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TEMPORAL DISSOCIATION BETWEEN SODIUM DEPLETION AND SODIUM APPETITE APPEARANCE: INVOLVEMENT OF INHIBITORY AND STIMULATORY SIGNALS

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Abstract—Our aim was to analyze the participation of inhibitory and stimulatory signals in the temporal dissociation between sodium depletion (SD) induced by peritoneal dialysis (PD) and the appearance of sodium appetite (SA), particularly 2 h after PD, when the rats are hypovolemic/natremic but SA is not evident. We investigated the effects of bilateral injections of the serotonin (5-HT) receptor antagonist, methysergide, into the lateral parabrachial nucleus (LPBN) on hypertonic NaCl and water intake 2 h vs. 24 h after PD. We also studied plasma renin activity (PRA) and aldosterone (ALDO) concentration 2 h vs. 24 h after PD. Additionally, we combined the analysis of brain Fos immunoreactivity (Fos-ir) with the detection of double immunoreactivity in 5HT and oxytocinergic (OT) cells 2 h after PD. Bilateral LPBN injections of methysergide (4 µg/200 nl at each site) increased NaCl intake when tested 2 h after PD compared to controls. We found a significant increase in PRA and ALDO concentration after PD but no differences between 2 and 24 h after PD. We also found for the first time a significant increase 2 h after PD in the number of Fos-ir neurons in the brainstem nuclei that have been shown to be involved in the inhibition of SA. In summary, the results show that 5HT-mechanisms in the LPBN modulate sodium intake during the delay of SA when the renin angiotensin aldosterone system (RAAS) is increased. In addition, the activation of brainstem areas previously associated with the satiety phase of SA is in part responsible for the temporal dissociation between SD and behavioral arousal.

Key words: sodium depletion, sodium appetite, temporary dissociation, lateral parabrachial nucleus, serotonergic system.

INTRODUCTION

Thirst and *sodium appetite* (SA) are the motivational states in animals that lead to the search for and consumption of water and sodium (Na) respectively in order to reestablish hydroelectrolyte balance. There is a temporal dissociation between sodium depletion (SD) and the appearance of SA behavior. Previous studies from our laboratory have shown that acute SD by peritoneal dialysis (PD) produces a rapid and significant drop in volemia and Na concentration in serum and CSF within 1–4 h after PD. Sodium concentration rises gradually until 20 h later when the animals not only recover the normal blood volume and extracellular Na values (possibly by mobilizing body sodium reservoirs) but also the specific SA becomes evident (Ferreyra and Chiaraviglio, 1977).

It is widely known that the renin angiotensin aldosterone system (RAAS) is the principal system involved in the genesis of SA and thirst 24 h after PD or other SA induction procedures such as central injection of Angiotensin II (ANGII). However, the temporal pattern of this system activity after SD has not been described.

The lack of evidence for a specific stimulus that increases SA (in a physiologically relevant model) suggested that it is stimulated primarily by ANGII, but that the appetite for salt is usually held in check by a dominant inhibitory signal. In agreement with this hypothesis and with previous evidences, it has been shown that the

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Abbreviations: 5-HIAA, hydroxyindolacetic acid; 5HT, serotonergic system; 5-HT-ir, 5-HT immunoreactivity; AC, anterior commissure; ALDO, aldosterone; ANGII, Angiotensin II; AP, area postrema; BNSTL, lateral division of the bed nucleus of the stria terminalis; CD, control dialyzed or sham-depleted rats; CeA, central amygdaloid nucleus; CVOs, circumventricular organs; DAB, diaminobenzidine hydrochloride; DRD, dorsal subdivision of DRN; DRN, dorsal raphe nucleus; DRV, ventral subdivisions of DRN; DRVL, ventrolateral subdivision of DRN; EDTA, ethylenediaminetetraacetic acid; Fos-ir, Fos immunoreactivity; LC, locus coeruleus; LPBN, lateral parabrachial nucleus; Methy, methysergide maleate; MnPO, median preoptic nucleus; Na, sodium; NHS, normal horse serum; NTS, nucleus of the solitary tract; OT, oxytocinergic system; OT-ir, OT immunoreactivity; OVLT, organum vasculosum of the lamina terminalis; PaDC, dorsomedial cap; PaMM, medial magnocellular group; PaMP, medial parvocellular group; PaPo, posterior paraventricular subnucleus; PaV, ventral paraventricular subnucleus; PB, phosphate buffer; PD, peritoneal dialysis; PeM, periventricular nucleus; PRA, plasma renin activity; PVN, paraventricular nucleus; RAAS, renin angiotensin aldosterone system; RIA, radioimmunoassay; SA, sodium appetite; SD, sodium depletion; SFO, subfornical organ; SON, supraoptic nucleus; Veh, vehicle.

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satiety or inhibitory mechanisms of SA may involve: (1) *brainstem nuclei* (Contreras and Stetson, 1981; Watson, 1985; Curtis et al., 1996, 1999; Wang and Edwards, 1997; Franchini and Vivas 1999; Olivares et al., 2003; Callera et al., 2005; Godino et al., 2007); (2) *oxytocinergic (OT) neurons* (Stricker and Jalowiec, 1970; Stricker and Verbalis, 1987, 1996; Stricker et al., 1992; Franchini and Vivas 1999; Amico et al., 2001; Godino et al., 2007; Vivas et al., 2013); and (3) *serotonergic (5HT) neuron circuits* (Margatho et al., 2002; Castro et al., 2002, 2003; Franchini et al., 2002; Olivares et al., 2003; Tanaka et al., 2003, 2004; Cavalcante-Lima et al., 2005a,b; Reis, 2007; Badaue-Passos et al., 2007; Fonseca et al., 2009; Godino et al., 2007, 2010, 2013; Menani et al., 2014).

In our studies of the brain areas and neurochemical systems involved in the control of SA (Franchini and Vivas, 1999; Franchini et al., 2002; Godino et al., 2007; Vivas et al., 2013), the circumventricular organs (CVOs) of the lamina terminalis, subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT), were found activated (as shown by Fos immunoreactivity (Fos-ir)), during SA stimulation (24 h after PD). The brainstem nuclei (such as the nucleus of the solitary tract (NTS), area postrema (AP) and lateral parabrachial nucleus (LPBN)) and 5HT and OT neurons in the dorsal raphe nucleus (DRN) and hypothalamic nuclei respectively, were also found activated during the inhibition or satiety phase of SA (after sodium intake induced by 24 h after PD). Although the areas and neurochemical systems associated with SA appearance and satiation are well known, the neurochemical circuit responsible for the temporal dissociation between hyponatremia/hypovolemia and the appearance of SA behavior is still unknown.

