Distribution of Fos immunoreactivity in rat brain after sodium consumption induced by peritoneal dialysis

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Franchini, Lucia F., and Laura Vivas. Distribution of Fos immunoreactivity in rat brain after sodium consumption induced by peritoneal dialysis. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R1180–R1187, 1999.—Fos immunoreactivity was used to map the neuronal population groups activated after sodium ingestion induced by peritoneal dialysis (PD) in rats. Oxytocin immunoreactivity in combination with Fos immunoreactivity was also analyzed to evaluate whether the oxytocinergic neurons of the paraventricular nucleus of the hypothalamus (PVN) are activated during the satiety process of sodium appetite. Sodium ingestion stimulated by PD produced Fos immunoreactivity within defined cells groups of the lamina terminalis and hindbrain areas such as the nucleus of the solitary tract, area postrema, and lateral parabrachial nucleus. On the other hand, particular parvocellular and magnocellular oxytocinergic subdivisions of the PVN and supraoptic nucleus were double labeled after PD-induced sodium consumption. Approximately 27 and 2.1%, respectively, of the activated dorsomedial cap and parvocellular posterior subnuclei of the PVN, which project to the hindbrain, were oxytocinergic. Our data indicate that specific neuronal groups are activated during the satiety process of sodium appetite, suggesting they may form a circuit subserving sodium balance regulation. They also support a functional role for the oxytocinergic neurons in this circuit.

SODIUM APPETITE IS A homeostatic behavior resulting from a complex interaction of inhibitory and excitatory hormonal and neural signals. Numerous previous studies have indicated that the circumventricular organs (CVOs) of the lamina terminalis (the organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO)) play key functions as humoral receptive areas in the arousal of salt appetite through their angiotensin- and osmosodium-sensitive neurons (18, 40, 41). On the other hand, the area postrema (AP), the nucleus of the solitary tract (NTS), and the lateral parabrachial nucleus (LPBN) have been implicated in the central integration of the visceral and somatic sensory inputs related to the satiation of sodium appetite (6, 7). The afferent projections in the vagal and glossopharyngeal nerves conveying gustatory, baroreceptor, gastric, and hepatic osmosodium receptor influences terminate in these hindbrain areas. Thus, in this regard, the neural regulation of sodium homeostasis can be seen as a neuroendocrine reflex with two main afferent limbs located in the CVOs of the lamina terminalis and within the hindbrain structures. Moreover, whereas the stimulating hormonal signals are mainly comprised of increased ANG II levels acting on receptive cells located along the lamina terminalis (LT), the inhibitory system implicates, in part, central oxytocin (OT) pathways, projecting from the paraventricular nucleus of the hypothalamus (PVN) (31, 32). Examination of several models of sodium appetite in the rat has revealed that saline ingestion is manifest only when circulating levels of OT are suppressed, whereas salt consumption is inhibited when OT release is stimulated (30, 31). Systemic OT administration produces renal sodium excretion (39); however, it does not inhibit salt appetite in sodium-deficient rats (30). On the contrary, central OT administration may inhibit angiotensinergic and polyethylene glycol-induced sodium appetite (3, 4). Therefore, the oxytocinergic magnocellular supraoptic nucleus (SON) and PVN projections are involved in renal sodium excretion regulation through neurohypophysial OT release, whereas the oxytocinergic parvocellular pathways, projecting from the PVN to the NTS, AP, and LPBN, may be implicated in sodium appetite control. Several lines of evidence suggest that these hindbrain nuclei and the bidirectional connections with the PVN (2, 20, 28, 34, 35) may form part of the inhibitory circuit of sodium appetite (7, 21, 32).

