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Activation of lateral parabrachial afferent pathways and endocrine responses during sodium appetite regulation

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ABSTRACT

Modulation of salt appetite involves interactions between the circumventricular organs (CVOs) receptive areas and inhibitory hindbrain serotonergic circuits. Recent studies provide support to the idea that the serotonin action in the lateral parabrachial nucleus (LPBN) plays an important inhibitory role in the modulation of sodium appetite. The aim of the present work was to identify the specific groups of neurons projecting to the LPBN that are activated in the course of sodium appetite regulation, and to analyze the associated endocrine response, specifically oxytocin (OT) and atrial natriuretic peptide (ANP) plasma release, since both hormones have been implicated in the regulatory response to fluid reestablishment. For this purpose we combined the detection of a retrograde transported dye, Fluorogold (FG) injected into the LPBN with the analysis of the Fos immunocytochemistry brain pattern after sodium intake induced by sodium depletion. We analyzed the Fos-FG immunoreactivity after sodium ingestion induced by peritoneal dialysis (PD). We also determined OT and ANP plasma concentration by radioimmunoassay (RIE) before and after sodium intake stimulated by PD. The present study identifies specific groups of neurons along the paraventricular nucleus, central extended amygdala, insular cortex, dorsal raphe nucleus, nucleus of the solitary tract and the CVOs that are activated during the modulation of sodium appetite and have direct connections with the LPBN. It also shows that OT and ANP are released during the course of sodium satiety and fluid reestablishment. The result of this brain network activity may enable appropriate responses that re-establish the body fluid balance after induced sodium consumption.

Introduction

Sodium appetite is a motivational state that involves an important homeostatic behavior, the seeking out and ingestion of salty substances to compensate sodium losses, and is defined operationally by measuring hypertonic sodium solution consumption under specified experimental conditions.

The cerebral structures involved in controlling the excitatory appetitive and inhibitory or satiety phases of sodium intake are likely to be interconnected with one another, constituting a neural network that integrates associated information (Johnson & Thunhorst 2007; Fitzsimons, 1998). Our previous evidence indicates that modulation of salt appetite involves interactions between the circumventricular organs (CVOs) receptive areas and inhibitory hindbrain serotonergic circuits (Badaue-Passos et al., 2007, Godino et al., 2007). That is, for normal salt appetite sensation, and consequently for appropriate salt drinking after sodium depletion, the hyponatremia and the released ANG II should act centrally both to activate brain osmo-sodium and angiotensinergic receptors that stimulate salt appetite, and also to inhibit brain serotonin (5-HT) mechanisms that inhibit sodium appetite, thus removing a "braking" mechanism. The central 5-HT circuits underlying this interaction mainly include bi-directional connections between the CVOs, 5-HT neurons of the dorsal raphe nucleus (DRN) and 5-HT terminals within the lateral parabrachial nucleus (LPBN) (Colombari et al., 1996; Menani et al., 1996, 1998a,b, 2000; Menani and Johnson, 1995; Olivares et al., 2003; Castro et al., 2003; Lima et al., 2004; Tanaka et al., 1998, 2001, 2003, 2004; Cavalcante-Lima et al., 2005a,b). Numerous behavioral, lesion, pharmacological and electrophysiological findings show the involvement of the CVOs nuclei, namely, the subfornical organ (SFO) and the organum vasculosum of the lamina terminalis (OVLT), in the regulatory response of sodium appetite, as key areas sensing changes in plasma and cerebrospinal fluid sodium, osmolality and ANG II (Simpson et al., 1978; Sladek and Johnson 1983; Vivas et al., 1990; Ferguson and Bains, 1996). For example, a recent study demonstrates that selective knockdown of AT1 receptors in the SFO attenuates centrally mediated responses to increased circulating ANGII (Krause et al., 2008). Other results provide insight into how the CVOs may be the origin of a neural projection to the DRN that initiates an inhibitory process. The serotonin from DRN can be released into the LPBN and in

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turn facilitate this inhibitory pathway (Badaue-Passos et al., 2007, Colombari et al., 1996, Menani et al., 2000).

Regarding LPBN participation in this circuit, a microdialysis study, measuring extracellular 5-HT and its metabolite 5-hydroxyindolacetic acid (5-HIAA) in the LPBN, shows that acute water and sodium depletion induced by combined furosemide-captopril treatment causes a significant decrease in extracellular 5-HT and 5-HIAA. Sodium and water drinking stimulated by furosemide-captopril treatment produces the opposite effect on 5-HT release in the LPBN (Tanaka et al., 2004). Anatomical evidence also shows the LPBN as a key site within a central neural network related to sodium appetite control, since it is connected with forebrain and hindbrain areas that are involved in the control of water and electrolyte balance, such as the CVOs, paraventricular nucleus (PVN), central amygdaloid nucleus (CeA), bed nucleus of the stria terminalis (BNST), the nucleus of the solitary tract (NTS), DRN, granular insular cortex (GI), etc. (Norgren., 1978; Petrov et al., 1992, Saper & Loewy, 1980; Ciriello et al., 1984; Fulwiler & Saper, 1984; Lanca & Van der Kooy., 1985; Hebert et al., 1990; Jhamandas et al., 1992, 1996; Krukoff et al., 1993). Taken together, this evidence provides further support to the idea that LPBN plays an important inhibitory role in the modulation of sodium appetite and thirst.

In our previous work we have characterized the spatial brain pattern of c-fos expression during arousal and satiation of sodium appetite induced by peritoneal dialysis (Vivas et al., 1995; Franchini and Vivas, 1999; Franchini et al., 2002; Johnson et al., 1999; Godino et al., 2007). According to this, the aim of the present work was to identify the specific groups of neurons projecting to the LPBN that are activated in the course of sodium appetite regulation, and to analyze the associated endocrine response, specifically OT and atrial natriuretic peptide (ANP) plasma release, since both hormones have been implicated in the regulatory response to fluid reestablishment. For this purpose we combined the detection of a retrograde transported dye, Fluorogold (FG) injected into the LPBN with the analysis of the Fos immunocytochemistry brain pattern after hypertonic sodium intake induced by sodium depletion. We analyzed the Fos and FG immunoreactivity (Fos/FG-ir) double-labeled neurons along the forebrain and hindbrain areas after sodium ingestion induced by peritoneal dialysis treatment (PD). We also determined OT and ANP plasma concentration by radioimmunoassay (RIE) before and after sodium intake stimulated by PD.