Our recent results indicate that the LPBN appears to be an integrative region involved in the processing of information derived from receptive and integrative areas during the satiety or inhibition phase of SA, during blood volume expansion, and modulates the regulatory responses to achieve body fluid homeostasis under these stimuli (Margatho et al., 2008; Godino et al., 2010). Connectional studies also demonstrate that the LPBN is monosynaptically connected with the AP and DRN (Lanca and van der Kooy 1985; Petrov et al., 1992). Serotonin from these nuclei can be released into the LPBN and, in turn, can facilitate this inhibitory pathway, related to the modulation of sodium intake and excretion (Lanca and van der Kooy, 1985; Godino et al., 2010). In addition, a study by de Gobbi et al. (2008), suggests that the LPBN participates in the inhibitory process of SA during extracellular volume expansion, as the stimulation of cardiopulmonary receptors (simulating blood volume expansion) increased the number of Fos-ir neurons along the LPBN and reduced the sodium intake caused by furosemide-captopril treatment.

Taking this into account, the present work focused on the possible areas or systems involved in the temporary dissociation between SD and the appearance of SA. With this purpose, we analyzed the participation of the stimulatory and inhibitory signals of SA at 2 h after PD when the rats were hypovolemic/hyponatremia but SA was not evident, as follows: (1) plasma renin activity (PRA) and plasma aldosterone (ALDO) concentration 2 h vs. 24 h after PD; (2) the effect of serotonergic antagonism at the LPBN (Methysergide, a nonselective 5-hydroxytryptamine (5-HT)1/2 receptor antagonist), on sodium intake 2 h after PD; and (3) the brain Fos-ir pattern and the neurochemical (OT and 5-HT) mechanisms involved in SA regulation, 2 h after PD.

EXPERIMENTAL PROCEDURES

Animals

For the experiments, we used adult male Wistar rats, born and reared in the breeding colony at Instituto Ferreyra (INIMEC-CONICET-UNC, Córdoba, Argentina). Animals weighing 250–300 g were housed singly in metabolic cages with free access to normal sodium diet (Purina Rat chow), distilled water and hypertonic NaCl solution. Room lights were on for 12 h/day and temperature was controlled at 23 °C. All experimental protocols were approved by the appropriate animal care and use committee of our institute, following the guidelines of the international Public Health Service Guide for the Care and Use of Laboratory Animals.

SD

The PD technique, (described in Ferreyra and Chiaraviglio, 1977), consisted of an intraperitoneal injection of 5% glucose solution warmed to 37 °C, at a volume equivalent to 10% of the rat body weight. After 1 h, a needle was inserted into the peritoneal cavity and an equivalent volume of ascitic fluid was withdrawn. In control dialyzed or sham-depleted rats (CD), no injection was given but the needle was inserted into the peritoneal cavity.

PD and CD rats were returned to their individual metabolic home cages without food and with distilled water as the only drink for the following hours prior to the drinking test.

Electrolyte assays and protein determination

The blood was centrifuged and 1 ml of plasma was extracted and stored at −20 °C. The sodium concentration of these samples was analyzed by flame photometry (Hitachi 911, automatic analyzer). Plasma volume was inferred by the plasma protein concentration, measured according to Lowry et al. (1951).

Determination of PRA and ALDO concentration

The trunk blood was collected in chilled plastic tubes containing EDTA. PRA was measured by radioimmunoassay (RIA) of angiotensin I (DiaSorin, Saluggia, Italy) in the presence of reagents that inhibit angiotensin I-converting enzyme and angiotensinases. Intra- and inter-assay coefficients of variation were below 8% and below 6.66%, respectively.

ALDO was measured in plasma samples using a commercial kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). Intra- and inter-assay

coefficients of variation were below 3.3% and below 7.42%, respectively.

Immunohistochemistry

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 normal saline followed by ~400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH7.2). 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% H_2O_2 and 10% methanol until oxygen bubbles ceased appearing. They were incubated in 10% normal horse serum (NHS) in PB for 1 h to block non-specific binding sites.

All the series of the free-floating sections from each brain were first processed for Fos-ir using an avidin–biotin-peroxidase procedure. The sections of the midbrain were then also stained for 5-HT immunor eactivity (5-HT-ir) and those from the hypothal amus for OT immunoreactivity (OT-ir). The staining procedures followed the double-labeling proce dures described in Franchini and Vivas (1999) and Franchini et al. (2002). 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, Calbiochem, Darmstadt, Germany), diluted 1:10,000 in PB containing 2% NHS (Gibco, Auckland, NZ, USA) and 0.3% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA). The sections were then washed with PB and incubated with biotin-labeled anti-rabbit immunoglobulin and avidin–biotin-peroxidase complex (Vector Laboratories Inc., Burlingame, CA USA, 1:200 dilutions in 1% NHS-PB) 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 series of Fos-labeled sections, also processed for immunocytochemical localization of 5HT and OT, were incubated for 72 h at 4 °C with their corresponding antibodies: polyclonal rabbit anti-5HT antibody (ImmunoStar Inc, WI, USA, dilution 1:10,000) and polyclonal rabbit anti-OT antibody (Calbiochem, Darmstadt, Germany, dilution: 1:25,000). After incubation, the sections were rinsed and incubated with biotin-labeled anti-rabbit immunoglobulin and avidin–biotin-peroxidase complex for 1 h at room temperature. Cytoplasmic 5-HT-ir and OT-ir were detected with unintensified DAB that 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 which had been preadsorbed with an excess of Fos peptide, or by processing sections without the primary antiserum. No Fos-ir neurons were observed following 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 (1997). The different subnuclei of paraventricular nucleus (PVN) analyzed in the present work were counted at three different levels of PVN, anterior, medial and posterior (distance from the bregma of the corresponding plates: −1.30, −1.8 and −2.12 mm respectively). The distance from the bregma of the corresponding plates is indicated between brackets: supraoptic nucleus (SON), (−1.3 mm), central amygdaloid nucleus (CeA −2.3 mm), lateral division of the bed nucleus of the stria terminalis (BNSTL, −0.26 mm), SFO (−0.92 mm), organum vascul osum of the lamina terminalis (OVLT, −0.20 mm), median preoptic nucleus (MnPO, −0.30 mm), DRN (−8.00 mm), Locus coeruleus (LC: −10.04 mm), LPBN (−9.3 mm), the nucleus of the solitary tract (NTS intermediate −13.24 and caudal −13.68) and area postrema (AP −13.68 mm).