The aims of the present work were to evaluate whether the oxytocinergic parvocellular and magnocellular neurons of the PVN are turned on during the satiety process of sodium appetite. We also attempted to estimate the extent to which other areas that are known to participate in the central regulatory circuit of sodium appetite were activated. For that purpose, we analyzed the number of Fos-immunoreactive (Fos-ir) nuclei within the different subdivisions of the PVN, SON, NTS, AP, LPBN, SFO, OVLT, and the median preoptic nucleus (MnPO) after sodium ingestion induced by peritoneal dialysis (PD). In addition, we utilized OT immunohistochemical staining in combination with nuclear Fos-ir to provide a quantitative estimation of the oxytocinergic-activated neurons. This approach was selected because the presence of Fos, the nuclear protein product of the immediate-early gene c-fos, has been used as a marker of neural activation in response to a wide variety of stimuli, including various natrioregic procedures (17, 19, 24, 41). Therefore, in the present study, the analysis of c-fos expression pattern following sodium depletion-induced sodium ingestion potentially identifies the individual cellular components of functional neural networks mediating this behavioral phase.
MATERIALS AND METHODS

The experiments used 76 adult male albino rats weighing 250–320 g. The animals were housed singly in hanging wire cages for at least 1 wk before experiments and had free access to food, water, and 2% NaCl. Room lights were on for 14 h/day, and temperature was controlled at 23°C.

Sodium appetite was stimulated by an acute body sodium depletion induced by PD. The technique, described previously (9), consisted of an intraperitoneal injection of a 5% glucose solution warmed at 37°C, in a volume equivalent to 10% of rat body weight. After 1 h, the ascitic fluid was recovered by inserting a needle into the peritoneal cavity. The amount of NaCl withdrawn by this method in the dialyzed or experimental group (experimental) was 0.84 ± 0.02 meq/100 g body wt (mean ± SE, n = 18). In control sham-depleted rats (control sham), no injection was given but the needle was inserted into the peritoneal cavity. Dialyzed and control rats were caged individually without food and with distilled water as the only drink. Twenty-four hours after PD, an intake test of 2.0% NaCl solution was given, and the cumulative volume drunk was measured at 15, 30, and 60 min. The behavioral data were analyzed by one-way ANOVA, and a least-significant difference (LSD) test was used as follow-up. Ninety minutes after the termination of the intake test, the animals were perfused for immunohistochemical detection of Fos and OT.

To analyze comparatively the pattern of Fos-ir seen before and after the sodium consumption, we simultaneously ran a separate group of control animals, sodium depleted but without access to salt (control depleted).

Immunohistochemistry. After the completion of the different experimental procedures, the three groups of rats were anesthetized with thiopentone (100 mg/kg ip) and perfused transcardially with ~200 ml normal saline followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.2). The brains were removed, fixed in the same solution overnight, and then stored at 4°C in PB containing 30% sucrose. Coronal sections were cut into two series of 40 µm,

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Na, mmol/l</th>
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<tbody>
<tr>
<td>Baseline</td>
<td>11</td>
<td>131.2 ± 2.2</td>
</tr>
<tr>
<td>Control depleted</td>
<td>8</td>
<td>130.2 ± 0.7</td>
</tr>
<tr>
<td>Control sham without access to salt</td>
<td>8</td>
<td>131.6 ± 1.5</td>
</tr>
<tr>
<td>Experimental 30 min</td>
<td>7</td>
<td>134.7 ± 1.5</td>
</tr>
<tr>
<td>Control sham 30 min</td>
<td>8</td>
<td>129.5 ± 2.7</td>
</tr>
<tr>
<td>Experimental 150 min</td>
<td>12</td>
<td>130.8 ± 4.7</td>
</tr>
<tr>
<td>Control sham 150 min</td>
<td>7</td>
<td>130.3 ± 4.9</td>
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Values are means ± SE; n = no. of rats.