Materials and methods

Animals

Male, Wistar-derived rats from the colony of the Instituto de Investigación Médica Mercedes y Martin Ferreyra (INIMEC-CONICET, Córdoba, Argentina) were used. All animals (270–300 g) were housed individually in hanging wire cages for at least 1 week before the beginning of the experiments and had free access to food, water and 2% NaCl solution, except as noted. Room lights were on for 12 h/day, and the temperature was controlled at 23 °C. All experimental protocols were approved by the Ferreyra Institute animal care and use committee, and the guidelines of the International Public Health Service Guide for the Care and Use of Laboratory Animals were followed.

Sodium appetite studies

Sodium intake induced by peritoneal dialysis

Sodium appetite was stimulated by acute body sodium depletion induced by PD. The technique, described previously (Ferreyra & Chiaraviglio, 1977, Godino et al., 2007), consisted of an intraperitoneal injection of a 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 by inserting a needle into the peritoneal cavity. In control sham-depleted rats (CD), no injection was given but the needle was inserted into the peritoneal cavity. Peritoneal-dialyzed and CD rats were housed individually in metabolic cages without food and with distilled water as the only drink. Twenty-four hours later PD and CD groups were given access to the 2% NaCl and water intake test. The cumulative volume drunk was measured at 15, 30, 60 and 120 min. A one-way ANOVA with repeated measures was used for the analysis of sodium intake data. *Post hoc* comparisons were made using the least significant difference (LSD) test, with significance levels set at $p<0.05$.

Retrograde tracing and Fos immunohistochemical studies

Tract tracing injection into the LPBN

The animals were anesthetized with 2,2,2-tribromoethanol (Aldrich Chemical Co., St. Louis, MO; 200 mg/kg of body weight, i.p.) and secured prone in a flat skull position in a Kopf stereotaxic instrument (model 900). Micropipettes (20–25 μm outer diameter) were filled with 2% FG (FluoroChrome Inc, Denver, CO) in saline and lowered into the LPBN. The FG injection was performed bilaterally into the LPBN, according the coordinates suggested by Paxinos and Watson's rat brain stereotaxic atlas (1997). The coordinates used were: 9.4 mm caudal to the bregma, 2.2 mm lateral to the midline, and 5.7 mm below dura mater. Tracers were deposited in the brain by passing a positive current (5 mA; 7 s on/off) through a silver wire inserted into the micropipette for 1.5 min using a constant-current device (B & K Precision Single Output DC Power; Cole Parmer Instrument Corp., Vernon Hills, IL). The micropipette was left in place for 5 min after the delivery of the FG to minimize diffusion along the pipette track. Wounds were irrigated with sterile saline and closed with silk. After a survival of 7–8 days, the animals were subjected to the peritoneal dialysis procedure.

Staining procedure for Fos-/FG-ir

Ninety minutes after the termination of the intake test induced by PD, experimental and control animals were deeply anesthetized with 2,2,2-tribromoethanol (200 mg/kg i.p.) and perfused transcardially with 200 ml of normal saline, followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS), pH 7.2. The brains were removed, fixed overnight in the perfusion solution and stored at 4 °C in PBS containing 30% sucrose. Two series of 40 μm coronal sections were placed in a mixture of 10% H_2O_2 and 10% methanol until oxygen bubbles ceased appearing. They were then incubated in 10% normal horse serum (NHS) in PBS for 1 h to block non-specific binding.

Free-floating sections from each brain were processed first for Fos-ir and then for immunocytochemical localization of FG. The staining procedures followed the double-labeling procedure described in Badaue-Passos et al., 2007, Margatho et al; 2008. 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, USA), diluted 1:10,000 in PBS containing 2% NHS (Gibco, Auckland, NZ) and 0.3% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA). The sections were then washed with PBS and incubated with biotin-labeled anti-rabbit immunoglobulin and the avidin–biotin–peroxidase complex (Vector Laboratories Inc., Burlingame, CA, USA, 1:200 dilution in 1% NHS-PBS) for 1 h at room temperature. The peroxidase label was detected using diaminobenzidine hydrochloride (DAB, Sigma Chemical Co., St. Louis, MO, USA) intensified with 1% cobalt chloride and 1% nickel ammonium sulfate. This method produces a blue-black nuclear reaction product. The Fos-ir-labeled sections were then processed for immunocytochemical localization of FG. Sections were incubated

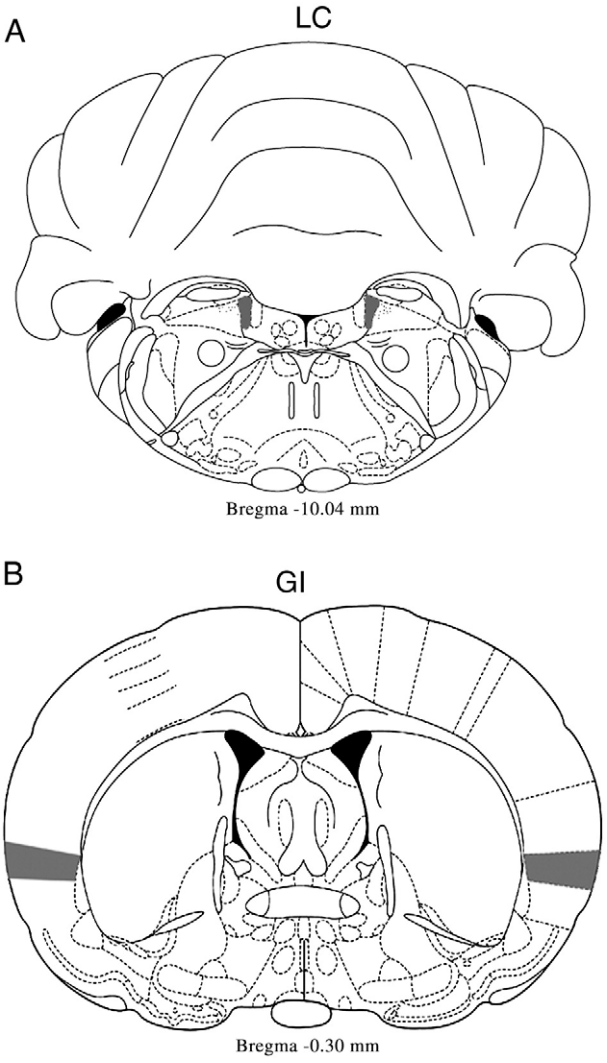

Fig. 1. Schematic representation of the locus coeruleus (LC) and granular insular cortex (GI) nuclei showing the specific areas that were evaluated in the analysis of Fos and Fos-FG immunoreactive pattern.

for 72 h at 4 °C in polyclonal rabbit anti-FG antibody (AB 153, Chemicon, Temecula, CA, in KPBS 0.01 M) diluted 1:3,000 in a solution of KPBS 0.01 M containing 2% NHS and 0.3% Triton X-100. After incubation, sections were rinsed and incubated in the appropriate biotinylated secondary antiserum and avidin–biotin–peroxidase complex (Vector, Burlingame, CA, 1:200 dilution in 1% NHS-PBS). Cytoplasmic FG-ir was detected with unintensified diaminobenzidine (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 DePeX (Fluka, Switzerland). Controls for Fos-ir were conducted by placing sections in primary Fos antibody that had been preabsorbed with an excess of the Fos peptide or by processing sections without the primary antiserum. No Fos-ir neurons were observed after either of these control procedures.