Fos-ir nuclei were quantified using a computerized system including a Zeiss microscope equipped with a DC 200 Leica digital camera attached to a contrast enhancement device. Images were digitized and analyzed using Scion Image PC, based on the NIH 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 at exactly the same level in each group were acquired with the aid of the Adobe Photoshop Image Analysis Program, version 5.5. The counting was done in six 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 researcher who conducted the counting of Fos-ir cells was not aware of the experimental condition.

LPBN cannulation

The rats were anesthetized with ketamine (80 mg/kg of body weight, Agener União, SP, Brazil) combined with xylazine (7 mg/kg of body weight, Agener União, SP, Brazil) and placed in a Kopf stereotaxic apparatus (model 900). The skull was leveled between the bregma and lambda. Stainless steel guide cannulas (0.4 mm ID, 0.6 mm OD) were implanted bilaterally in the LPBN using the coordinates: 9.4 mm caudal to the bregma, 2.2 mm lateral to the midline and 4.1 mm below the dura mater. The tips of the guide cannulas were targeted to terminate 2 mm above LPBN. Cannulas were fixed to the cranium using dental acrylic resin and two jeweler's screws. A 30-gauge metal wire filled the cannulas, except during injections. After surgery, the rats received a prophylactic injection of penicillin (20,000 units im) and they were allowed to recover for 7 days, during which they were handled daily and habituated to the removal of the obturator of the guide cannula.

Histology of LPBN

After the experiments, the animals received bilaterally 2% solution of Evans Blue (200 nl/site) into the LPBN. They were deeply anesthetized with sodium thiopental (80 mg/kg of body wt) and perfused transcardially with isotonic NaCl followed by 10% formalin. The brain was removed, fixed in 10% formalin, frozen, cut in 50-μm coronal sections, stained with Cresyl Violet and analyzed by light microscopy (Axioskope 35 M; Zeiss) to locate the injection site in the LPBN.

Drugs

Methysergide maleate (Methy, 4 μg, Tocris, nonselective 5-HT1/2 receptor antagonist) was dissolved in a solution of propylene glycol/water 2:1. The dose of methysergide used in the present study was selected based on a previous report (4 μg/200 nl per animal, Margatho et al., 2002). The LPBN injections were performed using a 10-μl Hamilton syringe connected by polyethylene tubing (PE-10) to an injector needle (0.3 mm OD). Each rat received only one LPBN treatment.

Statistical analysis

The data of brain activity pattern and the activity of OT and 5HT systems were analyzed using Student's *t*-test. A two-way ANOVA was used for the analysis of plasma electrolytes, protein and plasma PRA and ALDO concentration. A one-way repeated measures ANOVA was used for the analysis of sodium and water intake. Post hoc comparisons were made using the Tukey test. All the results were shown as group mean values ± SE, and the significance level was set at $p < 0.05$.

Experimental protocols

As shown in Fig. 1, we ran three different experiments as follows:

Experiment 1: Plasma RAAS, sodium intake, volemia and natremia determinations 2 and 24 h after SD induced by PD. Animals were sodium depleted and 2 h or 24 h later they were decapitated and bled. The plasma was used to measure plasma sodium and protein concentration, and the plasma RAAS system by analyzing ALDO concentration and PRA. In order to confirm our previous studies, a group of PD and CD rats were housed individually in metabolic cages without food and with distilled water as the only drink. Two or twenty-four hours later PD and CD groups were given access to the hypertonic sodium and water intake test. The cumulative volume drunk was measured from graduated buret during 60 min.

Experiment 2: Effect of LPBN serotonergic antagonism on SA after PD. The PD rats were housed individually in metabolic cages without food and with distilled water as the only drink. Two or twenty-four hours later PD and control groups were given access to the hypertonic sodium and water intake test. The injection of Methy or Veh was performed bilaterally 10 min before the intake test. The cumulative volume drunk was measured from graduated buret during 60 min.

Experiment 3: Brain pattern of Fos-ir and double-immunolabeled (Fos-5-HT and Fos-OT) cells 2 h after PD. A separate group of animals were sodium depleted by PD and two hours later they were perfused for immunohistochemical detection of Fos and OT or 5-HT.

