pressure as a result of excess aldosterone[18]. The sensor in the brain for the increased aldosterone levels that leads to hypertension is unknown. Elucidating the specific central

role aldosterone plays in DOCA-salt hypertension would allow us to examine specific mechanisms and potential therapies.

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Figure 1 ***Effects of increased salt intake on blood pressure in DOCA and Sham treated rats*** on Days 0-1, 3-4, and 7-8 from biotelemetry measurements. Salt was administered on Day 0 in both models as indicated on graphs. As shown above, blood pressure increased in the DOCA rat 1 day after increased salt intake while no change was observed in the Sham rat.

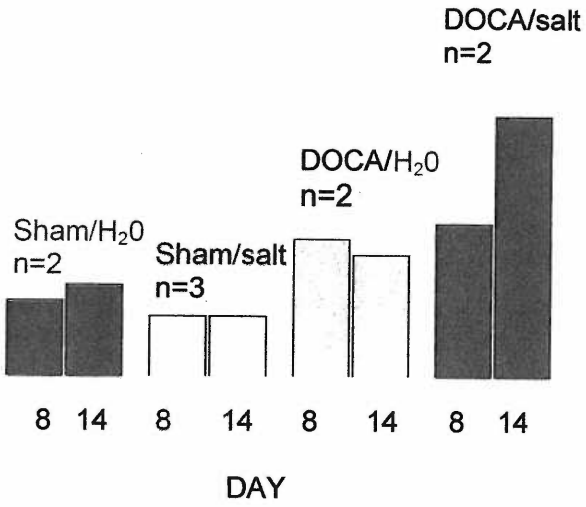


Figure 2 ***Plasma sodium concentrations on Day 8 and Day 14 of Sham/H_2O, Sham/salt, DOCA/H_2O and DOCA/salt rats***

Figure 3 ***Sections from Sham/H_2O and Sham/salt rat brains*** showing the MnPO (A,E), PVN (B,F), PAG (C,G) and LPB (D,H) regions. Fos activity is detected by black nuclear stainings. Sham/H_2O fos activity is at basal levels while Sham/salt fos activity is slightly elevated as shown in the photomicrographs.

MnPO, median preoptic nucleus; PVN, paraventricular nucleus; PAG, periaqueductal grey area; LPB, lateral parabrachial nucleus