Cytoarchitectural and quantitative analysis

The brain nuclei evidencing Fos- and FG-ir were identified and delimited according to the rat brain atlas (Paxinos and Watson, 1997). The number of Fos-ir nuclear profiles and Fos-FG neurons in the sections of all nuclei were counted at one level. The distance from the bregma of the corresponding plates is indicated between brackets: hypothalamic paraventricular nucleus (PVN, −1.8 mm), central amygdaloid nucleus (CeA, −2.3 mm), bed nucleus of the stria terminalis, lateral division, dorsal part (BSTLD, −0.26 mm) (Fig. 8), granular insular cortex (GI) (Fig. 1), subfornical organ (SFO, −0.92 mm), organum vasculosum of the lamina terminalis (OVLT, −0.11 mm) (Fig. 6), dorsal raphe nucleus (DRN, −8.00 mm) (Fig. 6), locus coeruleus (LC, −10.04 mm) (Fig. 1), the nucleus of the solitary tract adjacent to area postrema (NTS/AP, −13.68 mm) (Fig. 6).

Two series of sections were used in the quantitative analysis. In one series of sections, Fos-ir neurons were counted using a computerized system that includes a Zeiss microscope equipped with a DC 200 Leica digital camera attached to a contrast-enhanced device. Images were digitized and analyzed using Scion Image PC based on the NIH 1997 version. Single-labeled cells in each section were counted by setting a size range for cellular nuclei (in pixels) and a threshold level for staining intensity. In the second series of sections, Fos-/FG-ir-labeled neurons were hand counted.

Representative sections in control and experimental groups were acquired at exactly the same level with the aid of the Adobe Photoshop Image Analysis Program, version 7.0. The counting was done in four or five animals in each group and was repeated at least twice on each section analyzed to ensure that the numbers of profiles obtained were similar. The investigator who conducted the counting of Fos-ir positive cells was "blind" as to whether a given animal was in the control or experimental group. The immunohistochemical data were analyzed using Student's *t*-test, with significance levels set at $p<0.05$.

Determination of plasma OT and ANP concentration

Trunk blood was collected into chilled plastic tubes containing heparin for the measurement of OT and EDTA (2 mg/ml) and proteolytic enzyme inhibitors (20 µl of 1 mM phenylmethylsulfonyl fluoride and 20 µl of 500 µM pepstatin) for ANP. Plasma level of OT and ANP was measured by radioimmunoassay as described by Morris & Alexander (1989) and Gutkowaska et al. (1984), respectively. For the assay, OT was extracted from 1 ml of plasma with acetone and petroleum ether, and ANP was extracted from 1 ml of plasma in Sep-Pac C-18 cartridges (Waters Corporation, Milford, MA, USA). The percentage of recovery after extraction was 85% and 90% for OT and ANP respectively. The assay sensitivity and intra- and inter-assay coefficients of variation were 0.9 pg/ml, 7% and 12.6% for OT and were 7.0 pg/ml, 6.0% and 10.0% for ANP. The data were subjected to a two-way ANOVA. *Post hoc* comparisons were made using LSD tests with

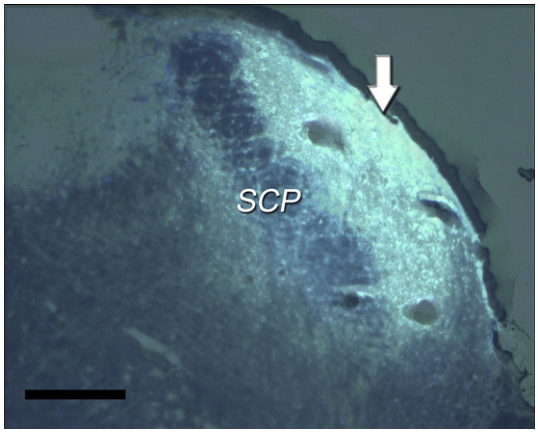

Fig. 2. Representative photomicrograph showing a Fluorogold injection into the lateral parabrachial nucleus (LPBN). The injection site was limited almost completely to above the superior cerebellar peduncle (SCP), as indicated by the arrow. Scale bar: 100 µm.

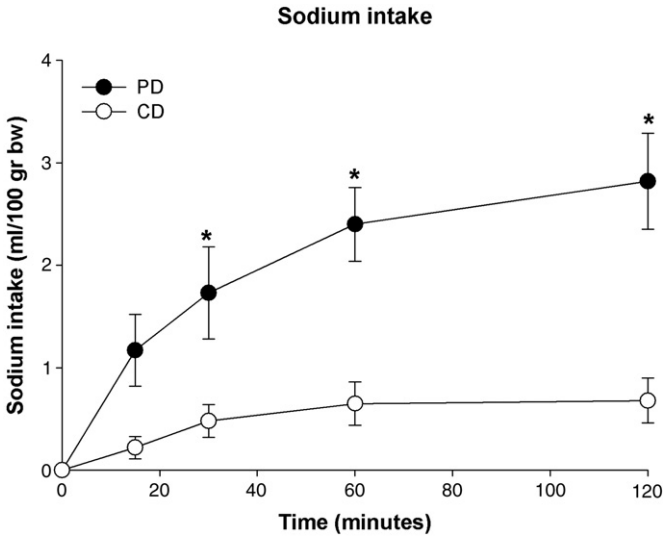

Fig. 3. Cumulative volume of 2% NaCl solution drunk during the intake test (2 h), 24 h after control (CD) or peritoneal dialysis (PD) treatment. Values are means ± SE. *$p<0.05$.

significance levels set at $p<0.05$. All the results were presented as group mean values ± S.E.

Experimental procedures

Experiment 1. Brain neuronal groups projecting to the LPBN activated by sodium-induced consumption

Animals previously injected bilaterally with FG into the LPBN were subjected to the PD protocol. Sodium-depleted and control animals were submitted to the intake test. Ninety minutes after finishing the intake test, the brains of experimental and control animals were collected, fixed and processed for immunohistochemical detection of Fos- and FG-ir. The counting was done in four or five animals in each group.

