Oxytocinergic and serotonergic systems involvement in sodium intake regulation: satiety or hypertonicity markers?

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Godino A, De Luca LA Jr, Antunes-Rodrigues J, Vivas L. Oxytocinergic and serotonergic systems involvement in sodium intake regulation: satiety or hypertonicity markers? Am J Physiol Regul Integr Comp Physiol 293: R1027–R1036, 2007. First published June 13, 2007; doi:10.1152/ajpregu.00078.2007.—Previous studies demonstrated the inhibitory participation of serotonergic (5-HT) and oxytocinergic (OT) neurons on sodium appetite induced by peritoneal dialysis (PD) in rats. The activity of 5-HT neurons increases after PD-induced 2% NaCl intake and decreases after sodium depletion; however, the activity of the OT neurons appears only after PD-induced 2% NaCl intake. To discriminate whether the differential activations of the 5-HT and OT neurons in this model are a consequence of the sodium satiation process or are the result of stimulation caused by the entry to the body of a hypertonic sodium solution during sodium access, we analyzed the number of Fos-5-HT- and Fos-OT-immunoreactive neurons in the dorsal raphe nucleus and the paraventricular nucleus of the hypothalamus-supraoptic nucleus, respectively, after isotonic vs. hypertonic NaCl intake induced by PD. We also studied the OT plasma levels after PD-induced isotonic or hypertonic NaCl intake. Sodium intake induced by PD significantly increased the number of Fos-5-HT cells, independently of the concentration of NaCl consumed. In contrast, the number of Fos-OT neurons increased after hypertonic NaCl intake, in both depleted and nondepleted animals. The OT plasma levels significantly increased only in the PD-induced 2% NaCl intake group in relation to others, showing a synergic effect of both factors. In summary, 5-HT neurons were activated after body sodium status was reestablished, suggesting that this system is activated under conditions of satiety. In terms of the OT system, both OT neural activity and OT plasma levels were increased by the entry of hypertonic NaCl solution during sodium consumption, suggesting that this system is involved in the processing of hyposmotic signals.

Moreover, each neuronal group may also have its own role within inhibitory systems. For example, sodium depletion and subsequent hypertonic NaCl intake, respectively, decrease and increase Fos expression in dorsal raphe nucleus (DRN) 5-HT neurons (13), but only induced hypertonic NaCl intake is capable of altering OT activity. Both Fos expression and oxytocin synthesis in supraoptic and paraventricular neurons increased after peritoneal dialysis (PD)-induced-2% NaCl intake (PD-2%) (14, 15).

Sodium appetite is typically represented by the avid ingestion of hypertonic NaCl, but the amount of sodium obtained as hypertonic NaCl is also ingested as isotonic NaCl because the rat adjusts the volume ingested according to the sodium concentration in solution (60). Thus recent evidence suggests that each solution (isotonic and hypertonic NaCl) has a different way to regulate the termination of sodium appetite, involving one or two presystemic factors, one related to the volume of ingested fluid (i.e., gastrointestinal distension) and one related to its concentration (i.e., increased osmolality of fluid in the small intestine and adjacent visceral tissue) (51).

Assuming that satiety of sodium appetite is finally reached regardless of the mechanism involved, one may predict that either isotonic or hypertonic NaCl intake activates OT and 5-HT neurons in sodium-depleted rats.

Because of the differences observed in the neural activity of 5-HT and OT cells in the PD model, and to know whether this activity is a consequence of the sodium satiation process or the result of stimulation caused by the entry of a hypertonic sodium solution into the body during sodium access, we analyzed the number of Fos-5-HT- and Fos-OT-immunoreactive neurons in the DRN and the paraventricular nucleus of the hypothalamus-supraoptic nucleus (PVN-SON), respectively, after isotonic vs. hypertonic NaCl intake induced by PD. We also studied the neural activity of other brain nuclei previously analyzed in this paradigm, since OT plasma release in other models has been associated with hypernatremic, hyposmolalic, and hypovolemic stimulation (20, 25, 52, 55).

MATERIALS AND METHODS

The experiments used 64 adult Wistar-derived male rats, born and reared in the breeding colony at the Instituto Ferreyra (Córdoba, Argentina). Animals weighed 250–300 g and were housed singly in hanging wire cages for at least 3 days before the experiments began.

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Animals had free access to food, water, and 2% or 0.9% NaCl solution, according to the experimental group. Room lights were on for 12 h/day, and temperature was controlled at 23°C.

All experimental procedures were approved and carried out in accordance with the guidelines of the Ferreyra Institute Ethical Committee for the use and care of laboratory animals.

