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AT_1 receptor role in the hypothalamic and renal function interaction

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Abstract

Angiotensin II (ANG II) is involved in renal sodium homeostasis under normal and pathological conditions in close relation with the sympathetic nervous system. Vasopressin, a hormone that modulates renal sodium and water reabsorption, is synthesized and released in the supraoptic and paraventricular nucleus under the influence of ANG II. We hypothesized that brain ANG II (AT_1) receptors regulate renal sodium and water reabsorption and excretion through the sympathetic nervous system. In this study, male Wistar rats with renal denervation/sham were fed a hypersodic (4%) or normal (0.4%) diet and evaluated during 5 days in metabolic cages. On day 5, they were injected in the lateral ventricle with an AT_1 receptor antagonist, losartan, and sacrificed 12 h later; blood samples and brains were obtained for evaluation. The urine was collected daily. The neuronal activation was analyzed in the nucleus of the supraoptic, paraventricular, subfornical, and organum vasculosum of the lamina terminalis. Activation of vasopressin neurons was evaluated in the supraoptic nucleus. Depending on renal nerve integrity, the hypersodic diet or losartan administration differentially affected neuronal activation. In sham animals, losartan prevented the stimulatory effects induced by the hypersodic diet in water intake and the neuronal activation in vasopressin-positive neurons. Renal denervation modified the effect of the hypersodic diet on water intake, urinary volume, and creatinine excretion, and losartan administration was able to prevent these alterations. Food intake was similar in all groups. Our results suggest that brain AT_1 receptors regulate renal sodium and water reabsorption through the sympathetic nervous system in close interaction with vasopressin.

Keywords: Renal denervation; Kidney; Vasopressin; Losartan; Sodium intake

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1. Introduction

Brain angiotensin II (ANG II) action through AT_1 receptors (AT_1-R) is involved in the regulation of cerebral blood flow, body fluids, and mineral balance and osmoregulatory homeostasis-associated behaviors such as thirst and sodium intake[1-4]. ANG II is a neuropeptide that when activating AT_1-R, plays a key role in the regulation of the sympathetic and neuroendocrine systems[5]. Moreover, peripheral ANG II acting

through AT_1-R is involved in renal sodium homeostasis under normal and pathological conditions in close relation to the sympathetic nervous system (SNS). The anatomical and physiological evidence shows that sympathetic nerves innervate the juxtaglomerular cells, renal tubules, and vasculature[6,7]. Hence, the frequency of the renal sympathetic nerve activation (RSNA) mediates the increase in sodium and water excretion regulating tubular renal water and sodium reabsorption in the nephron, promoting changes in renal function, blood flow, and glomerular filtration rate. However, any change in water and sodium intake can modify their excretion and absorption. The SNS regulates renal vasculature vasoconstriction and peripheral ANG II generation by renin release from juxtaglomerular cells[8,9].

Cardiovascular and hydrosaline homeostasis maintenance requires fine coordination between the neuroendocrine and autonomic systems. The central nervous system (CNS) receives information from multiple sensors (osmolarity, pH, pressure, blood volume, and circulating hormones) and integrates the signals generating adaptive responses[10]. The hydromineral and osmotic regulation depend on arginine-vasopressin (AVP) and oxytocin released from the supraoptic nucleus (SON) and paraventricular nucleus (PVN) which both playing a key role in sodium and water excretion[11]. The osmotic sensors are in the subfornical (SFO) and organum vasculosum of the lamina terminalis (OVLT), placed outside the blood-brain barrier[12-14]. Moreover, there are direct and indirect interactions between SNS and CNS through specific innervation or mediation by ANG II that regulate renal sympathetic activity. Evidence shows that the increase in the RSNA can be blunted with angiotensin-converting enzyme inhibitors (ACEI) or AT_1-R antagonists[6]. In the same way, the effects of ANG II intrarenal administration are decreased after renal denervation[6]. The interactions are modulated by changes in the renin-angiotensin system activation that control RSNA and its arterial baroreflex[15]. The kidney is the target for oxytocin and AVP hormones that control water and sodium excretion, released in response to hypovolemia or hyperosmolarity[16,17]. In addition to its neuroendocrine actions, AVP induces a fast and enduring increase in SNS activity[18,19].

In addition to the effects induced by circulating ANG II on circumventricular organs, the effects inside the blood-brain barrier are mediated by local ANG II[20,21]. It has been found that central administration of AT_1-R antagonist can modulate changes in brain ANG II activity induced by physiological alterations in sodium intake. This modulation was also observed in a physiopathological model (congestive cardiac insufficiency), where there is an increase in brain ANG II and SNS activity. Intra- and extra-renal interactions between the renin-angiotensin system and RSNA are involved in the neural control of renal function[6]. ANG II through AT_1-R placed in kidney tubules and vessels exert direct actions on sodium, chlorine, and water reabsorption, as well as in vascular constriction[6].