Experiment 2. Effect of sodium depletion and induced sodium consumption on plasma OT and ANP concentration in rats subjected to PD protocol

In order to analyze plasma OT and ANP concentrations, animals separate from those used in the immunohistochemical studies were divided into different treatment conditions as follows: control dialyzed with no access to 2% NaCl (CD-NA, $n=9$), peritoneal dialyzed with no access to 2% NaCl (PD-NA, $n=13$), control (sham) dialyzed with access to 2% NaCl (CD-A, $n=9$), and peritoneal dialyzed with access to 2% NaCl (PD-A, $n=13$). Blood samples were taken immediately after termination of the 60 minute-intake test in the PD-A and CD-A groups or after a comparable period in the PD-NA and CD-NA groups, which had no access to 2% NaCl.

Results

Histological analysis of the LPBN

The injection sites into the LPBN (Fig. 2) were centered in the central lateral and dorsal lateral portions of the LPBN. Injections also reached the ventral lateral and external lateral portions. The spread of the injections was almost completely limited to above the brachium (superior cerebellar peduncle).

Sodium intake in animals studied for Fos and Fos-FG-ir

Confirming our previous results (Franchini and Vivas, 1999, Godino et al., 2007), the cumulative volume of 2% NaCl solution drunk during the intake test (2 h) was statistically different between groups, namely PD versus CD, as shown in Fig. 3 (PD: 2.83 ± 0.47 ml/100 g bw vs. CD:0.68 ± 0.22 ml/100 g bw, $F_{3,48}=5.06$ $p=0.004$ two-way ANOVA, repeated measures, Scheffé test).

Brain pattern of For-ir and Double immunolabeled Fos-FG cells after 2% NaCl intake induced by PD

Brainstem

The double immunolabeled Fos-FG cells, increased after 2% NaCl intake in the NTS/AP ($p=0.01$) and DRN ($p=0.01$) (Figs. 4 and 6). Similarly, c-fos expression also increased by induced sodium consumption within the NTS/AP ($p=0.001$) and the DRN ($p=0.01$) of the PD group, as previously shown (Franchini & Vivas, 1999; Franchini et al., 2002; Godino et al., 2007). In contrast, along the LC nucleus, we did not find any significant difference between the number of cells immunoreactive for Fos ($p=0.43$) or Fos-FG ($p=0.55$), comparing control and dialyzed animals (see Fig. 1).

Circumventricular organs of the Lamina terminalis

The number of Fos-FG double-labeled cells following control or induced sodium ingestion along the circumventricular organs of the LT, namely the SFO and the OVLT, is shown in Figs. 5 and 6. Rats that drank 2% NaCl for 2 h following 24 h of sodium deficiency had a

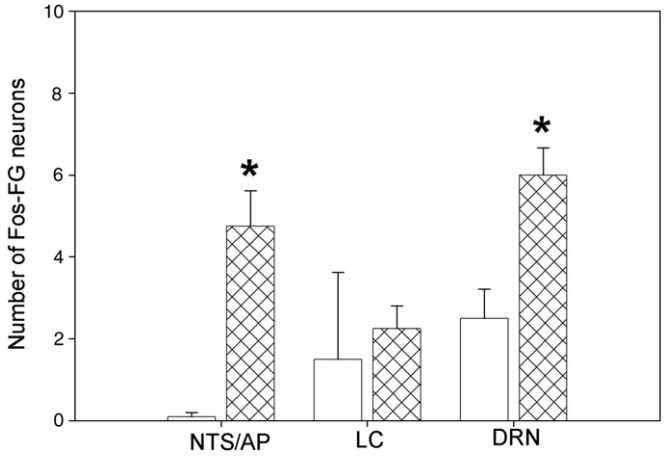

Fig. 4. Average number of Fos-FG in the nucleus of the solitary tract adjacent to the area postrema, NTS/AP; locus coeruleus, LC and dorsal *raphe* nucleus, DRN, after sodium ingestion induced by PD. Values are means ± SE; *$p<0.05$.

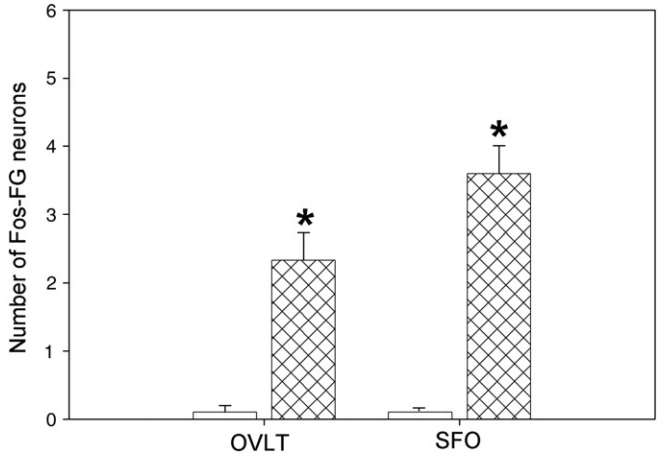

Fig. 5. Average number of Fos-FG immunoreactive neurons in the organum vasculosum of the lamina terminalis (OVLT) and subfornical organ (SFO), after sodium ingestion induced by PD. Values are means ± SE; *$p<0.05$.