**Sodium Intake Induced by PD**

Sodium appetite was stimulated by acute body sodium depletion induced by PD. This technique, described previously (9), consisted of an intraperitoneal injection of 5% glucose solution warmed to 37°C in a volume equivalent to 10% of rat body weight. After 1 h, an equal volume of ascitic fluid was recovered from a needle inserted into the peritoneal cavity; the recovered sample was then centrifuged and stored at −20°C to measure sodium concentration. In control sham-depleted (CD) rats, no injection was given, but the needle was inserted into the peritoneal cavity. PD and CD rats were housed individually in metabolic cages without food and with distilled water as the only drink. Twenty-four hours later, urine samples were collected and approximately half of each of these two (PD and CD) groups was given access to the 2% NaCl intake test and the other half of each group was given access to the 0.9% NaCl intake test. The cumulative volume ingested was measured at 15, 30, and 60 min. The groups were designated as PD and CD with access to 2% NaCl (PD-2% and CD-2%, respectively) and PD and CD with access to 0.9% NaCl (PD-0.9% and CD-0.9%, respectively).

**Sodium Balance: Electrolyte Assays**

To analyze the ascitic fluid and urine sodium concentration in PD-2% and PD-0.9% groups, samples of these fluids were centrifuged; 1 ml was extracted and stored at −20°C. Sodium concentration of these samples and of the solutions given during the intake test (2% and 0.9% NaCl) were analyzed by flame photometry (Hitachi 911, automatic analyzer).

**Plasma Electrolyte, Osmolality, and Protein Assays**

For the assay of plasma osmolality, sodium, and protein concentrations, we used separate groups of animals different from those used in immunohistochemistry studies. Animals from the different groups (PD-2%, CD-2%, PD-0.9%, and CD-0.9%) were decapitated and bled immediately after the sodium intake test ended. Trunk blood was collected into chilled tubes containing EDTA (final concentration of 2 mg/ml blood) for centrifugation at 3,000 g for 10 min at 4°C. Plasma sodium concentration was analyzed by flame photometry (Hitachi 911, automatic analyzer), plasma osmolality was determined by vapor pressure osmometry (VAPRO 5520), and plasma volume was indirectly inferred by the plasma protein concentration, measured according to Lowry et al. (27).

**Immunohistochemistry**

Ninety minutes after the intake test had ended, the animals were perfused for immunohistochemical detection of Fos and OT or Fos and 5-HT. For this purpose, the groups of rats were anesthetized with thiopentone (100 mg/kg ip) and perfused transcardially with ~100 ml of 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 using a freezing microtome and were placed in a mixture of 10% H2O2 and 10% methanol until oxygen bubbles ceased appearing. Samples were incubated in 10% normal horse serum (NHS) in PB for 1 h to block nonspecific binding sites.

All of the series of the free-floating sections from each brain were first processed for Fos immunoreactivity (Fos-ir) using an avidin-biotin-peroxidase procedure. Sections of the midbrain and hypothalamic neurons were then stained, respectively, for 5-HT immunoreactivity (5-HT-ir) and OT immunoreactivity (OT-ir). The staining procedures followed the double-labeling procedures described in Franchini and Vivas (14) and Franchini et al. (13). Briefly, the free-floating sections were incubated overnight at room temperature in a rabbit anti-fos antibody (produced in rabbits against a synthetic 14-amino acid sequence, corresponding to residues 4–17 of human Fos) (AB-5; Oncogene Science, Manhasset, NY), diluted 1:10,000 in PB containing 2% NHS (GIBCO, Auckland, NZ) and 0.3% Triton X-100 (Sigma, St. Louis, MO). The sections were then washed with PB and incubated with biotin-labeled anti-rabbit immunoglobulin and the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA; 1:200 dilution in 1% NHS-PB) for 1 h at room temperature. The peroxidase label was detected with diaminobenzidine hydrochloride (Sigma), intensified with 1% cobalt chloride and 1% nickel ammonium sulfate. This method produces a blue-black nuclear reaction product. The sections of Fos-labeled sections, also processed for immunocytochemical localization of 5-HT and OT, were incubated for 72 h at 4°C with their corresponding antibodies: polyclonal rabbit anti-5-HT antibody (Immunostar; 1:10,000 dilution) and polyclonal rabbit anti-OT antibody (Peninsula Laboratories, San Carlos, CA; 1:25,000 dilution). After incubation, the sections were rinsed and incubated with biotin-labeled anti-rabbit immunoglobulin and the avidin-biotin-peroxidase complex for 1 h at room temperature. Cytoplasmic 5-HT-ir and OT-ir were detected with unintensified diaminobenzidine hydrochloride, which produces 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 DePeX (Fluka, Buchs, Switzerland).