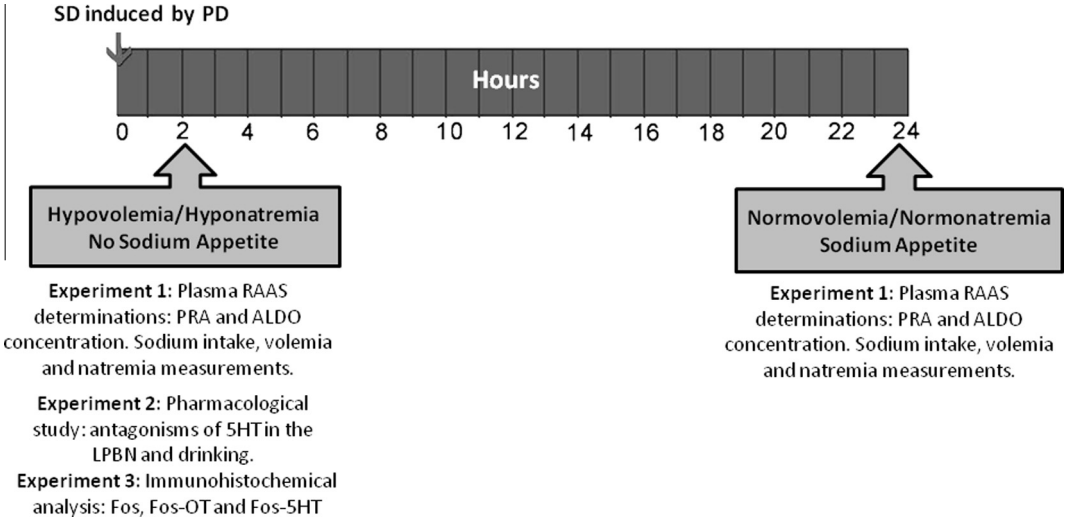

Fig. 1. Schematic diagram showing the time of manipulations after sodium depletion induced by peritoneal dialysis. Peritoneal dialysis was performed by an ip injection of 5% glucose solution, at a volume equivalent to 10% of the rat body weight. After 1 h, a needle was inserted into the peritoneal cavity and an equivalent volume of ascitic fluid was drawn. Experiment 1- Animals were sodium depleted and 2 h or 24 h later they were decapitated and bled. The plasma was used to measure plasma sodium and protein concentration, and plasma RAAS by analyzing ALDO concentration and PRA. Other PD and CD rats were housed individually in metabolic cages without food and with distilled water as the only drink. Two and twenty-four hours later, PD and CD groups were given access to the sodium and water intake test. Experiment 2- The rats with LPBN cannulas were submitted to PD procedure and sodium and water intake were analyzed 2 and 24 h later and 10 min after the injection of methysergide or vehicle in the LPBN. Experiment 3- animals were sodium depleted and two hours later they were perfused for immunohistochemical detection of Fos and OT or 5-HT.

RESULTS

Experiment 1: Plasma RAAS, sodium intake, volemia and natremia determinations 2 and 24 h after SD induced by PD

Plasma electrolytes, blood volume and sodium intake. As shown in Table 1, during the first 2 h after SD induced by PD, plasma sodium concentration and blood volume were reduced, and both parameters were re-established without any access to sodium solution 20–24 h later. The ANOVA for plasma sodium and protein concentration indicated that there was a significant interaction between both factors analyzed, i.e., depletion (PD) vs. sham depletion (CD) and 2 h vs. 24 h after PD, (Plasma sodium concentration $F1,22 = 5.34$, $p = 0.03$; plasma protein concentration $F1,20 = 4.77$, $p = 0.041$). On the other hand, sodium intake was not evident 2 h after PD when hyponatremia and hypovolemia occurred, but appeared when these parameters were re-established 24 h after PD (Table 1).

PRA and ALDO concentration. As expected, sodium-depleted animals showed an increase in PRA and ALDO concentration. An ANOVA for PRA (Fig. 2A) and ALDO (Fig. 2B) indicated that there was a significant difference in both parameters related only to the SD factor (PRA: $F1,27 = 45.53$, $p < 0.0001$; ALDO: $F1,29 = 103.02$, $p < 0.0001$). The time after PD factor and the interaction of both factors did not show statistically significant levels (Fig. 2). The PD groups had higher levels of PRA and ALDO concentration than CD groups, regardless of time after SD.

Table 1. Plasma sodium, protein concentrations and sodium intake measured in SD and CD animals 2 and 24 h after sodium depletion

Groups	Plasma Na+ concentration mEq/l	Plasma protein concentration g/dl	Sodium intake ml/100 gbw
SD 2 h	135.80 ± 4.77*	9.016 ± 0.26*	0.000 ± 0.20
SD 24 h	144.11 ± 0.48	7.791 ± 0.16	1.331 ± 0.18*
CD 2 h	143.88 ± 2.20	7.910 ± 0.26	0.000 ± 0.20
CD 24 h	143.70 ± 1.83	7.677 ± 0.21	0.227 ± 0.19

Values are mean ± SE; $n = 5$.
* $P < 0.05$ significantly different between groups.

Experiment 2: Effect of LPBN serotonergic antagonism on SA after PD

Histological analysis. In the present study, the injection sites were, as usual, centered in the central lateral and external lateral portions of the LPBN. Injections reaching the ventral lateral and dorsal lateral portions, as well as the Kölliker-Fuse nucleus, were observed in some rats and these results were also included in the analysis. The spread of the injection was almost completely above the brachium (superior cerebellar peduncle), Fig. 3C.

Effects of bilateral injections of methysergide into the LPBN on hypertonic NaCl and water intake. Bilateral injections of methysergide (4 μg/200 nl each site) into the LPBN, in the absence of any other treatment, produced no effect on the ingestion of 0.3 M NaCl (0.21 ± 0.01 ml/h, $n = 6$ rats) and a slight effect on water intake (0.88 ± 0.11 ml/1 h).

Bilateral injections of Methy (4 μg/0.2 μl each site) into the LPBN significantly increased hypertonic NaCl intake 2 h after PD compared to the control treatment (2 h Veh). Post hoc comparisons also showed significant differences between 24 h Veh and 2 h Methy for 0.3 M NaCl ($F(2, 24) = 55.0$; $p = 0.05$) intake at 45 and 60 min but not at the beginning of the intake test (15 and 30 min) Fig. 3A.

As shown in Fig. 3B, bilateral injections of Methy did not produce any significant changes in water intake 2 h after PD (interaction: SD vs. time factors $F(1, 26) = 12.984$, $p = 0.00130$, pos-hoc Tukey).

Experiment 3: Brain pattern of Fos-ir and double-immunolabeled (Fos-5-HT and Fos-OT) cells 2 h after PD

Brain pattern of double-immunolabeled (Fos-5HT and Fos-OT) cells 2 h after SD. DRN: The study analyzed the DRN at its anterior level since this was previously implicated in SA regulation (Franchini et al., 2002;

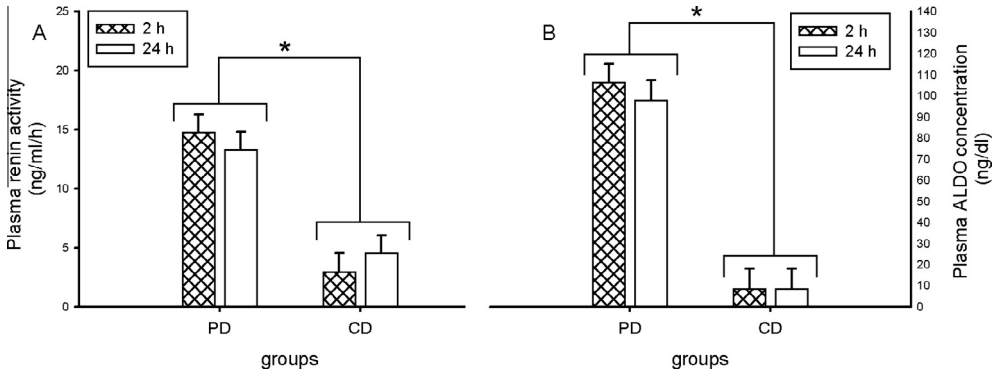

Fig. 2. Plasma renin activity (A) and plasma aldosterone concentration (B) 2 and 24 h after sodium depletion induced by PD and control depletion. Values are means ± SE. (2-way ANOVA, $n = 8$) *$p < 0.05$ in relation to CD groups.