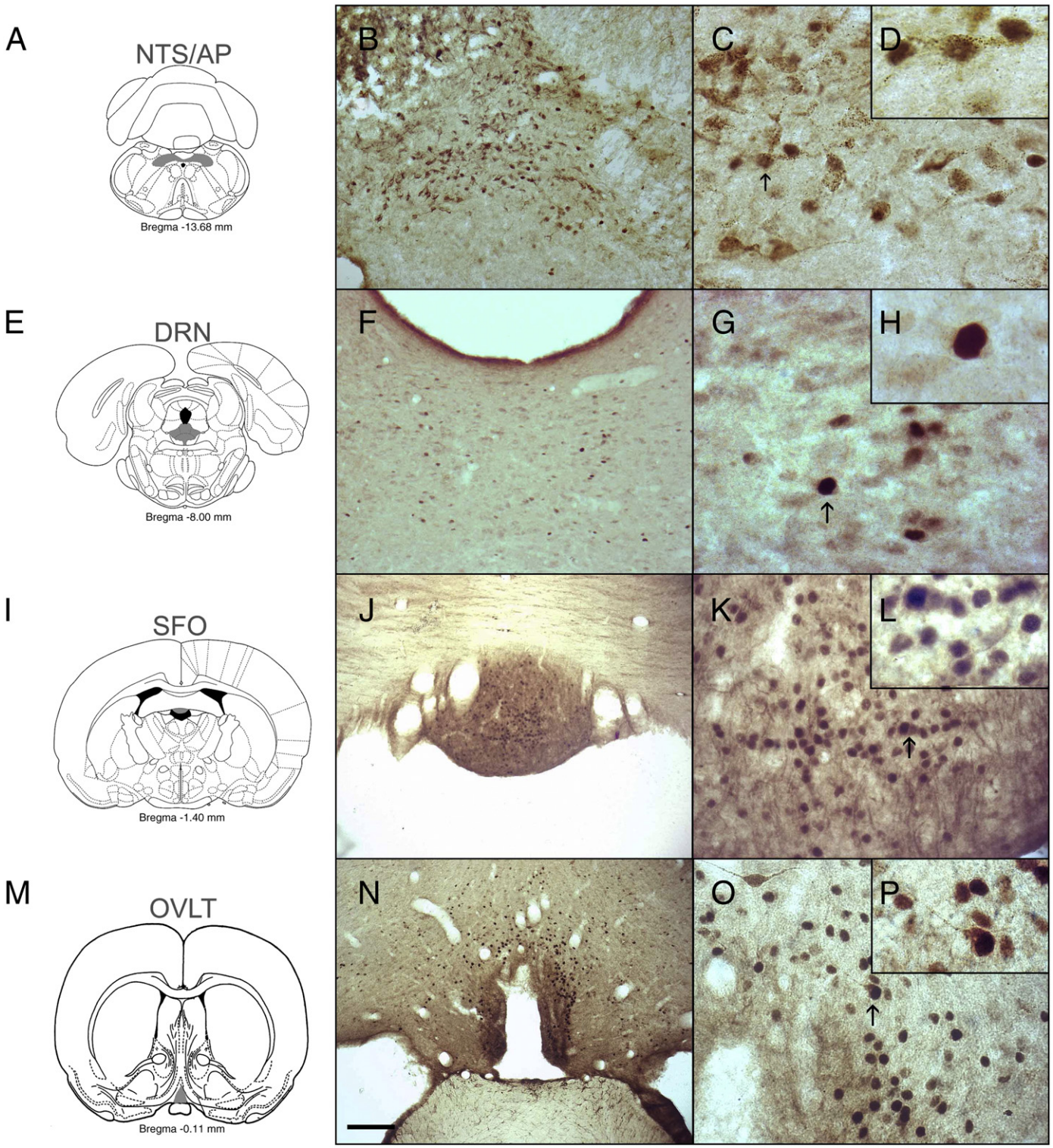

Fig. 6. Double immunoreactivity (Fos-FG) in the brainstem nuclei (NTS/AP and DRN) and circumventricular organs of the lamina terminalis (SFO and OVLT) after sodium intake induced by peritoneal dialysis. Plates A, E, I, M are a schematic representation of the corresponding nuclei sections. Plates B, F, J, N show double immunolabeling in the NTS/AP, DRN, SFO and OVLT respectively. Plates C, G, K, O are higher magnification (40X) of the previous plates and in plates D, H, L P are higher magnification (100×) of the double cells indicated by arrows. Scale bar = 100 μm.

significant increase in Fos-FG immunoreactivity in the SFO ($p = 0.03$) and the OVLT ($p = 0.01$) compared with control dialyzed animals. As previously shown (Franchini & Vivas, 1999; Godino et al., 2007), the number of Fos-ir neurons in the above-mentioned nuclei also increased after sodium consumption induced by PD (PD vs. CD; SFO: $p = 0.04$; OVLT: $p = 0.01$).

PVN/CExA/GI

Along the PVN subdivisions, the ventral and posterior subnuclei, which are included in the nonendocrine projection neurons of the PVN, showed an increase in the number of Fos ($p = 0.05$) and Fos-FG ($p = 0.015$) immunoreactive cells, after hypertonic sodium intake induced by PD (Figs. 7 and 8).

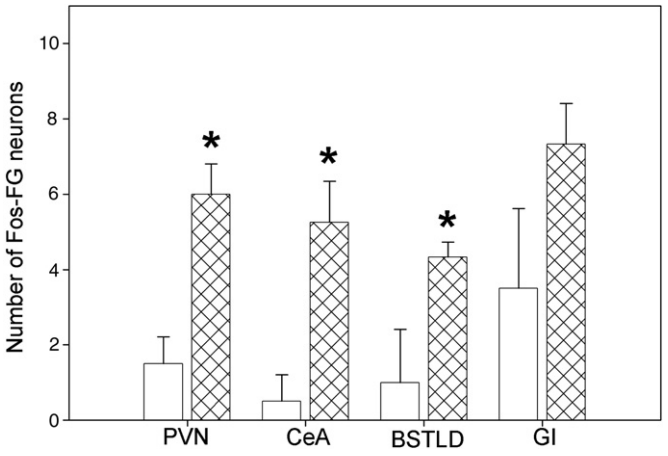

Fig. 7. Average number of Fos-FG-ir neurons in the paraventricular nucleus (PVN); lateral division of the central amygdaloid nucleus, (CeA); lateral division of the bed nucleus of the stria terminalis (BSTLD) and granular insular cortex (GI), after sodium ingestion induced by PD. Values are means ± SE; *$p<0.05$.

The central extended amygdala complex (CExA), more precisely the lateral division of the bed nucleus of the stria terminalis (BSTLD) and the central amygdala (CeA), also showed a significant increase in the number of Fos and Fos-FG-ir cells after sodium consumption induced by PD (CeA Fos: $p=0.04$; Fos-FG: $p=0.03$ and BSTLD Fos: $p=0.02$, Fos-FG: $p=0.03$; Figs. 7 and 8).

The granular insular cortex (GI) previously associated with the regulation of ingestive behaviors also showed an increased number of Fos cells in the PD group (Fos: $p=0.006$); however the number of double immunoreactive neurons Fos-FG tended to increase but did not reach significant levels (Fos-FG: $p=0.09$) (Figs. 1 and 7).

Effect of PD-induced sodium depletion and 2% NaCl intake stimulated by PD on plasma OT and ANP concentration

Statistical comparisons of the means clearly demonstrated that induced sodium ingestion in peritoneal dialyzed rats produced a significant increased in plasma OT and ANP concentrations compared with control (sham) dialyzed animals with access to 2% NaCl (OT: $p=0.024$, ANP: $p=0.0066$). On the other hand, rats that had no