Considering the above-mentioned evidence, we aimed to evaluate the brain AT_1-R's role in renal function mediated by its interaction with SNS under two independent conditions: Sham or renal denervation. The hypersodic diet was used to inhibit peripheral ANG II while stimulating brain ANG II. This condition can unmask the brain AT_1-R influence over SNS under surgical renal denervation[22].

2. Materials and methods

2.1. Animals

Male Wistar rats (250–300g) were used. The animals were maintained under controlled environmental conditions (20–24°C, 12 h light/dark cycle with lights on at 7 a.m.) with *ad libitum* access to food and water and were randomly housed in groups of 5 per cage (34 × 48 × 19 cm). All procedures were approved by the Animal Care and Use Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina (Res. No. 270/18), in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

The selective AT_1-R antagonist, losartan (Los, Sigma Aldrich), was dissolved in artificial cerebrospinal fluid (ACF). The solution was freshly used, protected from light, and kept at 4°C. The dose (4 µg/µL) was selected considering previous studies[23-25].

2.3. Experimental design

In this work, a 6-day protocol with animals receiving normal sodium (0.4%) or high sodium (4%) diet was performed. Stereotaxic surgery was executed on day 1, Los was administered on day 5 in the right and left lateral ventricles, and 12 h after Los administration, plasma, kidney, and SNC samples were taken (Figure 1).

2.4. Surgery for cannulae implantation and denervation

Animals were anesthetized with ketamine (75 mg/kg, Holliday)/xylazine (5 mg/kg, Köing) intraperitoneal administration (i.p.). In aseptic conditions, rats' skulls were exposed, and a bilateral stainless steel cannula (23 gauge) was implanted using a stereotaxic device fixed with dental cement (Subiton, Argentina). Furthermore, one

stainless steel screw was anchored to the skull. Cannulae were placed 2 mm above the final place of injection. According to Paxinos and Watson Atlas[26], coordinates for lateral ventricle concerning bregma were: Anteroposterior (AP) = –0.92 mm, L = ±1.5 mm, dorsoventral (DV) = –6.8 mm. The cannulae were implanted in the right and left sides of the lateral ventricle.

In the anesthetized animals, the surgical denervation was made under a magnifier. First, the right kidney was exteriorized, the lipid and connective tissue from vessels at the renal pelvis level was separated, and the nerve was dissected, leaving the renal artery and vein intact. The denervation phenol 10% (neurolytic) was applied to make it more accurate. The kidney was introduced, and the same procedure was performed in the left kidney. Finally, the surgery was finished with muscle and skin sutures[27-30]. The sham animals underwent the same procedure until renal nerve visualization for each kidney. The renal denervation was verified in kidney slices with a hematoxylin-eosin stain (Figure 2). Moreover, renal denervation did not affect the food, sodium, and water intake (Figure 3).

2.5. Losartan cerebral microinjection

The animals were placed in metabolic cages from day 1 and were bilaterally administered on day 5 with Los (4 µg/1 µL)

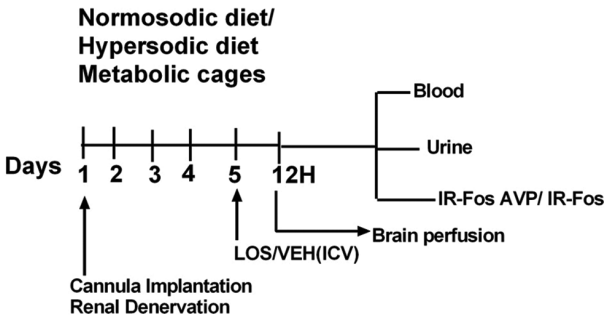

Figure 1. Experimental design: 6-day protocol with normosodic and hypersodic diet. The stereotaxic surgery was performed at the beginning of the protocol, and vehicle/losartan was administered on day five. Twelve hours later, the animals were sacrificed.

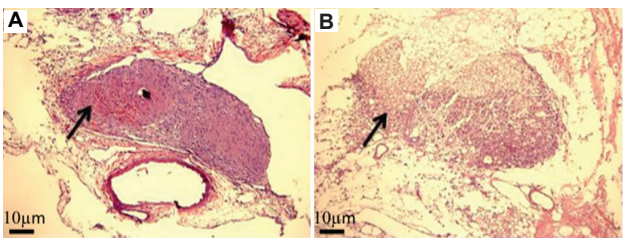

Figure 2. Representative microphotographs of histological sections of the kidney. (A) SHAM, renal nerve intact, (B) RDN, renal nerve lesioned. Renal nerve lesion verification in hematoxylin-eosin stained ×100 sections.

or artificial cerebral fluid (ACF), inserting a stainless steel injection cannula (30 gauge) into the guide cannula. The cannula was attached through a polyethylene catheter (P20) to a 25 µL microsyringe (Hamilton). Volumes of 1 µL of ACF or Los solution were gradually injected over a 1-min period into the left and right sides using an infusion bomb (HARVARD, model 22). The injection cannula was left in place for an additional 20 s to allow complete liquid diffusion[23,31,32].