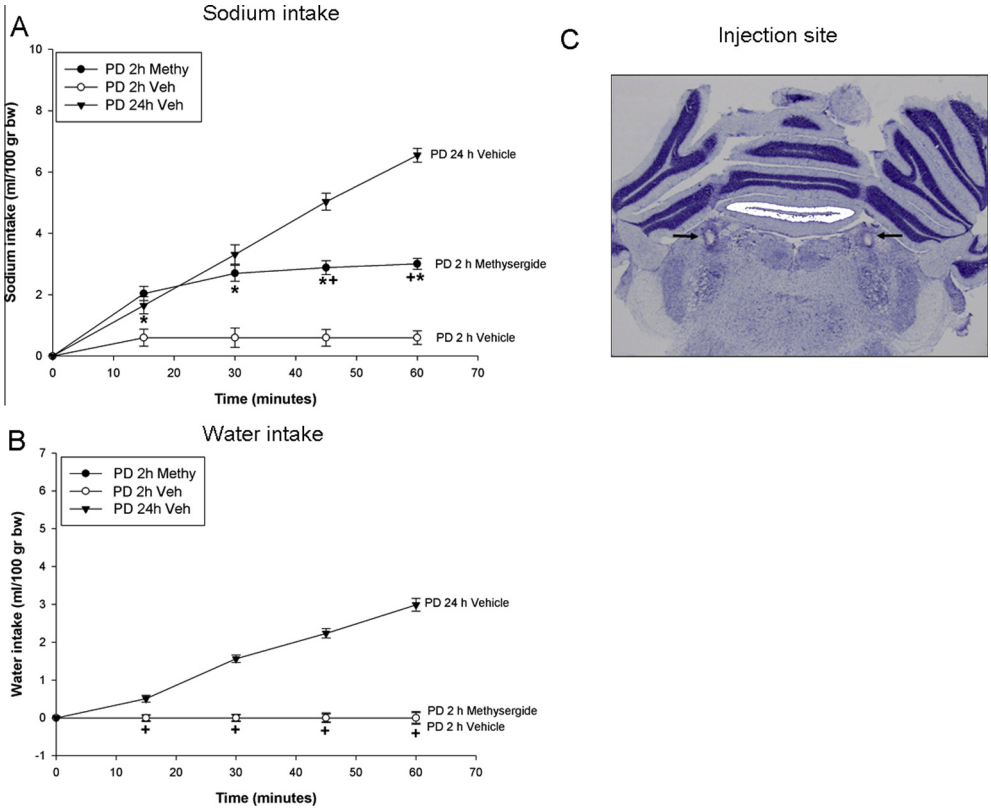

Fig. 3. Cumulative sodium intake (A) and water intake (B) at 2 and 24 h after PD in rats previously injected with vehicle (Veh) or methysergide (Methy, 4 μg/0.2 μl) into the LPBN. The results are represented by means ± S.E.M (one-way ANOVA repeated measures, Veh, $n = 6$ and Methy $n = 9$). $^{*}P < 0.05$ vs. 2 h Veh and $^{+}p < 0.05$ vs. 24 h Veh. (C) Photomicrography of the LPBN section showing the injection site.

Godino et al., 2007). At this level, this nucleus is composed of dorsal (DRD), ventrolateral (DRVL) and ventral (DRV) subdivisions. Two hours after SD, no significant change was induced in the number of Fos immunoreactive neurons within the DRD, DRV or DRVL subdivisions of DRN in relation to the control group (DRD: $t = 0.74$, $p = 0.48$; DRV: $t = 1.27$ $p = 0.24$; DRVL: $t = 0.12$ $p = 0.91$) (Fig. 4A). Also no significant difference was observed between PD and CD conditions in the number of Fos-5HT positive cells within these subdivisions (DRD: $t = -0.53$, $p = 0.61$; DRV: $t = -0.79$, $p = 0.46$; DRVL: $t = -0.33$, $p = 0.75$) (Fig. 4B, C). Despite the hyponatremic and hypovolemic states induced by SD at 2 h after PD, there was no change in the activity of DRN neurons in relation to normovolemic/normonatremic rats, suggesting that at this point in time DRN-serotonergic cells in the PD group had the same tonic activity as in the control group. It is important to note that, in previous studies, we have observed that this tonic activation of serotonergic cells (Fos-5HT), which is postulated as exerting a tonic inhibition of SA (Franchini et al., 2002; Godino et al., 2007, 2010; Reis, 2007; Menani et al., 2014), is reduced 24 h after PD, allowing SA to become evident.

SON and PVN: C-fos expression did not show any significant difference in the SON, 2 h after SD induced by PD, compared to the CD group ($t = -0.51$, $p = 0.62$) (Table 2). Likewise, we did not find any difference between the PD and CD groups in the number of Fos-OT positive neurons ($t = 1.11$, $p = 0.30$), which was almost zero.

Within the magnocellular subdivisions of the PVN, the medial magnocellular group (PaMM, mostly OT neurons), the lateral magnocellular group (PaLM, mostly AVP neurons) and the accessory magnocellular nuclei, such as the anterior commissure (AC) and periventricular nucleus (PeM), the number of Fos-ir neurons and Fos-OT neurons showed no statistically significant difference between experimental and control groups (Table 2) (Fos-ir, PaMM: $t = 11.67$, $p = 0.56$; PaML: $t = 2.19$, $p = 0.054$; AC: $t = -0.17$, $p = 0.86$; PeM: $t = 1.75$, $p = 0.12$) (Fos-OT, PaMM: $t = 0.41$, $p = 0.69$; PaML: $t = -1.32$, $p = 0.22$; AC: $t = -0.87$, $p = 0.40$; PeM: $t = -1$ $p = 0.34$).