Controls for Fos-ir were conducted by placing sections in primary Fos antibody that had been preadsorbed with an excess of Fos peptide or by processing sections without the primary antisem. No Fos-ir neurons were observed after either of these control procedures.

**Cytoarchitectural and Quantitative Analysis**

The brain nuclei exhibiting Fos-ir were identified and delimited according to the rat brain atlas of Paxinos and Watson (40). The numbers of Fos-ir nuclear profiles in the sections were counted at three levels for different PVN subnuclei, i.e., medial magnocellular (PaMM), the dorsomedial cap, lateral magnocellular (PaLM), and parvocellular posterior (distance from the bregma of the corresponding plates: from −0.92 to −2.12 mm). The Fos-ir neurons of all of the other nuclei were counted at one level. The distance from the bregma of the corresponding plates is as follows: for SON = −1.3 mm, for central lateral amygdaloid nucleus (CeL) = −2.3 mm, for bed nucleus of the stria terminalis (BNST) = −0.26 mm, for subfornical organ (SFO) = −0.92 mm, for organ vasculosum of the lamina terminalis (OVLT) = −0.20 mm, for DRN = −8.00 mm, for lateral parabrachial nucleus (LPBN) = −9.3 mm, and for the nucleus of the solitary tract adjacent to area postrema (NTS/AP) = −13.68 mm.

Fos-ir nuclei were quantified with a computerized system that included a Zeiss microscope equipped with a DC 200 Leica digital camera attached to a contrast enhancement device. Images were digitized and analyzed by Scion Image PC, based on the National Institutes of Health Image 1997 version. Fos-ir cells in each section were counted by setting a size range for cellular nuclei (in pixels) and a threshold level for staining intensity. Representative sections in each group were acquired at exactly the same level, with the aid of the Adobe Photoshop image analysis program (version 5.5). The counting was done in four animals of each condition and was repeated at least twice on each section analyzed, to ensure that the number of profiles obtained was similar. The investigator who conducted the counting of Fos-ir cells was blinded to the experimental groups.
Determination of Plasma OT Concentration

For the assay of plasma OT concentration, we used separate groups of animals different from those used in immunohistochemistry studies. The animals were decapitated and bled immediately after the sodium intake test ended. Trunk blood was collected in chilled plastic tubes containing heparin. Plasma level of OT was measured by radioimmunoassay as described by Morris and Alexander (33). For the assay, OT was extracted from 1 ml of plasma with acetone and petroleum ether. The percentage of recovery after extraction was 85%. The assay sensitivity and intra- and interassay coefficients of variation were 0.9 pg/ml, 7%, and 12.6%.

Statistical Analysis

A two × two factorial experimental design with sodium-replete or sodium-depleted animals (sodium depletion factor), with access either to 2% NaCl or to 0.9% NaCl (solution tonicity factor), was used for the analysis of immunohistochemical results and blood parameters. A two-way ANOVA with repeated measures was used for the analysis of sodium-intake data. Post hoc comparisons were made with the least significant difference test. Sodium balance data were analyzed by Student’s t-test. The correlation of oxytocin neuron activity and volume of hypertonic sodium solution consumed was analyzed by Pearson’s analysis. All results are presented as group mean values ± SE, and the significance level was set at P < 0.05.

RESULTS

Cumulative Hypertonic or Isotonic Sodium Intake Induced by PD

As expected, during the 60-min test, the PD rats consumed more saline solution than the CD rats (F1,50 = 70.76, P = 0.0000001, Fig. 1). The volume of sodium solution consumed in relation to solution tonicity factor (2% NaCl vs. 0.9% NaCl) was significantly different (F1,50 = 14.29, P = 0.000429), and the interaction of these two factors with time (sodium depletion and solution tonicity) was also statistically significant (F1,50 = 3.38, P = 0.038, two-way repeated-measures ANOVA, least significant difference test). Consequently, the cumulative volume ingested in the PD-0.9% group was significantly higher than that ingested in the PD-2% group (6.62 ± 0.48 vs. 3.54 ± 0.53 ml/100 g body wt, respectively), and the cumulative volume drunk by both dialyzed groups was significantly higher than that drunk by the CD groups, which did not differ significantly among each other (CD-2%: 1.01 ± 0.33 vs. CD-0.9%: 1.87 ± 0.4953 ml/100 g body wt).