2.6. Biochemistry

2.6.1. Urine collection and blood samples

The daily urine production was collected and registered, and 12 h of urine production was collected after Los/ACF injection and centrifuged at 10,000 G for 10 min. The supernatant was kept at –20°C until analysis.

At the end of the experiment, blood samples were taken from the heart's left ventricle in anesthetized animals. The samples were centrifuged at 3000 rpm for 5 min and kept at –80°C until analysis (Figure 1).

2.6.2. Biochemical determinations in urine and plasma

The urinary volume was registered, and the parameters such as creatinine, osmolarity, sodium, and potassium were determined. The urine samples were diluted in water 1:20 and analyzed for creatinine using a modified Jaffe reaction under spectrometry at 510 nm. The osmolarity was measured by an osmometer 3.300 ADVANCE (System), and the ions were measured using a selective electrode.

The parameters analyzed in the plasma were: Creatinine, sodium potassium, and chlorine. Creatinine was measured using a modified Jaffe reaction. Briefly, plasma samples (0.5 mL) were mixed with trichloroacetic acid 5% in equal volume and centrifuged at 5000 rpm for 10 min, and the ions were measured using a selective electrode.

2.7. Neuronal activation analysis

2.7.1. Immunostaining procedure

Twelve hours after Los/ACF administration (Figure 1), the animals were anesthetized with Urethane 50% (100 mg/kg) and transcardially perfused with 200ml of 0.9% saline and heparin (200 µL/L), followed by 200ml of 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS, pH 7.4). After removal, the brains were stored at 4°C in a 30% sucrose solution. Coronal sections of 40 µm were cut using a freezing microtome (Leica CM1510S) and collected in 0.01 M phosphate-buffered saline (PBS, pH = 7.4). They were placed in a mixture of 10% H_2O_2 and 10% methanol, washed 3 times with PBS 0.01 M, and then in a 10% normal goat serum (NGS; Natocor, Córdoba,

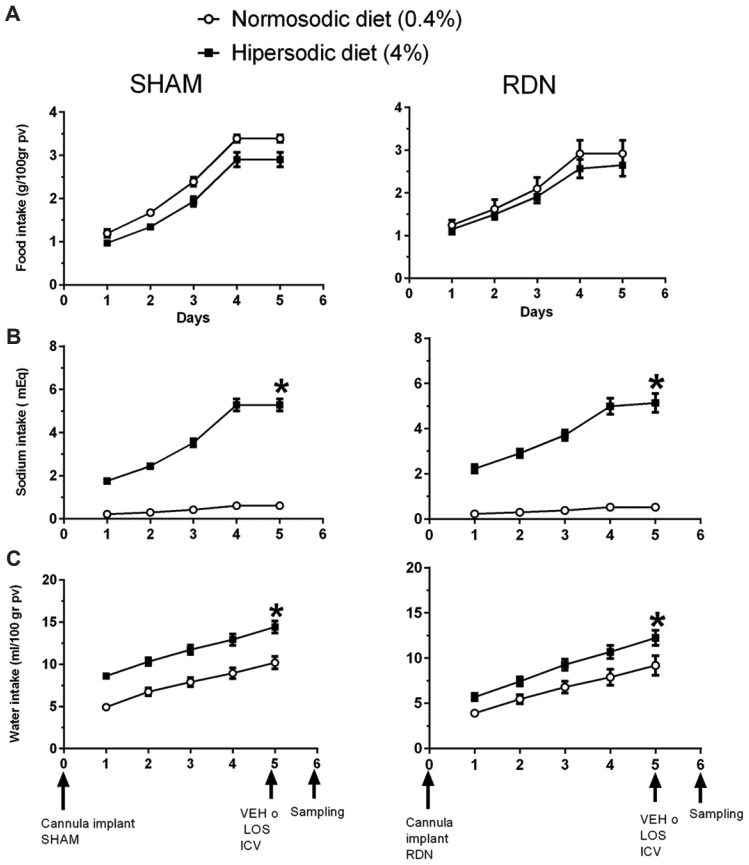

Figure 3. Food, sodium, and water intake in rats treated with a 0.4% normosodic diet (white) and with a 4% hipersodic diet (black) for 5 days prior to intracerebroventricular administration with vehicle/losartan (left panel: SHAM) and (right panel: RDN). (A) Food intake (g/100 g/bw), (B) Sodium intake (mEq), and (C) Water intake (mL/100 g/bw).