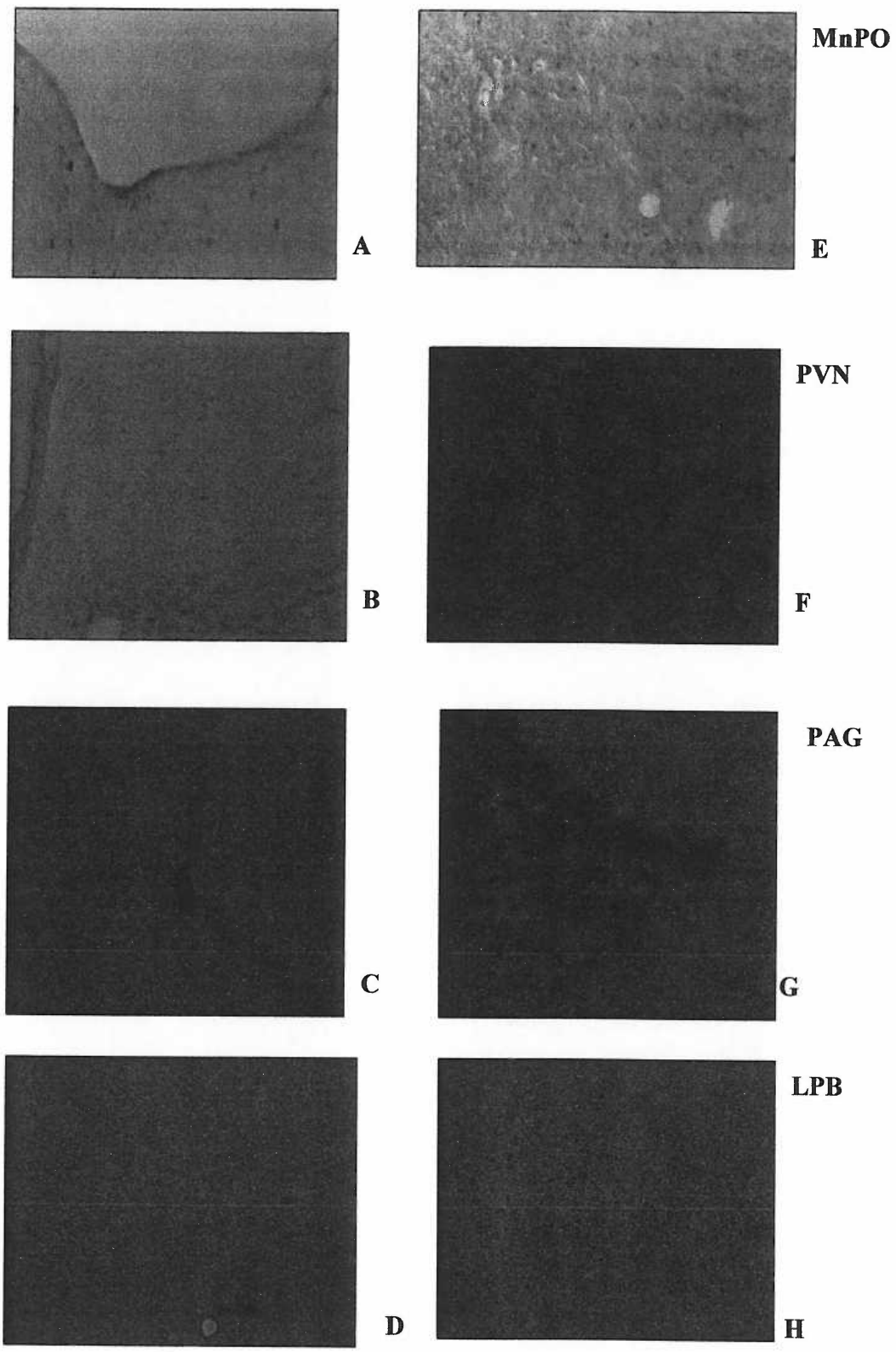


Figure 3

Figure 4 ***Sections from DOCA/H_2O and DOCA/salt rat brains*** showing the PVN (A,E), PAG (B,F), LPB (C,G) and IO (D,H) regions. Fos activity is detected by black nuclear stainings. DOCA/H_2O fos levels are slightly elevated and DOCA/salt fos levels are highly elevated as shown in the photomicrographs.

PVN, paraventricular nucleus; PAG, periaqueductal grey area; LPB, lateral parabrachial nucleus; IO, inferior olive