Sodium Balance After Isotonic vs. Hypertonic Sodium Intake Induced by PD

As shown in Table 2, the plasma sodium concentration reached after the termination of the intake test did not differ significantly among any of the groups analyzed (F1,14 = 0.0425, P = 0.839), suggesting that the amounts of saline that the animals consumed (which were comparable to the urinary sodium losses) in fact repaired plasma sodium concentration. However, PD-induced isotonic or hypertonic NaCl intake produces a significant increase in plasma osmolality and a significant decrease in plasma protein concentration of the PD-2% and PD-0.9% animals, compared with the control groups, CD-0.9% and CD-2% (sodium depletion factor of osmolality F1,14 = 8.94, P = 0.0097 and plasma protein concentration F1,14 = 7.04, P = 0.019), independently of the sodium concentration of the solution consumed (Table 2). The changes observed in these blood parameters (osmolality and protein concentration) possibly reflect an ongoing process of restoring osmolality and extracellular volume homeostasis that occurs immediately after stimulated consumption of large volumes of NaCl solutions in this particular animal model.

It is important to note that our preliminary experiments (data not shown) analyzing the volume of sodium ingested in a longer intake test period of 2 h indicate that ~70% of the animals did not consume any additional saline and the remaining animals drank ~10% more saline solution, giving support to the idea that the animals are almost satiated in a 1-h intake test.

Brain Pattern of Fos-ir and Double-Immunolabeled (Fos-5-HT and Fos-OT) Cells After Isotonic vs. Hypertonic Sodium Intake Induced by PD

DRN. As observed in our previous studies (13), greater activation was found at medial level in the DRN, where we...
Table 1. Sodium balance in PD-2% and PD-0.9% animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Sodium Exit</th>
<th>Sodium Enter</th>
<th>Sodium Balance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺ withdrawn by PD, meq·100 g body wt⁻¹·24 h⁻¹</td>
<td>Na⁺ excretion, meq·100 g body wt⁻¹·24 h⁻¹</td>
<td>Sodium intake, meq·100 g body wt⁻¹·24 h⁻¹</td>
</tr>
<tr>
<td>PD-2%</td>
<td>1.16±0.06</td>
<td>0.16±0.029</td>
<td>1.31±0.07</td>
</tr>
<tr>
<td>PD-0.9%</td>
<td>1.13±0.055</td>
<td>0.15±0.031</td>
<td>1.31±0.155</td>
</tr>
</tbody>
</table>

Values are means ± SE. PD-2%, peritoneally dialyzed animals with access to 2% NaCl; PD-0.9%, peritoneally dialyzed animals with access to 0.9% NaCl.

measured the Fos-ir and double immunoreactivity pattern for Fos and 5-HT. The sodium intake induced by PD significantly increased the number of Fos-ir neurons (F₁,₁₂ = 9.76, P = 0.0088) and the number of 5-HT-activated cells (F₁,₁₂ = 16.42, P = 0.0016), independently of the sodium concentration of the solution consumed (Fig. 2 and Fig. 4, respectively). Both dialyzed groups (PD-2% and PD-0.9%) had significantly higher levels of Fos-ir and Fos-5-HT than the CD-2% and CD-0.9% groups.

SON and PVN. c-fos expression had significant differences in the SON in both factors analyzed, i.e., sodium depletion (F₁,₁₂ = 11.38, P = 0.0055) and solution toxicity (F₁,₁₂ = 31.74, P = 0.00011), but it did not show interaction with each other (Fig. 3A). However, an ANOVA for the number of Fos-OT-positive neurons indicated that there was a significant interaction between factors in the SON (F₁,₁₂ = 4.88, P = 0.047). There was a synergic effect of both factors, which significantly increased the number of OT neurons activated after hypertonic sodium intake induced by PD compared with other animals (Fig. 3B and Fig. 4). That is, the highest number of Fos-OT-positive neurons within the SON was observed in the animals that were induced to consume hypertonic sodium solutions by PD.

Within the magnocellular subdivisions of the PVN (PaMM and PaLM), an increase was found in Fos-ir neurons in the 2% NaCl access rats compared with the 0.9% NaCl access groups (PaLM: F₁,₁₂ = 17.65, P = 0.0012; and PaMM: F₁,₁₂ = 9.4, P = 0.0098), but the sodium depletion factor did not have a statistically significant effect (Fig. 3A). Just as with the c-fos expression, we also observed a significant main effect of the solution toxicity factor on the double immunoreactivity of Fos-OT neurons, which increased after the 2% NaCl intake (PaLM: F₁,₁₂ = 8.97, P = 0.0111; and PaMM: F₁,₁₂ = 9.09, P = 0.041) (Fig. 3B and Fig. 5), but the sodium depletion factor had no effect on Fos-OT neurons. In addition, we have confirmed that particular parvocellular OT subdivisions of the PVN, i.e., the dorsomedial cap and parvocellular posterior subnuclei of the PVN, were activated after PD-induced hypertonic sodium consumption (14); however, the isotonic sodium solution consumption did not induce any change in the activity of these nuclei (dorsomedial cap interaction F₁,₁₂ = 6.42, P = 0.026; and parvocellular posterior interaction: F₁,₁₂ = 5.77, P = 0.033).