The medial portion of the PVN included in the parvocellular neurosecretory (PaMP) component as well as three subdivisions of the nonendrocrine projection neurons, the dorsomedial cap (PaDC) and the ventral and posterior paraventricular subnucleus (PaV and PaPo respectively), did not show a significant increase in the number of Fos-ir and Fos-OT neurons in PD compared with the control group (Ir-Fos, PaMP/PaV: $t = 1.82$, $p = 0.098$; PaPo: $t = 1.81$ $p = 0.099$; PaDC: $t = 0.91$, $p = 0.38$) (Fos-OT, PaPo: $t = 0$, $p = 0.99$ PaDC: $t = 0.41$, $p = 0.69$). In summary, the number of activated OT neurons in the PVN and SON is almost zero in both groups (CD and PD). The possible interpretations of these results are detailed in the discussion section.

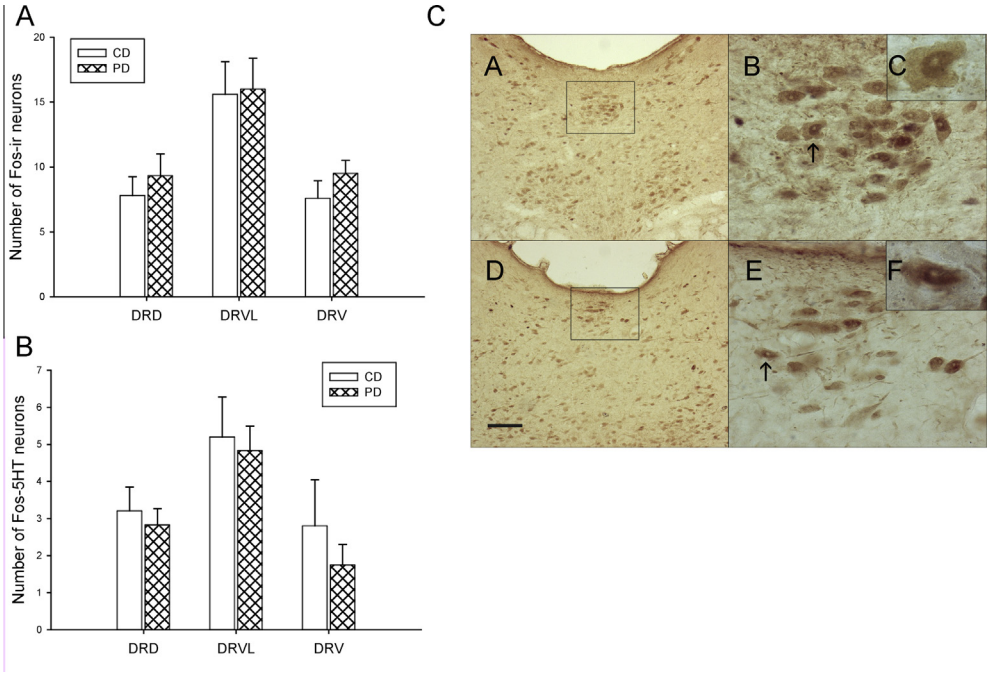

Fig. 4. Average number of Fos-ir neurons (A) and of double-immunolabeled Fos-5HT neurons (B) in the dorsal raphe nucleus subdivisions (DRD, DRV, DRVL) 2 h after sodium depletion. Values are means ± SE. Tukey test PD $n = 6$ and CD $n = 5$, $^{*}p < 0.05$ vs. CD. (C) Photomicrography representing the pattern of double immunoreactive Fos-5HT cells within DRN (A–F) 2 h after sodium depletion, in the CD (A–C) and PD groups (D–F). Plates B, E, are higher magnifications (40×) of the sectors indicated in plates A and D. Plates C and F are higher magnifications (100×) of cells indicated by arrows in plates B and E. Scale bar = 100 μm.

Table 2. Hypothalamic pattern of Fos-ir and double-immunolabeled (Fos-OT) cells 2 h after sodium depletion

Area	Fos-ir		Fos-OT	
	PD	CD	PD	CD
SON	2.20 ± 1.14	3.00 ± 0.65	0.60 ± 0.67	0.00 ± 0.00
PaMM	16.67 ± 8.45	11.67 ± 3.43	0.50 ± 0.37	0.33 ± 0.23
PaML	14.33 ± 2.90	7.17 ± 2.10	0.17 ± 0.18	0.83 ± 0.52
AC	5.83 ± 0.82	6.17 ± 1.92	0.17 ± 0.18	0.50 ± 0.37
PeM	9.83 ± 2.55	5.17 ± 1.42	0.00 ± 0.00	0.17 ± 0.18
PaMP/PaV	34.67 ± 6.82	19.50 ± 6.03	–	–
PaDC	9.83 ± 1.42	7.83 ± 1.92	0.67 ± 0.36	0.50 ± 0.24
PaPo	49.17 ± 10.18	28.67 ± 7.02	0.50 ± 0.25	0.50 ± 0.37

Values are means ± SE; $n = 5$.

Brain pattern of Fos immunoreactive neurons induced by SD at 2 h after PD in other nuclei involved in sodium balance regulation. Brainstem: The study shows that 2 h after PD, the number of Fos-ir neurons increased within the NTS, AP and LPBN in relation to the CD group (NTS intermediate: $t = 2.8$, $p = 0.025$; NTS caudal: $t = 5.51$ $p = 0.0004$; AP: $t = 3.95$, $p = 0.003$ and LPBN: $t = 2.44$, $p = 0.034$) (Fig. 5A, D). Fos-ir in the LC tended to increase in the PD group, although this difference did not reach a significant level (LC: $t = 1.91$, $p = 0.09$) (Fig. 4A). It is important to note that, in previous studies, we have observed that NTS, AP and LPBN activity decreased during the appearance of SA 24 h after PD (Franchini and Vivas, 1999) and increased after sodium consumption, suggesting their involvement in the inhibition of SA.

Lamina terminalis: Two hours after SD, the PD group showed an increased number of Fos-ir cells in the three structures of the lamina terminalis, OVLT, MnPO, SFO (OVLT: $t = 3.88$, $p = 0.003$; MnPO: $t = 2.56$, $p = 0.03$; SFO: $t = 2.97$, $p = 0.014$), compared to the CD group (Fig. 5B). We analyzed the ventral and dorsal part of MnPO, and the higher Ir-Fos level was localized in the ventral part of this nucleus.

Central extended amygdala: Two components of the central extended amygdala were analyzed in this study: CeA and BNSTL. As in our previous data 24 h after SD (Johnson et al., 1999), we found CeA and BNSTL neuronal activity 2 h after SD is increased in comparison with the CD group (Fig. 5C).