Argentina) in 0.1M PBS, for 2 h each. The free-floating slices were incubated overnight, at room temperature, in a primary antibody solution (2% NGS and 0.3% Triton X-100 (Flucka Analytical)) containing rabbit anti-c-Fos antibody (1:3000; Abcam), in 0.1M PBS. The following day, the slices were incubated in biotin-labeled anti-rabbit secondary antibody (1:1000; Vector Laboratories) and Avidin-Biotin-peroxidase Complex (ABC; 1:500; Vector Laboratories) for 2 h each, at room temperature. The peroxidase label was detected with diaminobenzidine hydrochloride (0.5 mg/mL, Sigma-Aldrich) and hydrogen peroxide; the solution was intensified with 1% cobalt chloride and 1% nickel ammonium sulfate. This method produces a violet nuclear reaction product[33,34]. Furthermore, the series of c-Fos-labeled sections containing SON were processed for arginine-vasopressin (AVP) immunohistochemically localization. They were incubated overnight, at room temperature, with polyclonal rabbit anti-AVP antibody (1:3000; PS 41 Vasopressin-NP; Rockville, MD, USA) in primary antibody solution. After incubation, the sections were rinsed and incubated with biotin-labeled anti-mouse secondary antibody (1:3000 Jackson Immune Research) and ABC for 2 h each, at room temperature. Cytoplasmic vasopressin immunoreactivity (AVP-IR) was detected with diaminobenzidine hydrochloride, which produces a brown reaction product. Finally, they were mounted on gelatinized slides, air-dried overnight, dehydrated, cleared in xylene, and placed under a coverslip with DPX mounting medium (Flucka Analytical).

2.7.2. Cytoarchitectural and quantitative analysis

Images containing c-Fos-IR nuclei and c-Fos-AVP-IR were obtained using a computerized system that included a Leica DM 4000B microscope equipped with a DFC Leica digital camera attached to a contrast enhancement device. The brain nuclei evidencing c-Fos-IR were identified and delimited according to the atlas of Paxinos and Watson[26]. The numbers of c-Fos-IR nuclear profiles in the sections were counted at a tone level; the distance from the bregma of the corresponding plates is as follows: for the dorsomedial region of PVN = −0.92 mm, SON = −1.30 mm, SFO and OVLT = −1.40 mm. The number of c-Fos-AVP-IR neurons

was counted in SON = −1.30 mm. The image analysis was accomplished using ImageJ software from the National Institutes of Health (NIH). The threshold was fixed between intervals of 0–150 in black-and-white conditions; all higher values were considered background. C-Fos-IR neurons were identified by the dense black nucleus and counted by setting a size range for cellular nuclei (8 –12 µm of diameter), and AVP-IR neurons were identified by dense brown staining of the cytoplasm. The counting of c-Fos-AVP-IR cells was manually performed. The final value for each brain area is the average of 4 images (bilaterally, in two sections). The counting was made on a 0.37 mm^2 area (corresponding to ×200 magnification). Since nuclei size and section thickness did not change between the experimental and control groups, any systematic error could be identical for all groups. The counting was made by two operators on each section analyzed to ensure that the number of profiles obtained was similar, but only one counting was used. Counting of c-Fos-IR and c-Fos-AVP-IR cells was performed blinded to the observer.

2.8. Statistical analysis

The experiments were designed to evaluate the brain AT_1-R role in renal function through SNS under two independent conditions: sham or renal denervation. The data were reported as mean ± SEM and analyzed using two-way ANOVA considering the following factors: Diet (normosodic/hypersodic) and treatment (Los/vehicle) followed by *post hoc* analysis Student-Newman Keuls in each condition. $p < 0.05$ was considered significant. The analyses were performed by using GraphPad Prism® 6.03 software.

3. Results

3.1. c-Fos-IR expression

SON: The hypersodic diet increased the number of c-Fos-IR cells, and Los blunted this increase in sham animals (diet factor $F_{(1,9)} = 106.6$, $p < 0.0001$; treatment factor $F_{(1,9)} = 76.35$, $p < 0.0001$; interaction $F_{(1,9)} = 84.20$, $p < 0.0001$). Although the diet did not affect c-Fos expression in renal denervated animals (RDN), Los decreased the number of Fos-IR cells (diet factor $F_{(1,14)} = 0.11$, p = not significant (NS); treatment factor $F_{(1,14)} = 4.81$, $p < 0.05$; interaction $F_{(1,14)} = 0.74$, p = NS) (Figure 4, left panel).

PVN: In sham animals, no statistical differences were found in c-Fos expression in diet factor, treatment factor, or interaction between both factors (diet factor $F_{(1,24)} = 0.04$, p = NS; treatment factor $F_{(1,24)} = 0.001$, p = NS; interaction $F_{(1,24)} = 0.30$, p = NS). In the RDN group, Los decreased the number of c-Fos-IR cells in animals with the hypersodic diet)diet factor $F_{(1,14)} = 0.71$, p =NS; treatment factor $F_{(1,14)} = 5.63$, $p < 0.05$; interaction $F_{(1,14)} = 1.34$, p = NS) (Figure 4, right panel).

