Fos expression following isotonic volume expansion of the unanesthetized male rat

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Randolph, R. R., Q. Li, K. S. Curtis, M. J. Sullivan, and J. T. Cunningham. Fos expression following isotonic volume expansion of the unanesthetized male rat. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R1345–R1352, 1998.—Cardiopulmonary afferents, baroreceptor afferents, or atrial natriuretic peptide binding to circumventricular organs may mediate the central response to volume expansion, a condition common to pregnancy, exercise training, and congestive heart failure. This study used Fos immunocytochemistry to examine brain regions activated by volume expansion. Male Sprague-Dawley rats were infused with isotonic saline equal to 10% of their body weight in 10 min followed by a maintenance infusion of 0.5 ml/min for 110 min. Control animals received 2-h infusions at 0.01 ml/min. Five minutes after the start of volume expansion, central venous pressure of expanded animals was significantly greater than control animals. The volume-expanded group exhibited significantly greater Fos activation (P < 0.05) in the area postrema, nucleus of the solitary tract, caudal ventrolateral medulla, paraventricular nucleus, supraoptic nucleus, and perinuclear zone of the supraoptic nucleus. Double labeling indicates that oxytocinergic neurons in the supraoptic nucleus are activated. Neurons in brain regions known to inhibit both sympathetic activity and vasopressin release show increased Fos expression following isotonic volume expansion.

THE CONTROL OF PLASMA VOLUME IS TIGHTLY REGULATED BY BOTH RENAL AND NEURAL MECHANISMS. CHRONIC ALTERATIONS IN PLASMA VOLUME OCCUR DURING PREGNANCY, CONGESTIVE HEART FAILURE (1), AND EXERCISE TRAINING (9); THEREFORE, THE CONTROL OF BLOOD VOLUME CAN HAVE IMPORTANT PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL IMPLICATIONS. RENAL RESPONSES TO VOLUME EXPANSION INCLUDE INCREASED GLOMERULAR FILTRATION RATE, DIURESIS, AND NATRIURESIS (14, 25, 41). ALTHOUGH SEVERAL STUDIES HAVE EXAMINED THE CENTRAL PATHWAYS THAT MODULATE CHANGES IN BLOOD PRESSURE, BLOOD OSMOLALITY, AND DECREASES IN BLOOD VOLUME, THE NEURAL MECHANISMS INVOLVED IN THE REGULATION OF AND ADAPTATION TO VOLUME CHANGES HAVE NOT BEEN DEFINED.

When an animal is volume expanded, central venous pressure (CVP) increases, stimulating cardiopulmonary receptors in the atria and ventricles and perhaps arterial baroreceptors in the aortic arch and carotid sinus. Volume expansion or stimulation of atrial receptors has been shown to inhibit vasopressin release (14, 27, 37, 41), decrease sympathetic nerve activity (17, 19, 39, 40), and attenuate drinking (26) in experimental models. Atrial distension stimulates secretion of atrial natriuretic peptide (ANP) from the atria leading to natriuresis (10, 16, 25). Thus it is possible that ANP may act at circumventricular organs to inhibit vasopressin release, reduce blood pressure, decrease drinking, and lead to natriuresis and diuresis via central pathways (20). In addition, ANP receptors have been found in circumventricular organs, brain regions lacking a blood-brain barrier that are able to detect changes in circulating factors (20).

Several studies have implicated different brain regions that may be important to the neural response to volume expansion. Bealer et al. (5) lesioned the anteroventrolateral third ventricular region (AV3V), an area vital to body fluid and cardiovascular regulation (23), and found that ablation of this region attenuated the natriuretic response to volume expansion. The involvement of the paravascular nuclei of the paraventricular hypothalamus (PVN) in isotonic volume expansion has also been examined. Lesions of the PVN have been shown to attenuate the decrease in renal sympathetic nerve discharge in response to volume expansion (17).