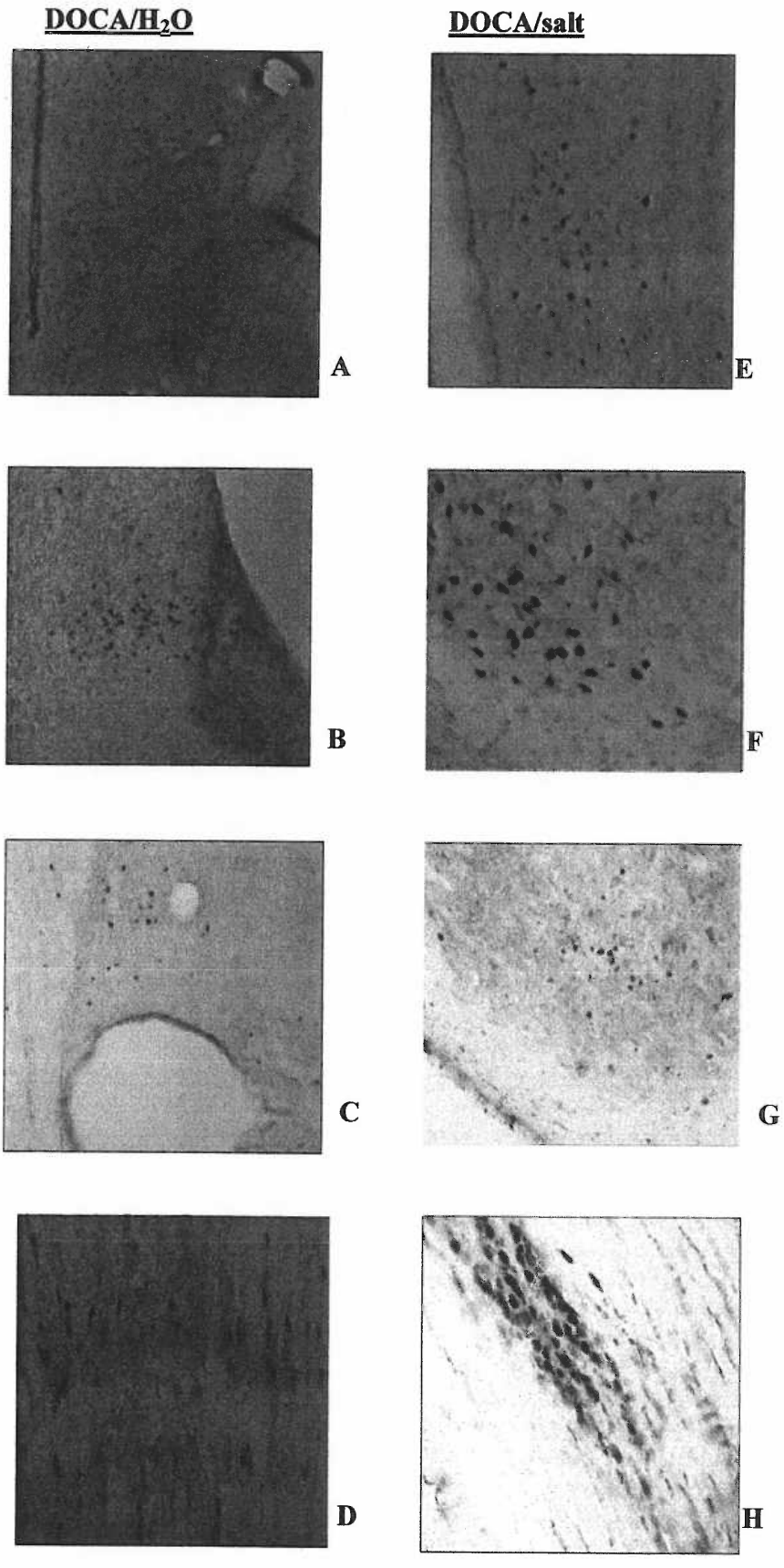
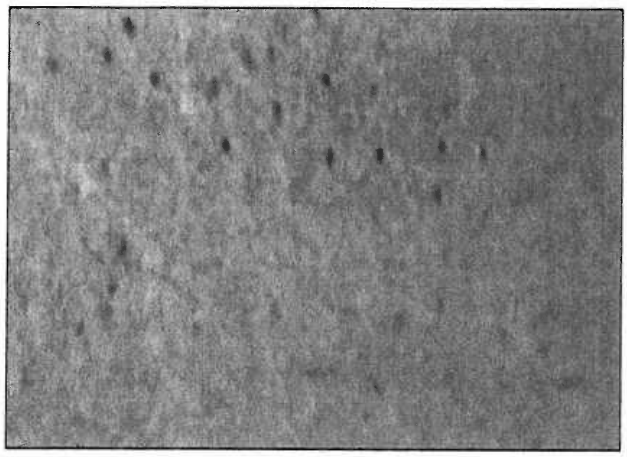
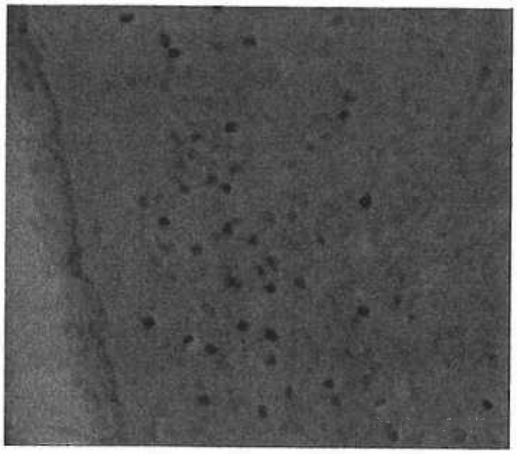