SFO: The diet or treatment did not affect c-Fos expression in sham animals (diet factor $F_{(1,25)} = 0.33$, p = NS; treatment factor $F_{(1,25)} = 0.19$, p = NS; interaction $F_{(1,25)} = 0.01$, p = NS). Although, in RDN animals, the

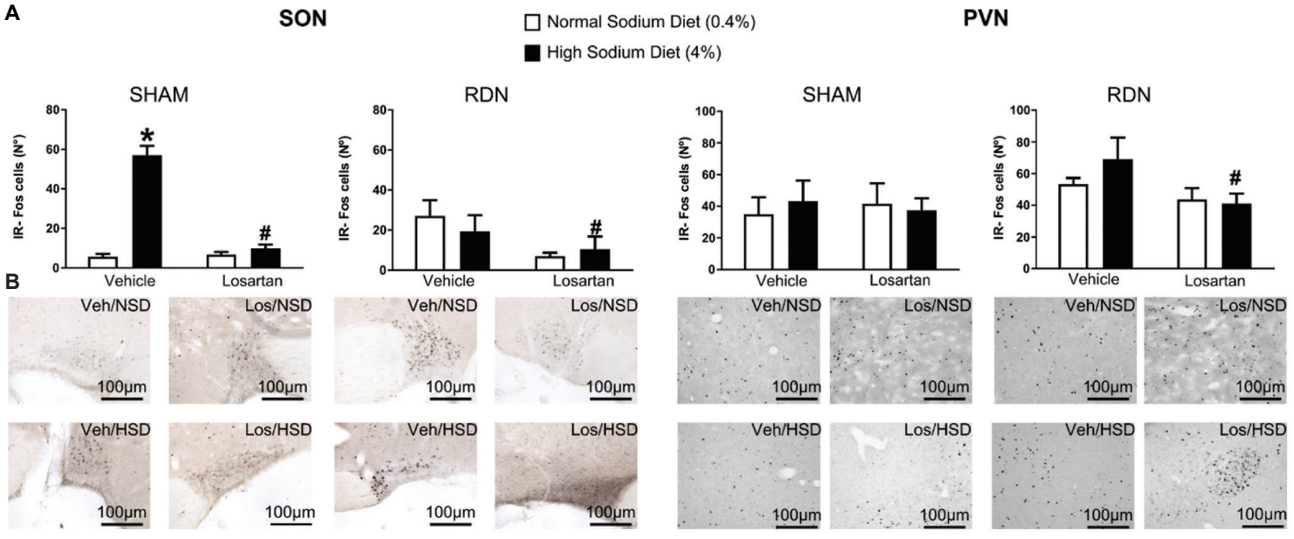

Figure 4. Immunostaining anti-c-Fos in the supraoptic nucleus (SON), left panel, and supraoptic nucleus (PVN), right panel. (A) Number of c-Fos-IR cells in rats treated with a normal sodium diet (0.4%) and with a hypersodium diet (4%) and injected intracerebroventricularly with vehicle or losartan (4 µg/µl). *$p < 0.05$ compared with the normal sodium diet. #$p < 0.05$ compared with the animals injected with vehicle, in SHAM control animals (n=7) and RDN denervated animals (n =6). Mean ± SEM. (B) Representative microphotograph of coronal sections c-Fos labeled in the SON and PVN (×200 magnification). The bar represents 100 µm. Abbreviations: Veh: Vehicle; Los: Losartan; NSD: Normosodic diet; HSD: Hypersodic diet.

hypersodic diet induced an increase in the number of c-Fos-IR cells, and Los administration did not affect it (diet factor $F_{(1,14)} = 7.54$, $p < 0.05$; treatment factor $F_{(1,14)} = 0.01$, p = NS; interaction $F_{(1,14)} = 0.12$, p = NS) (Figure 5, left panel).

OVLT: Neither diet nor treatment affected c-Fos expression in sham animals (diet factor $F_{(1,23)} = 0.44$, p = NS; treatment factor $F_{(1,23)} = 0.001$, p = NS; interaction $F_{(1,23)} = 0.34$, p = NS). However, the hypersodic diet decreased the number of c-Fos-IR cells, and Los did not affect it (diet factor $F_{(1,15)} = 9.30$, $p < 0.05$; treatment factor $F_{(1,15)} = 0.32$, p = NS; interaction $F_{(1,15)} = 0.41$, p = NS) (Figure 5, right panel).

3.2. c-Fos-AVP-IR expression

Hypersodic diet induced an increase in the number of c-Fos-AVP-IR cells in SON, and Los administration prevented it (diet factor $F_{(1,4)} = 15.37$, $p < 0.05$; treatment factor $F_{(1,4)} = 6.94$, p = NS; interaction $F_{(1,4)}$=23.22, $p < 0.01$) (Figure 6).