Fos, the protein product of the immediate-early gene c-fos, is expressed following synaptic activation and has been shown to be an effective tool to map regions of the central nervous system activated by various hemodynamic stimuli, including volume expansion (3, 34) and stimulation of atrial receptors (12). However, not all of the data in the literature are in agreement. Studies using Fos immunocytochemistry indicate that neuronal activity in the PVN is increased by atrial distension (12). Plasma expansion of conscious rabbits has been reported to increase the number of Fos-positive nuclei in the organum vasculosum laminae terminalis (OVLT), parvocellular PVN, nucleus of the solitary tract (NTS), and ventrolateral medulla, although Fos expression in the forebrain was dependent on the method of plasma expansion used (3).

Narvaez et al. (34) examined Fos immunoreactivity in urethane-anesthetized rats subjected to intravenous loads of PBS. They showed an increase in Fos expression in the NTS, caudal ventrolateral medulla (CVL), and rostral ventrolateral medulla (RVL). In contrast to the aforementioned studies, this group showed increased Fos in vasopressinergic neurons of the supraoptic nucleus of the hypothalamus (SON) (34). This latter finding contradicts the literature that demonstrates that vasopressin is inhibited by volume expansion. Yet, this discrepancy could be explained by the study’s use of anesthetized animals (34) because anesthetic has been shown to alter Fos expression (13).

The present study used Fos immunocytochemistry to study the central components of neural systems activated during isotonic volume expansion in unanesthetized, unrestrained rats. When the data were analyzed, particular attention was paid to areas of the central nervous system involved in the regulation of the
posterior pituitary hormones vasopressin and oxytocin and sympathetic outflow. We found increased Fos expression in brain regions associated with inhibition of sympathetic outflow, inhibition of vasopressin neurons, and activation of oxytocin neurons.

**Glossary**

- AP: Area postrema
- CVL: Caudal ventrolateral medulla
- DBB: Diagonal band of Broca
- NTS: Nucleus of the solitary tract
- OVLT: Organs vasculosum laminae terminalis
- PNZ: Perinuclear zone of the supraoptic nucleus
- PVN: Paraventricular nucleus
- RVL: Rostral ventrolateral medulla
- SFO: Subfornical organ
- SON: Supraoptic nucleus

**MATERIALS AND METHODS**

Surgery. All experiments were performed on male Sprague-Dawley rats (250–350 g body wt) purchased from Harlan (Indianapolis, IN). At least 1 wk before the experiment, rats were acclimated to a 12:12-h light-dark cycle. For catheterization surgery, animals were anesthetized with pentobarbital sodium (60 mg/kg ip). Catheters made of PE-10 tubing fused to PE-50 tubing were inserted into the femoral artery and vein for recording of mean arterial pressure (MAP) and heart rate (HR) and for saline infusion, respectively. A third catheter (PE-50 tubing) was inserted into the jugular vein and advanced to the right atria for recording of CVP. The catheters were filled with a saturated sucrose solution containing 1,000 U/ml heparin and 150 µg/ml gentamicin until the time of the experiment, and all catheters were externalized at the nape of the neck. The rats were returned to their individual cages, where they were allowed to recover.

Volume expansion protocol. Rats were randomly divided into two experimental groups: volume expanded (n = 12) and control (n = 5). Two days after the catheterization surgery, the arterial and central venous lines were attached to force transducers to record MAP, HR, and CVP with the Atlantis data acquisition program (Lakeshore Technologies, Chicago, IL) and an IBM personal computer. Once the readings stabilized, baseline recordings were taken for 30 min before the beginning of the volume expansion protocol. Infusion pumps (model 975; Harvard, Holliston, MA) were used to deliver isotonic saline via the femoral venous catheter. Experimental animals were expanded with isotonic saline equal to 10% of their body weight in the first 10 min of the protocol. Volume expansion was maintained by a 0.5 ml/min infusion for 110 min so that animals were expanded for a total of 2 h. The magnitude of the stimulus was chosen so that maximal expansion would be achieved. Control animals received an infusion of isotonic saline for 2 h at a rate of 0.01 ml/min. After completion of the protocol, the rats were anesthetized with pentobarbital sodium (60 mg/kg ip) and perfused transcardially with 0.1 M PBS followed by 4% paraformaldehyde (4°C). The brains were removed and placed in a PBS solution containing 30% sucrose for 2 days.