Table 1. Plasma sodium concentrations measured in different groups of sodium-depleted and sham-depleted rats with or without access to 2% NaCl solution

Fig. 1. Photomicrographs of coronal sections of nucleus of the solitary tract (NTS; A–D) and area postrema (AP; E and F) showing Fos-immunoreactive (Fos-ir) neurons after sodium ingestion in experimental (A, C, E) and control sham rats (B, D, F). Bars indicate 200 µm.
using a freezing microtome. Immediately before immunostaining, sections were placed in a mixture of 3% H$_2$O$_2$ and 10% methanol until oxygen bubbles ceased appearing and then were incubated in 10% normal horse serum (NHS) in PB for 1 h to block sites of nonspecific binding of serum products. Fos-ir was detected using the standard avidin-biotin peroxidase protocol. The free-floating sections were incubated overnight at room temperature in an antibody, raised in rabbits, against a synthetic 14-amino acid sequence corresponding to residues 4–17 of human Fos (Ab-5, batch no. 60950101; Oncogene Science, Manhasset, NY) diluted 1:30,000 in a solution of PB containing 2% NHS and 0.3% Triton X-100. After washes in PB, sections were subsequently incubated in biotin-labeled anti-rabbit immunoglobulin and ExtrAvidin peroxidase complex (Sigma, 1:20 dilution in 2% NHS-PB). Cytoplasmic OT immunoreactivity was detected using diaminobenzidine hydrochloride (DAB, Sigma) intensified with 0.5% cobalt chloride and 0.5% nickel ammonium sulfate. This method produces a blue-black nuclear reaction product. One series of c-fos-labeled sections was processed subsequently for immunocytochemical localization of OT. Sections were incubated for 72 h at 4°C in polydonal rabbit anti-OT antibody (Peninsula Laboratories). After incubation, sections were rinsed and incubated in the appropriate biotinylated secondary antiserum and ExtrAvidin peroxidase complex (Sigma, 1:20 dilution in 2% NHS-PB). Cytoplasmic OT immunoreactivity was detected with unintensified DAB to produce a brown reaction product. Finally, the free-floating sections were mounted on gelatinized slides, air-dried overnight, dehydrated, cleared in xylene, and placed under a coverslip with DPX. For one series of sections, an immunofluorescence procedure was used to stain for OT immunoreactivity to verify the accuracy of the DAB procedure described above. These sections were first stained for Fos-ir using the same procedure as above, but without intensification (staining cells brown). The brain sections were then incubated for 72 h at 4°C with polydonal rabbit anti-OT antibody (1:200 dilution). After washes in 1% NHS-PB, the sections were incubated with FITC-labeled anti-rabbit antibody (1:20, ICN Biomedicals) for 1 h at room temperature. The sections were mounted onto slides subbed with gelatin-chrome, dried, and placed under a coverslip with bicarbonate-buffered glycerol (pH 8.2). Controls for the Fos-ir included placing sections in primary c-fos antibody that had been preadsorbed with an excess of the Fos peptide or processing sections without the primary antisera. No Fos-ir neurons were observed in either of these control sections.

Cytoarchitectural and quantitative analyses. Sections were microscopically examined using low-power magnification (×10), and, on the basis of prior observation of c-fos staining after distinct treatments, the following areas of the brain were chosen for quantification: PVN, SON, NTS, AP, LPBN, OVLT, SFO, and MnPO. Subnuclei of the OVLT, PVN, and SON were identified according to the Swanson atlas (33); the other brain structures were identified and delimited using the Paxinos and Watson atlas (25). Numbers of Fos-ir nuclear profiles were counted in sections of the OVLT, PVN, and SON subdivisions, corresponding to plates 18, 25, and 26, respectively, in the Swanson atlas; the NTS (medial division at two levels), plates 50 and 72; the LPBN, plate 74; the LEB (external division), plate 55; the MnPO, plate 20; and the SFO, plate 25, in accordance with the Paxinos and Watson atlas.

Fos-positive nuclei were quantified using a computerized system that included a Zeiss microscope equipped with a video camera attached to a contrast enhancement device. Video images were digitized and analyzed using Scion Image PC, based on NIH version 1997. Fos-ir cells in each section were counted by setting a size range for cellular nuclei (in pixels) and threshold level for staining intensity. Representative sections in control and experimental groups were acquired at exactly the same level, with the aid of the Photostyler image analysis program. Sections processed according to the immunofluorescence procedure were examined with a Zeiss microscope fitted with a fluorescence attachment. The counting procedure was done in four animals of every condition, and it was repeated at least twice on every section analyzed to ensure that the numbers of profiles obtained did not change significantly. The data, expressed as the mean ± SE, were subjected to a one-way ANOVA. Tukey’s test was used as follow-up to analyze differences between groups. Results were considered to be significantly different at P < 0.05. The data were not corrected for double counting or by stereological technique. However, because the objects we were counting (nuclei) did not change in size and section thickness did not vary between experimental and control groups, any systematic error should be identical for both groups. Hence, the results are meant to provide relative data on expression of Fos-ir but are not meant to be accurate estimates of absolute cell counts.