3.3. Water, food, sodium, and potassium intake

As expected, the hypersodic diet increased the water intake in sham animals, and Los prevented it (diet factor $F_{(1,27)} = 6.03$, $p < 0.05$; treatment factor $F_{(1,27)} = 0.01$, p = NS; interaction $F_{(1,27)} = 6.08$, $p < 0.05$). In the RDN group, the hypersodic diet increased the water intake in the group treated with LOS (diet factor $F_{(1,20)} = 7.10$, $p < 0.05$; treatment factor $F_{(1,20)} = 0.27$ p = NS; and interaction $F_{(1,20)} = 2.26$, p = NS) (Figure 7).

Food intake expressed as 100 g per animal weight was similar in all groups (Table 1). For sham group, the results were found as follows: Diet factor $F_{(1,27)} = 0.07$, p = NS; treatment factor $F_{(1,27)} = 0.008$, p = NS; and interaction $F_{(1,27)} = 3.85$, p = NS (Table 1). For RDN group, the results were found as follows: diet factor $F_{(1,20)} = 7.09$, $p = 0.01$; treatment factor $F_{(1,20)} = 0.27$, p = NS; and interaction $F_{(1,20)} = 0.14$, p = NS (Table 1).

Sodium intake was high in animals exposed to a hypersodic diet in sham and RDN groups, and Los did not modify it. For sham group, the results were found as follows: Diet factor $F_{(1,27)} = 201.90$, $p < 0.0001$; treatment factor $F_{(1,27)} = 0.20$, p = NS; and interaction $F_{(1,27)} = 0.54$, p = NS (Table 1). For RDN group, the results were found as follows: Diet factor $F_{(1,20)} = 99.77$, $p < 0.0001$; treatment factor $F_{(1,20)} = 0.02$, p = NS; and interaction $F_{(1,20)} = 0.08$, p = NS (Table 1).

No differences in potassium intake induced by diet or treatment in either the sham or RDN group were found. For sham group, the results were found as follows: Diet factor $F_{(1,27)} = 0.27$, p = NS; treatment factor $F_{(1,27)} = 0.27$, p = NS; and interaction $F_{(1,27)} = 1.64$, p = NS (Table 1). For RDN group, the results were found as follows: Diet factor $F_{(1,20)} = 0.06$, p = NS; treatment factor $F_{(1,20)} = 0.06$, p = NS; and interaction $F_{(1,20)} = 0.24$, p = NS (Table 1).