Fos immunoreactivity. Brains were cut into 40-µm sections with a cryostat and collected in 0.1 M PBS. Sections were treated with 0.3% hydrogen peroxide in distilled water for 30 min at room temperature then rinsed for 30 min in 0.1 M PBS. Sections were then incubated for 2 h at room temperature in PBS diluent (3% normal horse serum (Sigma, St. Louis, MO) in 0.1 M PBS containing 0.25% Triton 100 (Sigma)). Sections were incubated in a sheep polyclonal anti-Fos antibody (Genosys Biotechnologies, The Woodlands, TX) diluted to 1:1,000 in PBS diluent for 2 days at 4°C. After two 30-min rinses in 0.1 M PBS, sections were incubated in a rabbit anti-sheep IgG (Vectorstain Kit; Vector, Burlingame, CA; diluted to 1:2,000 in PBS diluent) for 2 h at room temperature. The tissue was then reacted with an avidin-peroxidase conjugate (ABC-Vectastain kit, Vector) and PBS containing 0.04% 3,3'-diaminobenzidine hydrochloride and 0.025% nickel ammonium sulfate, and 0.025% cobalt. Sections were mounted on gel-coated slides, processed through a series of dehydrating alcohols followed by xylenes, and placed under a coverslip with Permount mounting medium.

Brain regions of interest were visualized using an Olympus microscope with a Dage charge-coupled device camera connected to an Apple Quadra 800 computer with National Institutes of Health Image software and identified using the stereotaxic atlas of Paxinos and Watson (36). Sections were visually examined using light microscopy, and at least two representative sections of each area were collected from each animal for statistical analysis. Care was taken to ensure that the sections used were taken from the same rostral-caudal plane in each brain. The number of Fos-positive cells was visually quantified by participants blind to the experimental conditions. The counts for each area were averaged for each animal.

Double-labeling with oxytocin antibody. The presence of Fos staining in the SON and the perinuclear zone of the SON (PNZ) prompted an additional, double-staining experiment for Fos and oxytocin to determine the phenotype of magnocellular neurons within the SON of the hypothalamus activated by isotonic volume expansion. For this staining protocol, a rabbit polyclonal Fos antibody (Oncogene, Cambridge, MA; diluted to 1:30,000 in PBS diluent) was used. After reaction in 0.04% 3,3'-diaminobenzidine hydrochloride and nickel, the tissue was incubated in a mouse oxytocin antibody supplied by Dr. A. J. Silverman (diluted to 1:1,000 in PBS diluent) for 5 days. Sections were incubated in anti-mouse IgG (Vector, 1:200 in PBS diluent) for 2 h. After two 30-min rinses in 0.1 M PBS, sections were reacted with an avidin-peroxidase conjugate (ABC-Vectastain kit, Vector) and PBS containing 0.04% 3,3'-diaminobenzidine hydrochloride without nickel. This protocol produced a black nuclear stain for Fos and a light brown cytosolic stain for oxytocin.

Statistics. All values are expressed as means ± SE. MAP, HR, and CVP data were analyzed using ANOVA followed by Dunnett's test to determine differences among the groups. Histological data were analyzed by Newman-Keuls test to determine differences among the groups. Data were deemed significant when P < 0.05.

**RESULTS**

CVP was significantly increased (P < 0.05) at 5 and 10 min of isotonic acute volume expansion compared with control values. As shown in Table 1, isotonic volume expansion did not significantly change MAP or HR.

Table 2 summarizes increases in Fos counts in several other brain areas associated with central control of body fluid homeostasis. Although Fos numbers tended to be increased in the diagonal band of Broca (DBB), OVLT, RVL, and subfornical organ (SFO) of volume-expanded rats, these differences were not statistically significant.
Table 1. MAP, HR, and CVP

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<tr>
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<td>Expanded</td>
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<tr>
<td>Baseline</td>
<td>115.1 ± 1.9</td>
<td>109 ± 1.8</td>
<td>364.7 ± 10.8</td>
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<td>5 min</td>
<td>119.0 ± 4.3</td>
<td>111 ± 3.3</td>
<td>365.9 ± 14.9</td>
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<tr>
<td>10 min</td>
<td>115.7 ± 4.6</td>
<td>112 ± 3.1</td>
<td>364.7 ± 11.8</td>
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<tr>
<td>30 min</td>
<td>114.4 ± 4.6</td>
<td>112 ± 2.4</td>
<td>351.8 ± 10.5</td>
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Values given for mean arterial pressure (MAP), heart rate (HR), and central venous pressure (CVP) are means ± SE; n = 12 volume-expanded rats, and n = 5 control rats. *P < 0.05 when compared with nonexpanded group.