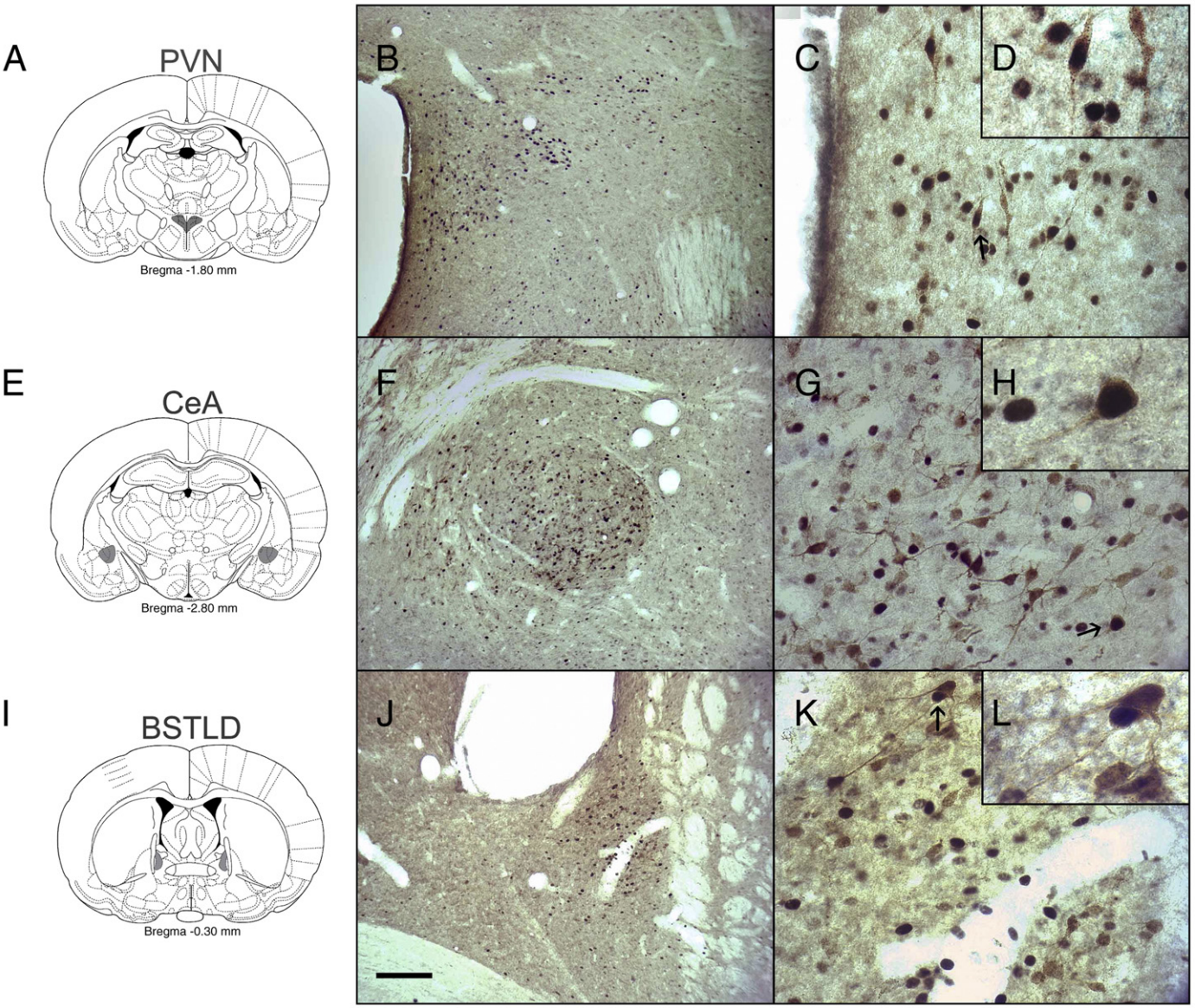

Fig. 8. Double immunoreactivity (Fos-FG) in the PVN, CeA and BSTLD after sodium intake induced by peritoneal dialysis. Plates A, E, I, are a schematic representation of the corresponding nuclei sections. Plates B, F, J show double immunolabeling in the PVN, CeA and BSTLD respectively. Plates C, G, K are higher magnification (40X) of the previous plates and in plates D, H, L are higher magnification (100X) of the double cells indicated by arrows. Scale bar = 100 μm.

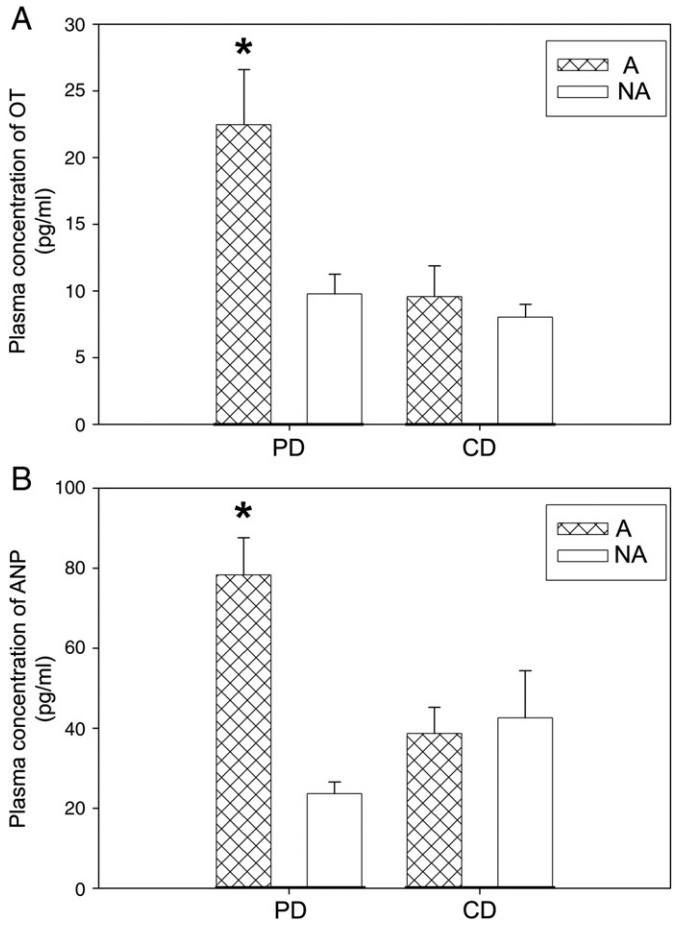

Fig. 9. Plasma ANP (A) and OT (B) concentrations in control dialyzed with no access to 2% NaCl (CD-NA), peritoneal dialyzed with no access to 2% NaCl (PD-NA), control (sham) dialyzed with access to 2% NaCl (CD-A), and peritoneal dialyzed with access to 2% NaCl (PD-A). Blood samples were taken immediately after termination of the intake test (60 min) in the PD-A and CD-A groups or after a comparable period in the PD-NA and CD-NA groups that had no access to 2% NaCl. Values are means ± SE; *$p<0.05$.

access to 2% NaCl, the sodium depletion induced by PD had no effect on plasma OT or ANP concentration; however, plasma ANP concentration tended to decrease after sodium depletion although this reduction did not reach a significant level (Fig. 9).