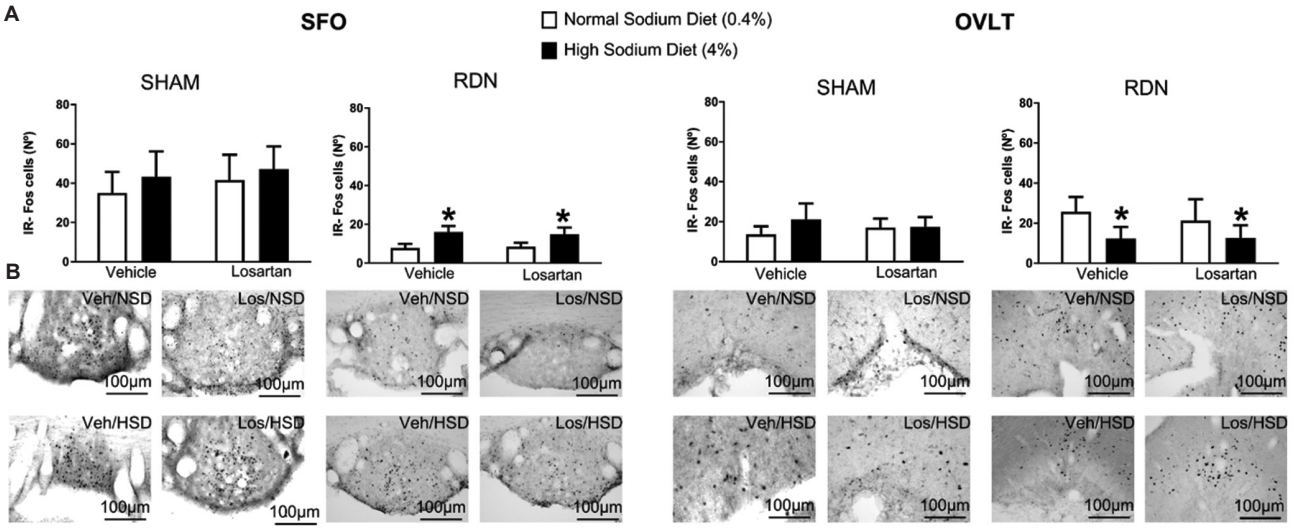

Figure 5. Immunostaining anti-c-Fos in the subfornical nucleus (SFO), left panel, and organum vascular lamina terminalis (OVLT), right panel. (A) Number of c-Fos-IR cells in rats treated with a normal sodium diet (0.4%) and with a hypersodium diet (4%) and injected intracerebroventricularly with vehicle or losartan (4 µg/µl). *$p < 0.05$ compared with the normal sodium diet, in SHAM control animals ($n = 7$) and RDN denervated animals ($n = 6$). Mean ± SEM. (B) Representative microphotograph of coronal sections c-Fos labeled in the SFO and OLVT (×200 magnification). The bar represents 100 µm. Abbreviations: Veh: Vehicle; Los: Losartan; NSD: Normosodic diet; HSD: Hypersodic diet.

3.4. Sodium, creatinine, chlorine, and potassium plasma levels

No differences were found in these parameters by diet or treatment, either sham or RDN group.

Concerning plasma sodium, in sham group, the results were found as follows: Diet factor $F_{(1,27)}$ = 1.07, p = NS; treatment factor $F_{(1,27)}$ = 0.76, p = NS; and interaction $F_{(1,27)}$ = 0.22, p = NS (Table 2). In RDN group, the results were found as follows: Diet factor $F_{(1,20)}$ = 0.17 p = NS; treatment factor $F_{(1,20)}$ = 1.63 p = NS; and interaction $F_{(1,20)}$ = 2.45 p = NS (Table 2).

Concerning plasma creatinine, in sham group, the results were found as follows: Diet factor $F_{(1,27)}$ = 0.028, p = NS; treatment factor $F_{(1,27)}$ = 1.62, p = NS; and interaction $F_{(1,27)}$ = 0.11, p = NS (Table 2). In RDN group, the results were found as follows: Diet factor $F_{(1,20)}$ = 0.41, p = NS; treatment factor $F_{(1,20)}$ = 0.001, p = NS; and interaction $F_{(1,20)}$ = 2.10, p = NS (Table 2).

Concerning plasma chlorine, in sham group, the results were found as follows: Diet factor $F_{(1,27)}$ = 2.16, p = NS; treatment factor $F_{(1,27)}$ = 0.50, p = NS; and interaction $F_{(1,27)}$ = 0.01, p = NS (Table 2). In RDN group, the results were found as follows: diet factor $F_{(1,20)}$ = 0.0009, p = NS; treatment factor $F_{(1,20)}$ = 0.58, p = NS; and interaction $F_{(1,20)}$ = 0.44, p = NS (Table 2).

Concerning plasma potassium, in sham group, the results were found as follows: Diet factor $F_{(1,27)}$ = 0.76, p = NS; treatment factor $F_{(1,27)}$ = 2.98, p = NS; and interaction $F_{(1,27)}$ = 6.41, p = NS (Table 2). In RDN group, the results were found as follows: Diet factor $F_{(1,20)}$ = 0.0002 p = NS; treatment factor $F_{(1,20)}$ = 0.75, p = NS; and interaction $F_{(1,20)}$ = 0.83, p = NS (Table 2).

3.5. Biochemistry

3.5.1. Urinary volume and sodium excretion

Regarding urinary volume, the increase induced by the hypersodic diet was blunted with Los in the sham group (diet factor $F_{(1,27)}$ = 36.83, p < 0.0001; treatment factor $F_{(1,27)}$ = 24.16, p < 0.0001; and interaction $F_{(1,27)}$ = 2.00, p = NS). In the RDN group, the hypersodic diet increased the urinary volume in animals administered with LOS (diet factor $F_{(1,16)}$ = 7.23, p < 0.05; treatment factor $F_{(1,16)}$ = 2.66, p = NS; and interaction $F_{(1,16)}$ = 3.97, p = NS) (Figure 7).

The sodium excretion in the sham group with a hypersodic diet was significantly high, and Los did not affect it (diet factor $F_{(1,27)}$ = 35.42, p < 0.0001; treatment factor

Table 1. Intake parameters

	SHAM				RDN			
	NSD-VEH	HSD-VEH	NSD-Los	HSD-Los	NSD-VEH	HSD-VEH	NSD-Los	HSD-Los
Body weight (g)	264.3±9.2	264.3±9.2	260.0±6.7	271.4±10.1	266.7±10.5	300.0±0.0	258.3±8.3	275.0±11.2
Food intake (g/100 g bw)	2.43±0.28	2.94±0.26	2.90±0.20	2.51±0.14	3.17±0.41	2.58±0.20	3.03±0.47	2.90±0.32
Na^+ (mEq)	0.38±0.03	4.09±0.45*	0.45±0.0.04	3.80±0.30*	0.58±0.08	5.28±0.40*	0.52±0.07	5.47±0.69*
K^+ (mEq)	2.29±0.18	2.46±0.27	2.71±0.25	2.29±0.18	3.49±0.49	3.18±0.25	3.18±0.46	3.28±0.41