Electrolyte analyses. To analyze serum sodium concentrations in sham- and sodium-depleted rats without access to the salt-intake test, blood samples were taken 24 h after PD. On the other hand, in sham- and sodium-depleted rats with access to the intake test, the blood samples were taken 30 and 150 min after the beginning of the test. To determine baseline values, blood samples were taken from untreated animals.

The animals were bled by cardiac puncture under light anesthesia with ether. The blood was centrifuged, and the serum sodium concentration was determined by flame photometry (Hitachi 911, automatic analyzer). The data were subjected to a one-way ANOVA. Post hoc comparisons were made using least significant difference (LSD) tests, with significance levels set at P < 0.05.

**RESULTS**

Sodium intake test. Cumulative volume of 2% NaCl drunk during the intake test (1 h) was 3.8 ± 0.2 and
0.6 ± 0.2 ml/100 g body wt in experimental (n = 13) and control sham (n = 13) animals, respectively [F(1,18) = 143.2, P < 0.0001; ANOVA, LSD test].

Serum sodium concentration. Serum sodium concentration values are shown in Table 1. Sodium serum levels of experimental rats, measured 30 min after the salt-intake test initiation, increased slightly. However, serum sodium concentration did not differ significantly among any of the seven groups analyzed [F(6,51) = 0.225, P < 0.97; ANOVA].

Fos-ir in the NTS, AP, and LPBN. Sodium ingestion induced by PD produced a pattern of highly localized and intense Fos-ir nuclei in the AP and the NTS, particularly in the medial subnucleus of the NTS (mNTS, Figs. 1 and 2). The largest c-fos activation in the mNTS was observed at two levels: the medial level (bregma −13.24 mm) and at a more posterior level in the NTS adjacent to the AP (NTS-AP; bregma −13.68 mm). The number of Fos-ir neurons in the mNTS and NTS-AP of experimental animals was significantly increased (P < 0.0001) compared with control sham rats (Fig. 2) and control depleted rats without access to the intake test (P < 0.0001).

In the LPBN, sodium ingestion enhanced the expression of c-fos above levels found in both control groups of animals (control sham and control depleted). The num-
number of Fos-ir neurons found in the external subdivision of the LPBN (plate 55 in the Paxinos and Watson atlas) was significantly increased in the experimental group (P < 0.05) compared with the control sham and control depleted groups of rats (Fig. 2).

Fos-ir in the CVOs and the MnPO. The data from the control depleted animals without access to the salt intake test confirmed our previous results showing activation of the SFO and OVLT neurons, but not of the MnPO cells, following sodium depletion (Figs. 3 and 4) (41). Induced sodium ingestion produces a significant increase in Fos-ir within the ventral and dorsal parts of the MnPO compared with both control groups (control sham and control depleted). However, the pattern of Fos activation along the SFO and OVLT did not change significantly 90 min after sodium repletion compared with the control depleted group but increased significantly in relation to the sham-depleted rats (Fig. 4).

Fos-ir in the SON and PVN. C-fos expression after salt intake induced by sodium depletion was observed in many neurons within the PVN and SON. As expected, all magnocellular subdivisions of the PVN were activated, namely the anterior, medial, and lateral magnocellular groups (P < 0.001, Figs. 5 and 6). Nevertheless, particular parvocellular subdivisions of the PVN were activated. The dorsal parvocellular group or dorsomedial cap (DC) and the posterior parvocellular group (PVPO) showed a significant increase in the number of Fos-ir neurons when compared with the control groups (P < 0.001 and 0.01, respectively, Fig. 6). The medial, ventral, and anterior parvocellular groups did not show a significant difference in c-fos activation. The entire SON showed a highly significant level of c-fos expression after sodium ingestion compared with both control groups of animals (P < 0.0001, Figs. 3 and 6).