In the dorsomedial medulla, isotonic volume expansion induced a significant increase in Fos-positive nuclei in the NTS (Table 2). Fos expression was distributed dorsally and bilaterally throughout the NTS (Fig. 1). The region of Fos activation correlates to the areas of the NTS that receive vagal afferent projections (24). Fos counts in the NTS were taken from rostral obex through area postrema (AP) (36) and averaged to yield the numbers shown in Table 2.

Volume expansion induced a significant increase in Fos-positive nuclei in the AP compared with control conditions (Table 2). The counts for this region were averaged from throughout the entire nucleus (between 13.68 and 14.08 mm posterior to bregma). Representative sections are illustrated in Fig. 1.

We examined CVL from the level of the caudal AP (13.80 mm posterior to bregma) to the level of the median accessory nucleus of the medulla (14.60 mm posterior to bregma). Volume-expanded rats had significantly more Fos-positive cells (Table 2) ventral to the nucleus ambiguus throughout this region of the medulla compared with control rats (Fig. 1). This region of the CVL contains A1 noradrenergic neurons that project to the forebrain as well as CVL neurons that project to the RVL (11).

Volume-expanded rats had a significant increase in Fos in the medial and dorsal parvocellular regions of the PVN (Ref. 36; Table 2). No increase in Fos-positive cells was observed in the magnocellular divisions of the PVN. This indicates that parvocellular neurons project to the brain stem and the median eminence were activated during volume expansion (Fig. 2).

Fos activation was observed in the lateral hypothalamus dorsal and medial to the SON (Fig. 2). This region corresponds to the PNZ (22, 44). The Fos activation following isotonic volume expansion was significantly greater than the number of Fos-positive nuclei in control animals (Table 2).

In the volume-expanded animals, a significant increase in Fos-positive cells was observed in the SON (Table 2). As shown in Fig. 2, the Fos-positive cells were also restricted to the anterodorsal aspects of the SON. This part of the nucleus has been traditionally described as containing mostly oxytocin-releasing magnocellular neurons that project to the posterior pituitary (2).

The presence of Fos-positive nuclei in both the SON and the PNZ prompted further examination of the phenotype of magnocellular neurons in the SON activated by volume expansion by labeling for oxytocin. As shown in Fig. 3, the Fos-positive nuclei are mainly colocalized with the cytoplasmic staining of oxytocin neurons. This observation indicates that the significant increase in Fos in the SON was due to an activation of oxytocin neurons. Examination of double labeling in the PVN did not illustrate this overwhelming colocalization of Fos-positive nuclei with oxytocin cells.

**DISCUSSION**

The present study used Fos immunocytochemistry to identify brain regions that respond to an acute increase in volume. A previous study by Hines and Mifflin (18) suggests that a change in right atrial pressure up to 60% may be required to increase the activity of NTS neurons. Thus the 60% increase in CVP produced by the volume expansion protocol used in the present study maximized the probability that a robust increase in Fos expression would be visualized in the central nervous system. In the present experiment, isotonic volume expansion significantly increased Fos immunoreactivity in the AP, CVL, NTS, PNZ, PVN, and SON. Previous studies indicate that the Fos technique is a useful index of synaptic activation of central neurons. However, it should be noted that this method has several limitations (13). First, because Fos can be activated by a wide variety of stimuli, appropriate controls must be used (13). Care was taken to ensure that animals did not experience undue stress during the protocol that might result in nonspecific Fos activation.
tion. Second, the failure to observe Fos activation in a neuron does not mean that the stimulus has not changed the activity of the neuron. For example, if the neuron is inhibited, there would be no change in Fos expression (13). The relatively low numbers of Fos-positive cells in control animals indicate that volume expansion can induce Fos expression in particular brain regions. The patterns of Fos activation following volume expansion occur in regions associated with inhibition of both sympathetic activity and vasopressin release.