In summary, hypertonic solution entering the body increased the activity of OT neurons in the SON and PVN in both depleted and nondepleted animals. In addition, isotonic sodium consumption, whether induced or spontaneous, did not produce any significant effect on the activity of OT cells at the PVN level and produced a minor increase in the SON.

We analyzed the correlation between the number of OT-activated cells and the volume of hypertonic sodium in the SON, PaMM, and PaLM (SON: r = 0.82, P = 0.012; PaLM: r = 0.76, P = 0.03; and PaMM: r = 0.63, P = 0.095) and found a significant, positive correlation in SON and PaLM nuclei and a tendency toward a positive correlation in the PaMM nucleus that did not reach a statistically significant level. This correlation might indicate that the number of Fos-OT-positive neurons increases when the animals drink higher volumes of sodium hypertonic solution.

Brain Pattern of Fos-ir Neurons in Other Nuclei Involved in Sodium Intake Regulation

Brain stem. The high increase of c-fos expression within the NTS/AP and LPBN found in PD-2% animals confirmed our previous results (14) (Fig. 6A). The present study shows how induced sodium consumption increased the number of Fos-ir neurons within the NTS/AP and LPBN (NTS/AP: F₁,₁₂ = 14.36, P = 0.0026; and LPBN: F₁,₁₂ = 7.12, P = 0.021). The toxicity factor had no significant effect on the Fos pattern in these nuclei; however, 2% NaCl-induced ingestion produced a tendency to increase the number of Fos-ir neurons in NTS/AP and LPBN in relation to PD-0.9% groups, but this did not reach a significant level (Fig. 6A).

Circumventricular organs of the lamina terminalis. The PD-2% and PD-0.9% groups showed an increased number of Fos-ir cells in the SFO (F₁,₁₂ = 4.75, P = 0.049) compared with CD groups (Fig. 6B). However, the number of activated neurons did not change in the SFO with the solution toxicity factor. In the OVLT, interaction between both factors, sodium depletion and solution toxicity, was statistically significant (F₁,₁₂ = 5.45, P = 0.037), with the highest significant increase of neuronal activity manifested in dialyzed rats with hypertonic sodium access compared with other groups (PD-0.9%, CD-2%, and CD-0.9%; Fig. 6B).

Central extended amygdala. Two components of the central extended amygdala were analyzed in the present study, the lateral division of the CeL and the lateral division of the

Table 2. Plasma osmolalities and sodium and protein concentrations measured in PD and CD animals with access to 2% NaCl or 0.9% NaCl solutions

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma Osmolality, mosmol/kgH₂O</th>
<th>Plasma Sodium Concentration, meq/l</th>
<th>Plasma Protein Concentration, g/dl</th>
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<tr>
<td>PD-2%</td>
<td>307.9±1.49*</td>
<td>148.66±1.31</td>
<td>5.907±0.292*</td>
</tr>
<tr>
<td>PD-0.9%</td>
<td>300.9±3.73*</td>
<td>148.88±2.09</td>
<td>6.159±0.171*</td>
</tr>
<tr>
<td>CD-2%</td>
<td>297.6±0.95</td>
<td>147.85±0.85</td>
<td>7.188±0.869</td>
</tr>
<tr>
<td>CD-0.9%</td>
<td>297±3.05</td>
<td>147.52±1.04</td>
<td>6.994±0.376</td>
</tr>
</tbody>
</table>

Values are means ± SE. CD-2%, control dialyzed animals with access to 2% NaCl; CD-0.9%, control dialyzed animals with access to 0.9% NaCl. *P < 0.05 significantly different from CD groups.
BNST (BNSTL), since previous studies had found them involved in sodium intake regulation (21).

The main effect of the sodium depletion factor indicates that the PD-0.9% and PD-2% groups showed higher levels of Fos-ir than the CD groups (Fig. 6C). The Fos-ir ANOVA analysis also indicated that the values obtained in the CeL and BNSTL almost reached a significant interaction between factors (sodium depletion × solution tonicity; CeL: $F_{1,12} = 3.90, P = 0.071$, and BNSTL: $F_{1,12} = 4.1, P = 0.065$). This interaction tendency indicates that neuron activity in dialyzed animals with hypertonic sodium access is higher than in animals dialyzed with isotonic sodium access in CeL and BNSTL.