Discussion

The present study identifies the specific groups of forebrain and hindbrain neurons that are activated during the course of body sodium depletion and sodium repletion and have direct connections with the LPBN. It adds new information concerning the anatomofunctional interrelationship between the LPBN cells and specific afferent areas, suggesting that these may form a neural network enabling the information that arrives at the LPBN to be integrated for the regulatory response to sodium appetite. It also contributes to characterizing the associated endocrine response before and after sodium-depletion-induced sodium intake, showing that oxytocin and atrial natriuretic peptide are released during the course of satiety of sodium appetite and fluid reestablishment. The present double-labeling study shows how a treatment that induces sodium balance reestablishment produces Fos activation in cells of the CVOs, PVN, CExA, GI, DRN, NTS/AP and that these cells project directly to the LPBN.

As mentioned earlier we have previously distinguished the spatial brain pattern of c-fos expression during the arousal or the satiation of sodium appetite stimulated by PD (Vivas et al., 1995; Franchini and Vivas, 1999; Franchini et al., 2002; Johnson et al., 1999; Godino et al., 2007). After sodium depletion or during the appetitive phase of sodium appetite the number of Fos-ir neurons increased in the SFO, OVLT, CeA, MeA, BNST while decreased within the DRN. On the other hand, after hypertonic sodium consumption induced by PD Fos activity increased in different cell groups of the NTS, DRN, LPBN, SON, and PVN. However, during this satiety phase the areas activated after sodium depletion also showed an increased activity. This evidence can be interpreted as the depletion-induced elevation of Fos still remains or the Fos activity is the result of stimulation caused by the entry to the body of a hypertonic sodium solution during sodium access. In any of these situations, the information should reach the LPBN in order to be integrated for the regulatory response to sodium depletion.

The SFO and OVLT are regions where the blood–brain barrier is absent and their cells are sensitive to humoral signals such as changes in plasma and cerebrospinal fluid, sodium concentration (Vivas et al., 1990), osmolality (Sladek & Johnson, 1983) and angiotensin II (ANG II) levels (Ferguson & Bains, 1996; Simpson et al., 1978). Such unique features make the SFO and OVLT key brain regions for sensing the status of the body fluids and electrolytes. Recently, Watanabe et al. (2000) have identified sodium-sensitive channels expressed in cells of the SFO and OVLT stimulated by a physiological extracellular sodium increase (Masaharu & Takeshi, 2005). On the other hand, the presence of ANP and ANG II receptors has been described in OVLT and SFO neurons (Brown & Czarnecki, 1990; Yoshimura et al., 1993; Allen et al., 1998). In our previous study, we observed that c-fos expression at OVLT level is dependent on the tonicity of the solution consumed, since depleted animals with isotonic solution access had the same neuronal activation as sham-dialyzed animals, while 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 (Godino et al., 2007).

Consistent with these data, OVLT lesion attenuated the oxytocin plasma increase observed after intra-atrial infusion of hypertonic solution (Negoro et al., 1988), suggesting that the OVLT plays a functional role in the osmoregulation of neurohypophyseal hormone release after hypertonic stimulation. In the present work, we observed c-fos expression in some OVLT cells projecting to the LPBN, suggesting that a rise of plasma tonicity during hypertonic sodium ingestion could be detected by OVLT cells, and the projections from its cells to the LPBN may contribute to inhibitory mechanisms involving neurons in the LPBN that limit the intake of sodium and prevent excess volume expansion.

The present study supports our previous results showing increased production of Fos within the SFO after peritoneal dialysis-induced sodium consumption and also shows how some neurons of this nucleus consistently displayed retrogradely labeled Fos-ir neurons after sodium-induced intake (Franchini and Vivas, 1999; Godino et al., 2007). As previously shown by Franchini et al. 2002 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 Godino et al., 2007. 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 (Houpt et al., 1998; Tordoff et al., 1991).

The brainstem nuclei are certainly involved in the reception of peripheral information from hepatoportal osmo-sodium receptors, gastric distension, and low- and high-pressure receptor stimulation. Vagal and splanchnic afferent nerves have been shown to carry information relative to hepatic osmo-sodium receptors after sodium intake, which is projected to the NTS/AP and the LPBN in the brainstem (Kharilas & Rogers, 1984; Tordoff et al., 1987; Kobashi et al., 1993). Previous studies also demonstrated that gastric distension and intragastric hypertonic sodium infusion increased the Fos-ir positive neurons and the electrical activity of NTS/AP and LPBN (Kharilas &

Rogers, 1984; Kobashi et al., 1993; Suemori et al., 1994; Carlson et al., 1997; Baird et al., 2001; Sabbatini et al., 2004, Hoffmann et al., 2006). In agreement with this evidence, AP/NTS lesion rats drank larger volumes of concentrated saline solutions than controls (Curtis et al., 1999). The NTS/AP lesion also attenuated the increase of vasopressin and oxytocin released in response to intravenous hypertonic saline infusion (Curtis et al., 1999; Huang et al., 2000; Stricker et al., 2001). In the DRN it has been shown that electrolytic or excitotoxic lesions of this nucleus produced an acute decrease of induced-ANP release and a significant enhancement of induced-sodium appetite (Reis et al., 1994; Olivares et al., 2003; Lima et al., 2004). As mentioned earlier our previous results demonstrate a significant increase in c-fos expression along the NTS/AP, the LPBN and the DRN only during the satiety phase of sodium appetite (Godino et al., 2005, 2007, Franchini & Vivas, 1999; Franchini et al., 2002). The present results show how neurons of the DRN and NTS/AP, activated by sodium ingestion, send projections to the LPBN, suggesting that these afferent projections may be important for carrying key signals to the LPBN, where the information is integrated and processed in order to trigger a homeostatic response, such as ANP release and sodium appetite inhibition.

Previous evidence has indicated that there are central serotonergic influences on endocrine, behavioral and renal response during body sodium disturbances (Margatho et al., 2007, 2008; Reis et al., 1991; Menani et al., 1996, 2000, Tanaka et al, 2004). The present results indicate that the DRN, an important serotonergic nucleus, sends a direct projection to the LPBN and that this pathway is activated during the satiety process of sodium appetite. In agreement with these data, several studies suggest that a serotonergic hindbrain circuit including the LPBN may normally exert an inhibitory action on several models of renin–angiotensin-dependent sodium and water intake (Menani et al., 1996, 1998a and b, 2000). According to this its important to consider that the SFO has direct, angiotensinergic projections to the DRN (Lind, 1986) that probably antagonize this inhibitory effect. Recent evidence also indicates that serotonergic modulation within the LPBN is implicated in OT and ANP release during sodium consumption or blood volume expansion models (Colombari et al., 1996; Menani and Johnson, 1995; Olivares et al., 2003; Castro et al., 2003; Lima et al., 2004; Menani et al., 1996, 1998a and b, 2000; Tanaka et al., 2004; Cavalcante-Lima et al., 2005a and b; Jonhson & Thunhorst, 2007; Margatho et al., 2007). Our previous results also indicated that the serotonergic system participated in the inhibition of sodium intake since we found an increase of activated serotonergic neurons in the DRN during the satiety phase of sodium consumption and also during extracellular volume expansion (Franchini et al., 2002; Godino et al., 2005).