DISCUSSION

This study demonstrates how the temporal dissociation between PD-induced SD and SA appearance is modulated by the interaction between the stimulatory

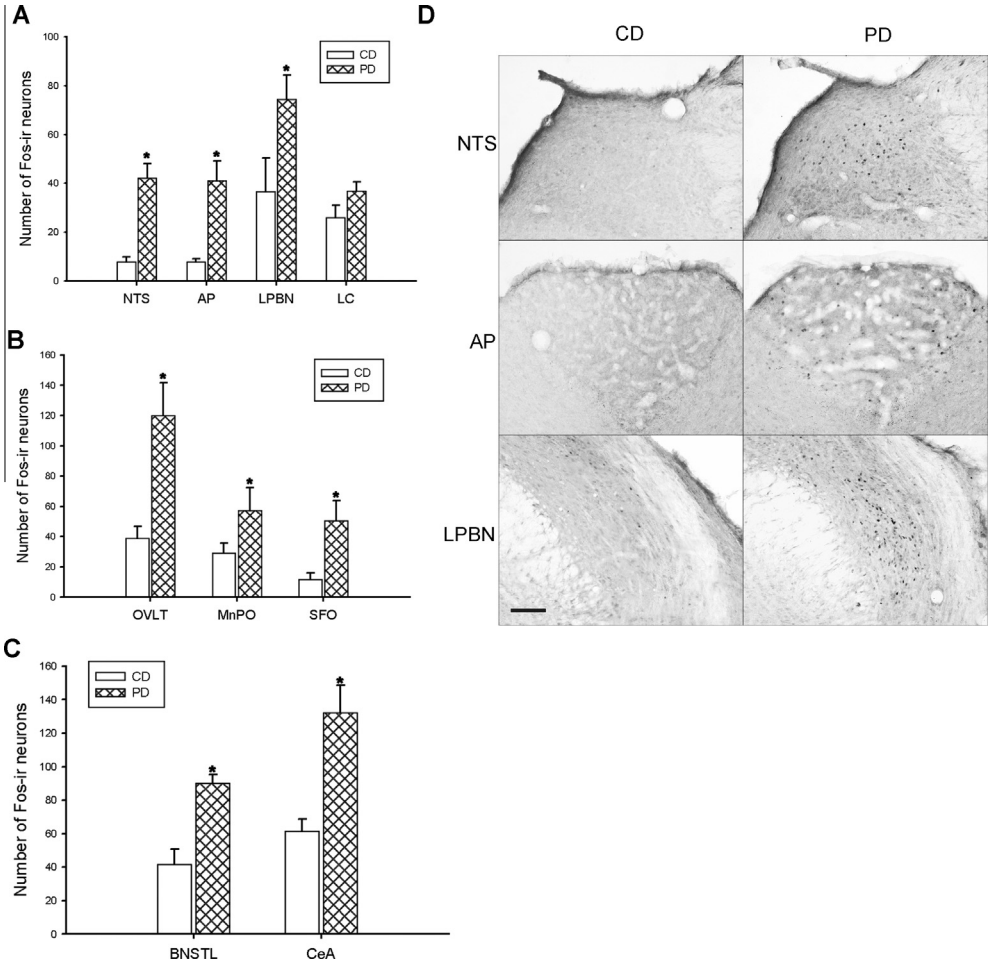

Fig. 5. Average number of Fos-ir neurons in the brainstem nuclei (NTS, AP, LPBN and LC) (A), in the lamina terminalis nuclei (OVLT, MnPO and SFO) (B) and in the central extended amygdala nuclei (BNSTL and CeA) (C) 2 h after PD. Values are means ± SE. Tukey test PD $n = 6$ and CD $n = 6$, $^{*}p < 0.05$ vs. CD. Photomicrograph representing the pattern of Fos-ir along the brainstem nuclei: NTS, AP and LPBN, in PD and control (CD) groups (D). Scale bar = 100 μm.

and inhibitory systems. The antagonism of 5HT at the LPBN indicates that the serotonergic system within the LPBN is involved in SA inhibition immediately after PD, when natremia and volemia are abruptly reduced and the PRA and ALDO plasma concentrations are increased.

The study also identifies the specific brain pattern of activity at forebrain and hindbrain level, 2 h after PD during the peak of hyponatremia and hypovolemia. Our results also demonstrated for the first time, during the delay of SA appearance, the activation of brainstem nuclei, i.e. the NTS, AP, and LPBN, which have been shown to be involved in the inhibition of SA during the satiety or consummatory phase (Houpt et al., 1998; Franchini and Vivas, 1999; Margatho et al., 2002). The double-labeling study suggests that: (1) the tonic activity of DRN 5HT cells, postulated to be inhibitory for salt appetite (Franchini et al., 2002; Godino et al., 2007, 2010; Reis, 2007; Menani et al., 2014), is still present; and that (2) there is no activation of the other hypothesized inhibitory system (i.e. the OT circuit), which may explain the delay in the appearance of SA, at least in this SD model and at the level of SON and PVN.

In a previous work using PD, SA behavior appeared 10 h after SD and was highly stimulated 20–24 h later (Ferreyra and Chiaraviglio, 1977). A work from our laboratory indicated that, after SD by PD, plasma and cerebrospinal fluid sodium levels decrease rapidly, particularly during the first 4 h after PD. This reduction in sodium levels was followed by a period of slow recovery, and sodium levels reached baseline by ~24 h when the specific Na appetite also became evident. Additionally, the results demonstrated that dialyzed animals exhibited a significant decrease in blood volume immediately after PD, which returned to control values 12 h later (Ferreyra and Chiaraviglio, 1977). The initial drop in blood volume and serum sodium immediately after PD stimulates body sodium release from reservoirs such as the skin (Schafflhuber et al., 2007; Dahlmann et al., 2015; Hofmeister et al., 2015). Consequently we may hypothesize that the relative increase in plasma osmolality and sodium concentration enables the stimulation of water intake in order to establish blood volume and natremia first, before SA appears and finally recovers the total amount of body sodium. In the present study, we also

demonstrated that, 2 and 24 h after PD, PRA and ALDO were significantly increased compared with control groups. This means that changes in plasma sodium concentration, plasma volume or PRA and ALDO concentration are not directly correlated with the onset of SA.