Effect of Induced Isotonic vs. Hypertonic Sodium Intake on Plasma Oxytocin Concentration

Clearly, statistical comparisons of the means demonstrated that induced hypertonic sodium ingestion in the PD-2% group produced a significant increase in plasma OT concentration compared with the other groups (PD-0.9%, CD-2%, and CD-0.9%). An ANOVA analysis of this data revealed that there was a significant interaction between both factors analyzed (sodium depletion and solution tonicity; $F_{1,35} = 4.13, P = 0.049$), indicating a synergic effect of both factors, which significantly increased the plasma levels of OT in the PD-2% groups compared with the others (Fig. 7).

DISCUSSION

5-HT DRN cell activity was not associated with the tonicity of the solution consumed because the number of Fos-5-HT-ir cells in the PD-0.9% and PD-2% groups were similarly increased compared with the CD groups. Activity of the OT PVN and SON nuclei neurons and the plasma OT release were directly correlated with the ingestion of an hypertonic sodium solution during induced consumption, independently of the satiety condition; only the number of Fos-OT-ir cells of the PD and CD groups with access to hypertonic solution was increased compared with the other groups (PD or CD groups

![Fig. 2](image-url) A: average number of Fos-immunoreactive (ir) neurons in the dorsal raphe nucleus (DRN) after sodium ingestion induced by PD. B: average number of double-immunolabeled Fos-serotonergic (5-HT) neurons in the DRN after sodium ingestion induced by PD. Values are means ± SE; $n = 4$. *$P < 0.05$, significantly different from CD groups [2-way ANOVA post hoc least significant difference (LSD) test].

![Fig. 3](image-url) A: average number of Fos-ir neurons in the supraoptic nucleus (SON) and magnocellular subdivisions of paraventricular nucleus of the hypothalamus (PVN) [lateral magnocellular (PaLM) and medial magnocellular (PaMM)] after sodium ingestion induced by PD. B: average number of double-immunolabeled Fos-oxytocingergic (OT) neurons in the SON and magnocellular subdivisions of PVN (PaLM and PaMM) after sodium ingestion induced by PD. Values are means ± SE; $n = 4$. *$P < 0.05$, significantly different from PD-2% group; #$P < 0.05$, significantly different from isotonic access groups (0.9% NaCl). +$P < 0.05$ significantly different from CD groups (2-way ANOVA post hoc LSD).
with access to isotonic sodium). Thus the present results extend previous studies with 5-HT and OT systems (13, 14), confirming the suspicion that these two sodium appetite inhibitory systems may operate at different levels. Whereas both systems are activated by hypertonic NaCl intake, only the 5-HT system is activated by either isotonic or hypertonic NaCl-induced intake and may be associated with the satiety condition.

The present data and previous reports regarding the 5-HT system involvement in sodium appetite control suggest that 5-HT cells of the DRN are markers of body sodium status, particularly when sodium is ingested in response to sodium depletion (5, 13, 43, 44, 46). Functional and neuroanatomic studies suggest that such markers present in the DRN might process information originated from primary visceral areas and in the forebrain areas subserving sodium appetite. The DRN has reciprocal connections with NTS/AP and LPBN, where early, peripheral, and visceral information arrives (18, 23, 24, 62). Electrolytic or excitotoxic lesions of the DRN increase spontaneous sodium intake and also enhance need-induced sodium intake (5, 38). 5-HT is released within the LPBN during sodium intake induced by furosemide and captopril treatment (59), corroborating the proposition that the 5-HT system in the LPBN plays an important inhibitory role in the modulation of sodium appetite, perhaps preventing an excess of sodium intake (7, 29, 30, 31). The raphe also has reciprocal functional connections with the SFO and OVLT (convergent sites for signals related to sodium and volume depletion innervated by 5-HT neurons and rich in 5-HT receptors), suggesting that the activity of a brain circuit formed by DRN 5-HT neurons and associated areas reflect the adjustments of volume, electrolytic, and cardiovascular variations that occur during sodium intake (3, 12, 13, 17, 26, 39, 49, 50, 63).

The PD-induced hypertonic sodium intake not only increased the number of OT neurons activated but also the OT plasma levels, whereas PD-stimulated isotonic intake did not produce any changes in OT plasma levels or in OT neuronal activation, suggesting that the satiety process is not being mainly regulated by the OT system. It is important to point out that there was a positive relationship between the number of OT-activated cells and the volume of hypertonic saline consumed, since the animals stimulated to drink, like the PD-2% group, had the greatest number of Fos-OT-ir cells. In accord

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**Fig. 4.** Pattern of double immunoreactivity cells (Fos-5-HT and Fos-OT) within DRN (A–F) and SON (G–L), respectively, in PD-2% group (A–C and G–I) and in PD-0.9% group (D–F and J–L). B, E, H, K are higher magnifications (×40) of the areas indicated in A, D, G, and J, C, F, and L are higher magnifications (×100) of cells indicated by arrows in B, E, H, and K. Scale bar = 100 μm.