The volume expansion-induced Fos activation observed in the NTS is consistent with previous studies (3, 34). Arterial baroreceptor and cardiopulmonary receptors, which may be stimulated by volume expansion, innervate the NTS through the IX and X cranial nerves (11). Whether the response to volume expansion, as quantified by changes in renal hemodynamics, is due to arterial baroreceptor (8) or cardiopulmonary afferents (4) is still controversial. Several studies have shown that blood pressure elevations increase Fos in the NTS, presumably from baroreceptor activation (15, 29, 30, 32, 33, 38). Stretch of cardiac receptors results in excitation of NTS neurons (19).

In the baroreflex pathway, the NTS sends excitatory projections to the CVL (11), another medullary region that exhibited a significant increase in Fos activation following volume expansion. Within the CVL, a population of depressor neurons exists that inhibits sympathetic motor outflow via GABAergic inhibitory projections to the RVL (11). Neurons in the RVL in turn project to sympathetic premotoneurons (11, 43). We saw no volume expansion-induced increase in Fos in the RVL, which is consistent with inhibition of neuronal activity in that region. However, as previously mentioned, Fos cannot be used as a marker for inhibition of neuronal activity.

The AP was the only circumventricular organ with a significant increase in Fos activity following volume expansion. As previously stated, volume expansion and atrial distension stimulate ANP release (10, 16, 25). Circumventricular organs, specifically the AP, SFO, and OVLT, have been shown to have ANP binding sites (20), so ANP could activate neurons in these areas that
have ANP receptors. However, although Fos numbers increased slightly in both the SFO and OVLT after volume expansion, the change was significant only in the AP. Fos expression in the AP may have been stimulated by ANP; however, the AP also receives afferent projections from the vagus (24). Therefore, vagal input from cardiopulmonary receptors may also be responsible for the increased Fos expression in the AP. The AP has multiple projections to brain regions important for cardiovascular regulation such as the NTS, lateral parabrachial nucleus, nucleus ambiguus, and ventromedullary catecholaminergic regions (42). Thus activation of the AP by volume expansion through ANP and/or vagal afferents may influence cardiovascular function through any of the aforementioned pathways.

We saw an increase in Fos-positive neurons in the parvocellular PVN following isotonic volume expansion. This finding confirms previous Fos studies that reported increased Fos-containing neurons in the PVN of rats subjected to right atrial stretch (12) or infusion of plasma volume expanders (3). The data are also consistent with the findings of Haselton et al. (17), who demonstrated that parvocellular PVN is essential for the inhibition of renal sympathetic nerve activity associated with volume expansion. Our Fos and double-labeling experiments do not indicate that oxytocin cells within the PVN were activated by volume expansion. Thus the apparent activation of the PVN may reflect the activity of neurons projecting to the brain stem that modulate autonomic function (6, 7) or it may indicate the release of another hypothalamic hormone.

We also observed a significant increase of Fos in the PNZ of the SON. Located in the lateral hypothalamus, the PNZ is rich in GABAergic neurons, which anatomic and electrophysiological studies have proven project to the SON (22, 44). Electrophysiological experiments have shown that excitotoxic lesions of the PNZ attenuate the inhibition of phasic, vasopressinergic SON neurons following baroreceptor activation (35). Therefore it is possible that information from arterial baroreceptors or cardiopulmonary receptors activated by volume expansion may relay afferent information from the periphery to the PNZ and inhibit the release of vasopres-
sin from the SON. Additional experiments are needed to determine whether the PNZ neurons activated by volume expansion innervate the SON and are necessary for inhibition of vasopressin release via volume expansion. Although the DBB has been suggested to be the source of input for baroreceptor information to the PNZ (11), the present study indicates that the DBB may not be involved in the response to volume expansion because Fos activity does not increase appreciably in this area. Fos in the DBB has been shown to increase following electrical stimulation of the aortic depressor nerve (32). Still, the absence of increased Fos in the DBB does not prove that the DBB was not activated by volume expansion. Additional experiments will be needed to determine whether the DBB is involved in the inhibition of vasopressinergic SON neurons by volume expansion.