The current evidence shows, as expected, that neurons within the CVOs of the lamina terminalis were active 2 h after PD, maybe sensing the acute drop in plasma Na concentration and the increased levels of angiotensin induced by PD, despite the fact that appetitive behavior is still absent. We also observed that the sodium-appetite inhibitory systems, such as the 5-HT system (but not the OT system), were active. More surprisingly, we found activation along brainstem nuclei, such as the NTS, AP and LPBN, that were previously shown to be active only in the satiety phase or during the inhibition of SA after ingestion, but not 24 h after PD when the SA appears (Franchini and Vivas, 1999; Franchini et al., 2002). These data are consistent with the possibility that circuits including population cells of the NTS, AP, LPBN and DRN are responsible for the delay in the appearance of SA during the first hours after body sodium deficit. Potts et al. (2000) demonstrated in rabbit that hypovolemia increased c-fos expression in these brainstem nuclei, suggesting that hypovolemia induced 2 h after PD may possibly activate these areas involved in the inhibition of SA.

Our previous studies demonstrated that both OT neural activity and OT plasma levels were increased by the entrance of hypertonic NaCl solution during sodium consumption, independently of the satiety conditions of the animals, i.e., whether the animals were in a sodium satiation process after depletion or were non-deprived animals during need-free sodium ingestion. Taken together, the results suggest that the OT system is involved in the inhibition of sodium intake as a marker of hypertonicity and not as a satiety marker (Godino et al., 2007). As mentioned earlier, Stricker and Verbalis' hypothesis proposes the OT system as a central mechanism that blocks SA (but not thirst) until a sufficient amount of water has been ingested to cause osmotic dilution of the extracellular fluid (Stricker and Jalowiec, 1970; Stricker and Verbalis 1987, 1996; Stricker et al., 1992). The present data do not show OT system activity to be involved in the inhibition of SA during the first hours after SD, since Fos-ir within the OT PVN and SON cells was almost absent. However, we cannot rule out the participation of another OT circuit that is possibly undetected by the immunohistochemical technique used in this work.

Our previous work showed 5-HT system involvement in the inhibition of SA, since the activity of DRN serotonergic cells decreased during the appetitive phase (24 h after PD before drinking) and increased during the satiety phase (after PD-induced sodium intake), when the animals are in the process of re-establishing body sodium status levels (Franchini et al., 2002; Godino et al., 2007, 2010). We recently demonstrated that sodium overload increased the firing frequency activity of 5HT-DRN neurons during extracellular recording (Godino et al., 2013). Following the same idea, in the present study, the identical activity observed in the PD and control animals may reflect the tonic activity still being present 2 h after PD, modulating the inhibition of SA and consequently the temporal dissociation between SD and SA.

This study supports the idea that there is a specific 5-HT tonic inhibition of SA at LPBN level during the hyponatremic/hypovolemic state, since SA was partially released by the administration of the nonselective 5-HT1/2-receptor antagonist, methysergide, into the LPBN. Menani et al., 2000, demonstrated that the antagonism in the LPBN is able to produce a rapid increase in SA induced by furosemide or isoproterenol treatment. In agreement with these data, a microdialysis study (Tanaka et al., 2004), measuring extracellular levels of 5-HT and its metabolite 5-hydroxyindolacetic acid (5-HIAA) in the LPBN, shows that acute SD, induced by combined furosemide-captopril treatment, causes a significant decrease in extracellular 5-HT and 5-HIAA concentration. Moreover, sodium drinking stimulated by furosemide-captopril treatment produces the opposite effect on 5-HT release in the LPBN (Tanaka et al., 2004). Our results also indicate that, when an ANGII signal - induced by PD - is present, the local LPBN 5HT antagonism is enough to induce SA. However, we also observed a partial hypertonic sodium intake release compared with the animals 24 h after PD, suggesting that there are possibly other mechanisms, such as visceral (baroreceptor or mechanoreceptor) or peripheral signals, reaching brainstem areas that might be involved in the inhibition of SA at 2 h PD (Potts et al., 2000). These studies strongly support the idea that the LPBN-5-HT system is in part involved in the inhibition of SA during the temporal dissociation between SD and SA.

There is also evidence that the LPBN has bidirectional connections with the BNSTL and CeA, two structures also involved in the control of SA. A recent study indicates that serotonergic mechanisms in the LPBN modulate the activity of these areas, since the antagonism of the 5-HT system at LPBN level during isoproterenol treatment (which typically stimulates only thirst) increased ingestion of hypertonic saline and increased the activity of these nuclei (Krukoff et al., 1993; Jhamandas et al., 1996; Davern and McKinley, 2010). These data confirm that the serotonergic system at LPBN level participates in the inhibition of SA when the RAAS is activated, and it also suggests that the LPBN has an important connection with areas involved in the modulation of SA arousal.

It has also been demonstrated that the RAAS in the SFO, which is a target organ for ANGII to induce ingestive behavior, has a functional antagonism with the serotonergic system. Likewise, the microinjection of ANGII in the SFO decreases local extracellular concentration of 5-HT and 5HIAA (Tanaka et al., 1998, 2003). Finally, it has been shown that the 5HT system at the LPBN blocks SA induced by ANGII injected into the SFO, while AT1 receptor blockade by losartan in the SFO blocks sodium intake induced by ANGII in the SFO combined with the 5-HT antagonist in the LPBN (Colombari et al., 1996). Together, these studies and the present results raise the possibility that, despite renin-angiotensin levels (as shown by PRA) being higher

2 h after body SD, this signal is not enough at this stage to decrease activity in the 5HT-LPBN system, which needs to be inhibited in order to release SA from inhibition.

The importance of the physiological role of these inhibitory mechanisms and neural circuits that promote a delay in the onset of SA after a body sodium deficit can be seen in clinical cases in which the rapid correction of chronic hyponatremia through the infusion of sodium chloride solutions causes severe damage such as cerebral edema, central pontine myelinolysis, coma or even death (Goh, 2004).

In summary, the results show that serotonergic mechanisms in the LPBN-DRN pathway modulate sodium intake during the delay of SA 2 h after PD when RAAS is activated. The results also suggest that the activation of areas previously associated with the satiety phase of SA, such as the NTS, AP, DRN and LPBN, are in part responsible for the temporary dissociation between SD and the arousal of SA.

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