Double immunolabeling in the SON and PVN. The pattern of double labeling cells in the PVN after sodium ingestion is shown in Fig. 5, E and F. Most of the oxytocinergic Fos-ir neurons in this nucleus were localized in the medial magnocellular subdivision; ~26 ± 5.62% of the activated neurons were oxytocinergic. However, two parvocellular subdivisions showed double staining cells: the DC and the PVPO. An important number of neurons of the DC subnuclei were both oxytocin and Fos positive (27.8 ± 9.7%); however, in the PVPO subdivision, there was a lower percentage of double-labeling neurons (2.1 ± 0.2%), in contrast to the high level of c-fos activation seen in this area (Fig. 6).

The double-immunolabeling experiments indicated that in the SON, the oxytocinergic neurons are placed dorsally, and presumably the ventrally located Fos-ir neurons are vasopressinergic.

DISCUSSION

Our results reveal that sodium ingestion stimulated by PD activates c-fos expression in different neuronal population aggregates along the brain stem level (NTS, AP, and LPBN) and the forebrain level (PVN, SON, OVLT, SFO, and MnPO), indicating they may form a central circuit involved in the reestablishment of sodium balance by regulating sodium intake and excretion.

A restricted population of neurons in the AP, the medial level of the NTS (mNTS and NTS adjacent to AP), and the LPBN (external subdivision) express c-fos following induced sodium consumption. These data are consistent with previous lesion studies showing that ablation of the AP and immediately adjacent medial NTS markedly enhanced the ad libitum intake of saline solutions (need-free sodium appetite) and increased stimulated sodium appetite (6, 7). In addition, recent studies indicate that pre-treatment with bilateral injections of serotoninergic receptor antagonist into the LPBN significantly increase salt intake induced by either intracerebroventricular ANG II or sodium depletion (21). Collectively, these results have lead to the hypothesis that the NTS, the AP, and the LPBN are components of a hindbrain inhibitory circuit modulating sodium and fluid ingestion.

Of the many hindbrain areas that potentially mediate the inhibition of sodium appetite behavior, it is likely that the NTS plays a central role. As the major relay site for taste, the NTS may exert control over both food and fluid ingestion through its neural projections to hindbrain somatic and autonomic motor nuclei, including the ventrolateral medulla, the vagal complex, and the LPBN (38). Thus induced expression of c-fos in the medial NTS following stimulated sodium ingestion could be correlated with the inhibition of sodium intake. The NTS receives viscero sensory inputs from

![Graph showing number of Fos-ir neurons](http://ajpregu.physiology.org/ by 10.220331 on November 7, 2017)
volume receptors in the atriovenous junction, the stomach, and other abdominal viscera, from baroreceptors via the carotid sinus, and from hepatic osmoreceptors via hepatic branch of the vagus. (5, 23, 38). Hence, it is plausible that, in our model, gastric mechanoreceptor activation, hepatic osmosodium receptor stimulation, and/or cholecystokinin release contribute to the satiety signal and mirror c-fos expression seen in these hindbrain structures. However, the brain stem neuronal activation observed in our model might also be at least partially due to the activation of descending projections from the forebrain, modulating in this way the autonomic responses associated with saline ingestion.

In this regard, the AP, the NTS, and the LPBN receive direct and relayed projections from the PVN, the central amygdala nucleus, the SFO, and the MnPO (20, 22, 26, 34, 37). These brain areas are all known to be involved in sodium appetite and body fluid balance (12).