The double-labeling study indicates that oxytocinergic cells of the SON are activated by isotonic volume expansion, data that contradict the study of Narvaez et al. (34). It has been established that stress induces oxytocin secretion (28), so the possibility that oxytocin activation is due to stress cannot be eliminated. In an attempt to reduce stress associated with the experimental protocol, all animals in this study were acclimated to their surroundings before the protocol began, and the animals remained in their home cages for the duration of the experimental procedures. Often the animals slept during the infusions, an observation that could indicate that stress was kept at a minimal level. Data suggesting that the PVN, not the SON, is responsible for stress-induced oxytocin release (21) further support the contention that volume expansion-induced activation of oxytocin neurons in the SON is a specific response to volume expansion rather than to stress.

Haanwinckel et al. (16) have shown that blood volume expansion of conscious rats with isotonic saline results in a significant increase in plasma oxytocin levels. This group has proposed that volume expansion activates an ascending catecholaminergic pathway involving serotoninergic neurons, the AV3V, neuronal ANP, and oxytocinergic neurons (31). They propose that oxytocin secreted in response to volume expansion is responsible for the natriuresis of volume expansion via a peripheral ANP mechanism (16). In support of this hypothesis, they have demonstrated that intraperitoneal administration of oxytocin significantly increases plasma ANP (16). When oxytocin was applied to the isolated rat atria, ANP secretion was increased. This effect could be blocked by an oxytocin antagonist (31). Oxytocin has been shown to act directly on the kidney to elicit natriuresis (45), which is consistent with oxytocin activation following volume expansion. Therefore, oxytocin may stimulate natriuresis and subsequent diuresis in an attempt to regain volume balance.

This study presents information concerning brain regions activated by volume expansion. The Fos technique indicates that regions of the central nervous system that are involved in the inhibition of sympathetic outflow and vasopressin release and stimulation of oxytocin release are activated by volume expansion. Our data also suggest that neural systems related to arterial and cardiopulmonary baroreceptors and the AP, a hindbrain circumventricular organ, are activated by volume expansion. Given the magnitude of the stimulus used for volume expansion, several neural and humoral signals may mediate the increased Fos expression observed in these regions. The afferent systems responsible for this pattern of central activation and how these systems may be altered by physiological or pathophysiological states remain to be defined.

**Perspectives**

The control of body fluid regulation and vasopressin release is a complex system that is not completely understood. Nonosmotic stimulation of vasopressin release and cardiopulmonary and arterial baroreceptor regulation of sympathetic outflow have been suggested to be important in several altered physiological states (1, 9). Pregnancy and cardiac failure cause prolonged volume expansion and decreased plasma osmolality, which would be expected to inhibit vasopressin release (1). However, vasopressin plasma levels are not reduced in these states; they are either normal or elevated. Because nonosmotic vasopressin release occurs during the aforementioned states of volume expansion, when vasopressin would normally be inhibited, adaptation of the system must be occurring. During pregnancy, it is possible that the central pathways controlling plasma volume are attenuated or reset by reproductive hormones, the levels of which are increased during gestation, so that the expanded volume can be maintained. Fos activation of the PVN following atrial stretch is attenuated in pregnant rats compared with virgin rats (12). There is also a blunted response of renal sympathetic nerve activity, HR, and MAP in pregnant animals when right atrial pressure is increased, suggesting that the cardiopulmonary reflex is attenuated by pregnancy (18). As of now, there is no evidence to indicate either at which point the central pathway may be blunted or what is responsible for its downregulation. Hines and Mifflin (18) suggest that atrial receptor function is altered by pregnancy. Data from the present study indicate that several areas in the central nervous system are activated during volume expansion, some or all of which may be modulated by reproductive hormones. The central pathways that control plasma volume must be defined before alteration of the pathways by pregnancy, cardiac failure, or other pathophysiological states can be understood.

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