**Fig. 5.** Pattern of double immunoreactivity cells (Fos-OT) in the PaLM (A–F) and PaMM (G–L) subdivisions of the PVN, in PD-2% group (A–C and G–I) and in PD-0.9% group (D–F and J–L). B, E, H, K are higher magnifications (×40) of the areas indicated in A, D, G, and J, C, F, and L are higher magnifications (×100) of cells indicated by arrows in B, E, H, and K. Scale bar = 100 μm.
with these results, there is evidence that intragastric hypertonic infusion enhances OT plasma levels and oxytocin mRNA expression in the hypothalamic nuclei in euhydrated and dehydrated rats, although this peptide does not increase after isotonic intragastric infusion, perhaps because it does not cause substantial hypernatremia or a hypertonic state (20, 36, 52). Together, these studies suggest that the OT system is a hypertonicity marker; thus the OT circuit may be signaling the entry to the body of a hypertonic sodium solution during sodium access, since 2% NaCl loading or ingestion increases OT neuron activity and OT plasma levels. A molecular mechanism supporting this hypothesis was given by Bourque and Qiu’s groups (42, 45, 64), demonstrating that the SON and PVN magnocellular cells are themselves osmotically and sodium-sensitive due to stretch-inactivated channels located in the body cells.

Our results are also consistent with previous data from Stricker and Verbalis’ studies (54–56), where osmotic dilution ultimately inhibits OT secretion, which coincides with the appearance of an enhanced intake of concentrated NaCl solution, indicating that changes in plasma sodium tonicity during the different stages of sodium appetite regulation are directly correlated with oxytocin system activity.

Several afferents to SON and PVN magnocellular groups also modulate oxytocin cell activation. Lesion studies have shown the participation of important brain nuclei in the oxytocin and vasopressin regulation. For example, the destruction of osmoreceptors in the OVLT and AP eliminates secretion of AVP and OT induced by the sodium loads administered (20, 35). SON and PVN receive afferent fibers from the brain stem, such as NTS, LPBN, and raphe nucleus (5-HT afferent), which carry visceral and cardiovascular information (1, 18, 41). The lamina terminalis nuclei, the BNST, and the central amygdaloid nucleus also send projections to these hypothalamic nuclei (1). The DRN has direct 5-HT projections to the PVN and SON; in addition, 5-HT innervations from the DRN are mainly received by SON OT cells (1, 41). Thus these pathways may communicate between the 5-HT and the OT systems; satiety and tonicity information is probably processed and integrated to regulate sodium excretion and ingestion through these systems.

This study also characterized the neural activity of other brain areas previously involved in sodium intake regulation, in response to sodium depletion and solution tonicity stimulation.

Fig. 6. Average number of Fos-ir neurons in brain stem nuclei [nucleus of the solitary tract adjacent to area postrema (NTS/AP) and lateral parabrachial nucleus (LPBN); A], circumventricular organs of the lamina terminalis nuclei [subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT); B], and central extended amygdala nuclei [CeL and lateral division of the bed nucleus of the stria terminalis (BNSTL); C], after sodium ingestion induced by PD. Values are means ± SE; n = 4. *P < 0.05 significantly different from PD-2% group, +P < 0.05 significantly different from CD groups (2-way ANOVA post hoc LSD).

Fig. 7. Plasma OT concentration immediately after end of sodium intake test. Values are means ± SE, *P < 0.05 significantly different from PD-2% group (2-way ANOVA post hoc LSD).
Regarding the brain stem nuclei, the present data confirm our prior results (14) showing a stimulated sodium intake-induced increase in Fos-ir among the NTS/AP and LPBN cells. The data are consistent with prior works in which gastric distension increased the Fos-positive neurons and the electrical activity of the NTS/AP and LPBN cells (2, 48, 57), suggesting that in our study the greater volume of sodium consumed by depleted rats possibly produced a higher increase in the number of Fos-ir neurons in these areas. However, in both nuclei, the highest increase of Fos-ir positive neurons was found in the sodium-depleted animals with access to hypertonic solution vs. in the sodium-depleted animals with isotonic access, although this difference did not reach a significant level. As mentioned earlier, a recent study demonstrated that hypertonic saline ingestion by DOCA-treated rats may be inhibited by two presystemic signals, i.e., gastrointestinal distension and osmolarity increase, whereas during DOCA-induced isotonic NaCl ingestion the gastrointestinal distension may provide the only signal that inhibits further intake (51). Our present results could be explained as an additive effect of gastric stretch and hyperosmotic stimulation in the PD-2% group, above all taking into account that vagal and splanchnic afferent nerves carry information sensed by hepatic osmo-sodium receptors, which is projected to the NTS/AP and the LPBN in the brain stem (23, 24, 62). Accordingly, vagotomized and NTS/AP-lesioned rats drank larger volumes of concentrated saline solutions than control animals. NTS/AP-lesioned rats also have an attenuated increase of vasopressin and oxytocin release in response to intravenous hypertonic saline infusion (6, 20, 53, 62). On the other hand, the c-fos expression in the NTS/AP and LPBN increased after intragastric hypertonic sodium infusion in non-deprived animals (4). Together, these results suggest that this brain stem neural pathway mediates peripheral satiety and osmoregulatory signals that modulate fluid intake and neurohypophyseal hormone secretion.