The present study confirms our previous results showing sodium depletion-induced production of Fos in cells of the SFO and OVLT (41) and shows that there is a similar number and spatial distribution of Fos-ir cells in these nuclei 90 min after sodium ingestion by sodium-depleted animals. We interpret these results to suggest that the depletion-induced elevation of Fos activity in the SFO and the OVLT might begin to decline 150–210 min after the NaCl intake test, as has been indicated by earlier reports (17, 41). In this regard, a possible explanation of the delay in the CVOs' deactivation process after sodium repletion can be associated with the gradual decrease in the circulating ANG II levels until they reach the baseline values (36). On the other hand, PD-induced sodium consumption stimulated the activation of Fos in the MnPO (Figs. 3 and 4). This result is consistent with lesion studies showing that ventral MnPO damage elicited hyperdipsia (13) and enhanced mineralocorticoid-induced sodium appetite (11).

Magnocellular and parvocellular OT neuronal activation observed in our work supports previous evidence...
that a direct correlation exists between sodium appetite inhibition and increased levels of OT in plasma (30, 31). These results also support the hypothesis that parvocellular oxytocinergic neurons projecting centrally from the PVN may subserve the satiation of sodium appetite (31, 32). It is of particular interest to note that specific parvocellular subnuclei are among those activated to express c-fos after sodium ingestion stimulated by PD.

The parvocellular division neurons project to different places within the brain; however, the specific parvocellular subdivisions of the PVN that were activated (the DC and the PVPO) project to the brain stem and spinal cord (2, 20, 28, 34, 35). The parvocellular projections to the brain stem mainly comprise the PVPO neurons and impinge on the NTS, the dorsal motor nucleus of the vagus, and the AP. As mentioned earlier, these regions have key roles on ingestive behavior and associated autonomic functions and may constitute specific components of the inhibitory control circuit of sodium appetite (3, 4, 31, 32). However, the low number of oxytocinergic activated neurons within the PVPO subdivision suggests that additional neuropeptides are contained in this pathway (28, 35). At least 88% of the DC neurons project to the spinal cord (27), and ~15% of these neurons send collaterals to the NTS and the motor nuclei of the vagus (dorsal vagal complex) (34). The functional significance of the oxytocinergic projections to the spinal cord is not well known; however, it has been suggested that this oxytocinergic pathway could play an important role in the release of atrial natriuretic peptide (ANP) from the atria (15). Recent experiments show that intraperitoneal injection of OT increases plasma ANP and urinary sodium excretion (16). Moreover, it is also well documented that when ANP or OT are centrally administered to sodium-depleted rats salt intake is inhibited (1, 10, 32).

In addition, the present work showed Fos-ir cells nuclei in many neurons within the magnocellular PVN and SON subnuclei. As expected, all magnocellular subdivisions of the PVN and the SON projecting to the neurohypophysis were activated, including ones that were immunoreactive for OT. Thus the increased pituitary secretion of OT, and presumably vasopressin, can be reasonably accounted for by the demonstrated activation of these forebrain regions. These findings support previous evidence indicating that both OT and arginine vasopressin, systemically administered, induce natriuresis (8, 39). The OT- and vasopressin-induced natriuretic effects can be explained either by their direct action on specific receptors located within the kidney tubular cells (29) or by their action on the atria, which evokes ANP release and mediates natriuresis (16).

**Perspectives**

The present work reveals the specific forebrain (PVN, SON, OVLT, SFO, and MnPO) and hindbrain (AP, NTS, and LPBN) neuronal groups that are activated during the satiety process of sodium appetite. Together, these structures may form a central circuit involved in the regulation of salt appetite. This circuit would contain sensory regions (AP, OVLT, SFO) with chemosensory and mechanoreceptors detecting humoral and neural signals conveying information about body fluid status as well as central integration sites (MnPO, PVN, NTS, and LPBN) of these visceral and somatic sensory inputs to modulate the appropriate behavioral and associated autonomic responses. The present findings also support a likely functional role for the parvocellular oxytocinergic neurons in this circuit, subserving the satiation of sodium appetite. The activation of magnocellular oxytocinergic neurons is consistent with a stimulated renal sodium excretion, although this needs further studies to be elucidated. Additionally, taking into account the overall activation of nonoxytocinergic neurons within the SON and PVN, this work implicates other neuropeptides located in these areas, such as ANP, ANG II, and vasopressin, that may participate in this regulatory behavioral response.

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