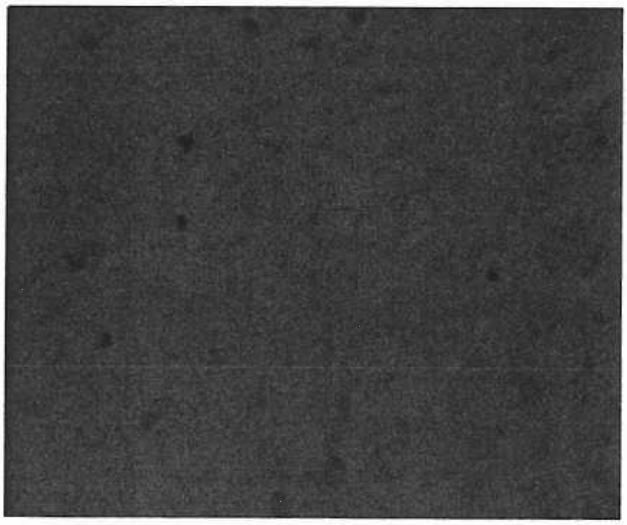
Figure 4



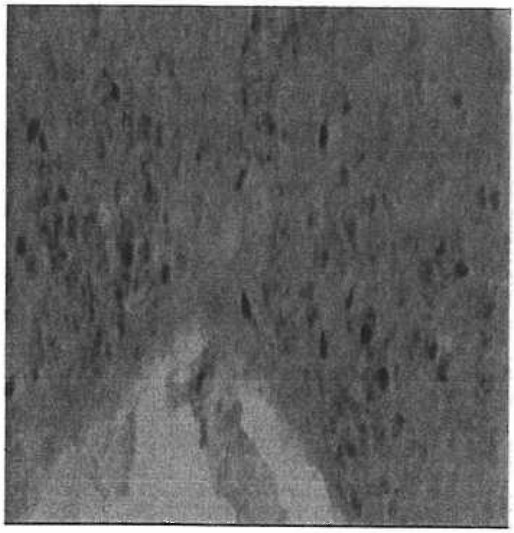
A MnPO



C BNST



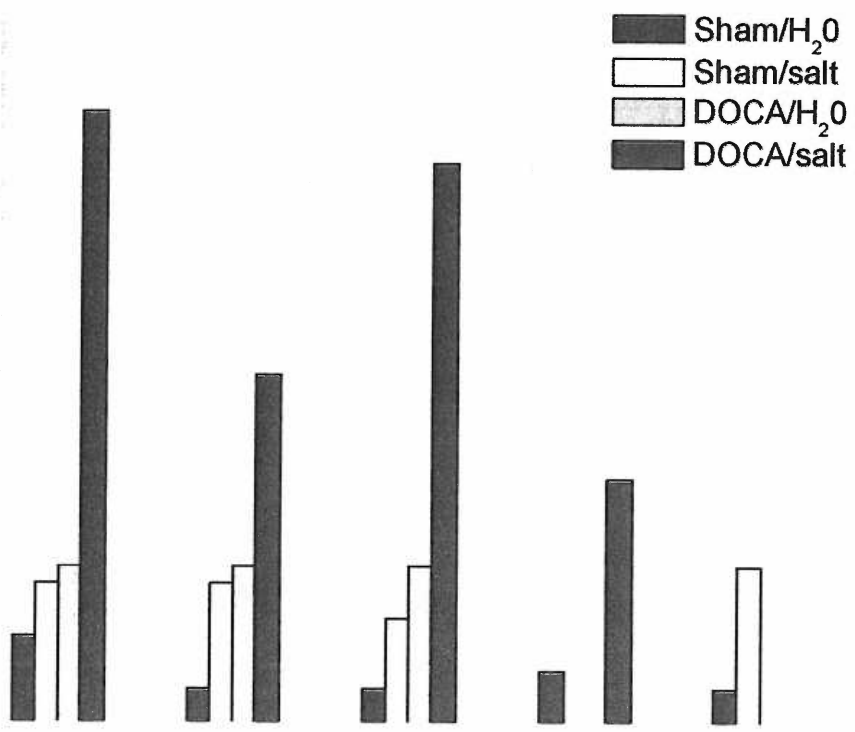
B LPB



D PVN

Figure 5 ***Sections from DOCA/1 day of salt rat brain*** showing the MnPO (A), LPB (B), BNST (C), and PVN (D) regions. Fos activity is detected by black nuclear stainings. Fos levels are slightly elevated as shown in the photomicrographs.

MnPO, median preoptic nucleus; LPB, lateral parabrachial nucleus; BNST, bed nucleus of the stria terminalis; *PVN, paraventricular nucleus;*



PVN, paraventricular nucleus; LPB, lateral parabrachial nucleus; PAG, periaqueductal grey area; BNST, bed nucleus of the stria terminalis; MnPO, median preoptic nucleus

Figure 6 ***Comparison of averages of Fos positive cells in sympathetic regions of the brain amongst all groups of rats***