Regarding the LPBN involvement, previous studies show that 5HT acts in the LPBN to inhibit water and sodium intake and to increase ANP and OT release facilitating renal excretion of sodium potassium and water (Margatho et al., 2007). For example it has been demonstrated that injecting methysergide, a nonselective 5-HT_1/5-HT_2 serotonergic receptor antagonist, into the LPBN increases the intake of water and NaCl induced by ANG II injected intracerebroventricularly or into the SFO, and by various systemic treatments including administration of the diuretic furosemide (FURO) together with the angiotensin-converting enzyme inhibitor, captopril (CAP) (Colombari et al., 1996; Menani et al., 1996,1998, 2000; Margatho et al., 2002, 2007). The increased drinking responses to these various treatments are blocked by pre-treatment of the SFO with the AT1 receptor antagonist, losartan (Rowland et al., 1996; Tanaka et al., 2001, 2003), these results indicate not only the involvement of 5-HT mechanisms in the LPBN in ANG II-induced water and sodium intake but also that the SFO is one site where serotonergic modulation occurs, mediated in part by AT1 receptors. Taking this together with the present data suggest that serotonergic cells are part of this functional projection to the LPBN, modulating sodium appetite, plasma OT and ANP release, and consequently the renal responses during hypertonic sodium intake induced by sodium depletion.

In the present work, peritoneal dialysis-induced sodium ingestion increased the number of Fos-FG immunoreactive neurons along the non-endocrine projection neurons of the PVN, as defined by Armstrong (1995). According with these data, the ventral and posterior subdivisions of this group of cells project caudally to the brainstem (Armstrong, 1995). Thus, the increased activity observed corroborates and supports physiological evidence suggesting the contribution of these PVN groups of neurons in the compensatory autonomic response to fluid volume regulation. (Lovick et al., 1993; Haselton et al., 1994; Badoer et al., 1997; Randolph et al., 1998; Deering & Coote, 2000; Pyner et al., 2002; Higa et al., 2002). Thus, our evidence suggests that this PVN-LPBN pathway carries sympathetic information to be processed and integrated in the control of sodium excretion/ingestion and endocrine responses during hypertonic sodium ingestion induced by peritoneal dialysis.

This study also shows two groups of neurons in the central extended amygdala, the CeA and the BSTLD (both lateral divisions), projecting directly to the LPBN, that are activated after PD-induced sodium intake, which suggests that this complex may be an important modulator of LPBN autonomic and behavioral functions. Our previous studies indicated that sodium depletion induces an increase in c-fos activation in the central ExA division, specifically in the central subdivision of the lateral part of the central amygdaloid nucleus and its continuation in the BSTLD (Johnson et al., 1999). Previous studies also indicated that the BNST and CeA make roughly equal contributions to different forms of experimentally induced salt intake (Galaverna et al., 1992, Zardetto-Smith et al., 1994, Reilly et al., 1994). The present data show how hypertonic sodium consumption activates cells of both subnuclei which project heavily to the LPBN, supporting the idea that these are corresponding areas that are interrelated and likely to have a strong influence on autonomic and behavioral aspects of sodium appetite.

As well as in the central extended amygdala nuclei, we have found increased activity shown by Fos immunoreactivity within the insular cortex, another forebrain station of a major gustatory projection from the NTS and PBN in the hindbrain (Norgren, 1976). This area receives inputs from receptors that monitor body fluid and electrolyte balance, such as taste receptors, modulating sodium intake in response to body sodium depletion (Flynn et al., 1991; Johnson et al., 1999; Pastukovas, et al., 2003). For example, the lesion of the GI disrupted conditioned taste aversion (Bermúdez-Ratonni and McGaugh, 1991) suggesting that this area is involved in the aversive memories. Confirming previous results from Pastukovas et al., 2003, we have found increased c-fos expression after induced sodium intake along the GI, suggesting that afferent activity due to oropharyngeal stimulation is received by the GI. However, only some activated cells project to the LPBN, maybe because the outputs of these cortical areas are directed to many other nuclei such as the amygdala, nucleus accumbens, hypothalamus, etc. (Yamamoto & Sawa, 2000).

As regards the associated endocrine responses analyzed in our study, we observed an increased OT and ANP plasma release after 2% NaCl intake induced by peritoneal dialysis. We have previously studied the involvement of the oxytocinergic system in sodium appetite regulation and the present results once more confirm our hypotheses about the role of the oxytocinergic system in body fluid regulation, signaling the entry to the body of a hypertonic sodium solution during sodium intake (Godino et al., 2007). The released oxytocin would then act at cardiac level to stimulate ANP release (Haanwinckel et al., 1995) with both hormones acting at kidney level inducing renal diuresis/natriuresis and also antagonizing the central and peripheral ANGII system, thus preventing sodium overload. With regard to involvement of the ANPergic system, peripheral and central ANP are also seen to be modulated by the renin–angiotensin system (SRA) antagonism (Zavala et al., 2004). As previously mentioned, the serotonergic system within the LPBN area also regulates ANP and OT release (Reis et al., 1994, Margatho et al., 2007). Thus, taking the

previous evidence into account, the plasma ANP level increase observed in the present study would indicate that the ANP system in the present model may be modulated by different systems at different levels.

In summary, the present study indicates that sodium consumption induced by sodium depletion is modulated by a complex neuroendocrine network involving different afferent signaling systems that trigger various efferent autonomic, endocrine and behavioral responses to achieve body fluid balance. In this network the LPBN is the key site since it receives and integrates information from different brain levels. At the brainstem, the neurochemical groups directly connected with the LPBN, the NTS/AP and the DRN contribute sensory signals coming from hepatoportal osmo-sodium receptors, gastric distension and inhibitory serotonergic modulation among others.

At telencephalic level, the circumventricular organs of the lamina terminalis, the OVLT and SFO, directly connected with the LPBN, contribute by sending signals from their osmo-sodium receptors, AII and ANP receptors. The LPBN also receives information from the extended amygdala complex and the PVN, crucial for elaborating effective autonomic, endocrine and behavioral responses. The result of this brain network activity, together with the release of ANP and OT, may enable appropriate natriorexigenic, renal and cardiovascular responses that prevent extracellular volume expansion and re-establish the body fluid balance after induced sodium consumption.

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