Hypertonic sodium intake induced by PD also increased the production of Fos in cells of the OVLT and SFO in relation to sham-depleted animals, as expected (14). Recent works have identified sodium-sensitive channels expressed in cells of the SFO and OVLT stimulated by an extracellular sodium increase (28, 65). In the present work, we observed that c-fos expression at the OVLT level is dependent on the solution tonicity consumed, since depleted animals with isotonic solution access had the same neuronal activation as sham-dialyzed animals, whereas sodium-depleted rats that consumed a higher volume of 2% NaCl had the highest level of activation, probably because of the stimulation of sodium-sensitive cells. Consistent with these data, the OVLT lesion attenuated the oxytocin plasma increase observed after intra-atrial infusion of hypertonic solution (35), and the electrical activity of the supraoptic nucleus increased after hyperosmotic OVLT stimulation but not after isosmotic stimulation (45), suggesting that the OVLT plays a functional role in the osmoregulation of neurohypophysial hormone release after hypertonic stimulation. On the other hand, lesion studies have also suggested that the SFO and OVLT sometimes play and sometimes do not play the same roles (10, 11, 47). As previously shown by Franchini and Vivas (14), the sodium-depletion enhancement of Fos expression observed in SFO cells remains until sodium access and does not change after hypertonic or isotonic sodium consumption, as shown in the present experiment. A possible explanation of this delayed deactivation process after sodium repletion may be associated with the gradual decrease in the circulating ANG II levels until they reach the baseline values (19, 22, 60, 63). A well-characterized function of the SFO is its role as a central nervous system target site for circulating ANG II; this octapeptide activates ~70% of all SFO neurons with very few inhibitory neural responses (8).

Among other brain areas, we found the CeL and the BNST (lateral division) were activated after sodium intake induced by PD. Our previous studies indicated that PD sodium depletion also produced a pattern of highly localized and intense Fos-ir cells within this specific portion called the “central division of the extended amygdala,” particularly, the central subdivision of the lateral part of the central amygdaloid nucleus and its continuation of the dorsal part of the lateral bed nucleus of the stria terminalis. Several studies indicate that destruction of the central amygdaloid nucleus (CeA) impairs salt appetite induced by DOCA, sodium depletion, systemic yohimbine treatment, and intracerebroventricular ANG II (16, 66). Similarly to CeA lesion, ablation of the BNST also significantly attenuates salt appetite induced by various treatments (43, 66). The BNST and CeA lesion produced a salt appetite decrease in the order of 50–65% compared with controls. The present data seem to indicate that the BNST and CeA make roughly equal contributions to these forms of experimentally induced salt intake. In addition, the amygdala and bed nucleus of the stria terminalis also receive afferent input from the structures of the lamina terminalis (26, 32) as well as visceral and somatic inputs via LPBN (21, 58). According to the latter, there was a major increase in c-fos expression in depleted animals with access to 2% NaCl compared with the 0.9% NaCl access group, suggesting that signals coming from osmo-sodium receptors arrive and are integrated in these neuronal groups of the extended amygdala. Together, the functional and anatomic findings provide support for the contribution of these nuclei in the process of integrated signals related to sodium appetite behavior.

In summary, the present study suggests further insights for the consummatory phase of sodium appetite, particularly of 5-HT and OT system involvement in sodium-depleted animals. Although both systems may restrain sodium intake, only the activity of DRN was related to the body sodium status reestablished, reached through the ingestion of both NaCl solutions (isosmic or hypertonic). This suggests, although does not prove, that DRN neurons have a more general role in the inhibitory control of sodium appetite and specifically with the satiation process. The role of the OT system is perhaps more restricted to the inhibition of hypertonic NaCl intake. This confirms the hypothesis proposed in the introduction that the two systems might have differential functions in their inhibitory role of sodium appetite; however, both systems may participate in body sodium homeostasis, avoiding excess sodium ingestion and volume expansion.

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