Parameters (food intake, sodium, and potassium) following intracerebroventricular injection (ICV) of losartan (Los) or vehicle (VEH; saline, 0.9% NaCl) in adult male rats that underwent renal denervation (RDN), or renal nerve intact (SHAM), and kept in a normal sodium (NSD) or high-sodium (HSD) diet during 5 days. Body weight (g), food intake (g/100 g bw), sodium intake (mEq), and potassium intake (mEq) were recorded. Groups: NSD-VEH, HSD-VEH, NSD-Los, and HSD-Los. Animals with RDN=7 and SHAM=6 in each group. Values are expressed as means±SEM. *p<0.05 with respect to the normal sodium diet.

Table 2. Plasma parameters

	SHAM				RDN			
	NSD-VEH	HSD-VEH	NSD-Los	HSD-Los	NSD-VEH	HSD-VEH	NSD-Los	HSD-Los
Na^+ (mmol/L)	136.7±3.3	135.3±1.7	135.7±2.2	131.9±2.7	141.2±4.4	135.8±2.3	128.0±6.4	137.2±4.4
Creatinine (mg/dL)	0.21±0.02	0.24±0.04	0.29±0.06	0.28±0.04	0.38±0.09	0.32±0.06	0.27±0.05	0.43±0.09
Cl^- (mmol/L)	95.1±2.3	92.0±1.4	93.5±2.0	90.9±1.6	54.6±21.3	67.8±19.5	81.7±5.3	69.7±19.2
K^+ (mmol/L)	7.2±0.7	5.7±0.2	6.9±0.3	7.6±0.5	8.2±0.5	7.1±0.4	8.6±1.2	9.2±1.1

Plasma parameters after an intracerebroventricular injection (ICV) of losartan (Los) or vehicle (VEH; saline, 0.9% NaCl) in adult male rats that underwent renal denervation (RDN) or renal nerve intact (SHAM), and that were kept in a normal sodium (NSD) or high-sodium (HSD) diet during 5 days. Sodium (Na^+), creatinine (mg/dL), chlorine (Cl^-), and potassium (K^+) were recorded. Groups: NSD-VEH, HSD-VEH, NSD-Los, and HSD-Los. Animals with RDN=7 and SHAM=6 in each group. Values are expressed as means±SEM.

$F_{(1,27)}$ = 0.34, p = NS; and interaction $F_{(1,27)}$ = 0.55, p = NS). The same phenomenon was observed in the RDN group (diet factor $F_{(1,16)}$ = 15.31, $p < 0.001$; treatment factor $F_{(1,16)}$ = 1.35, p = NS; and interaction $F_{(1,16)}$ = 1.39, p = NS) (Figure 7).

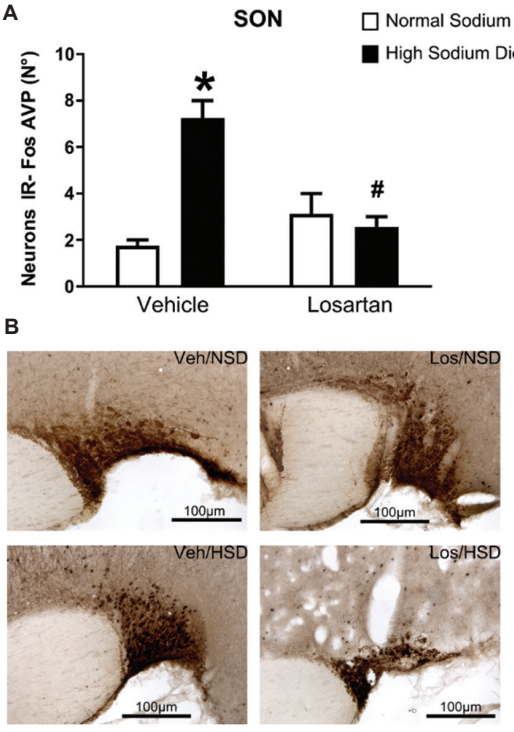

Figure 6. Double immunostaining anti-c-Fos and AVP in the SON of control animals (SHAM). (A) Number of c-Fos-AVP-IR cells in rats treated with normal sodium diet (0.4%) and with a hypersodium diet (4%) and injected intracerebroventricularly with vehicle or losartan (4 µg/µL). *$p < 0.05$ compared with the normal sodium diet. #$p < 0.05$ compared with those animals injected with vehicle (n = 6). Mean ± SEM. (B) Representative microphotograph of coronal sections c-Fos and AVP labeled (×200 magnification). The bar represents 100 µm. Abbreviations: Veh: Vehicle; Los: Losartan; NSD: Normosodic diet; HSD: Hypersodic diet.

In the sham group, the hypersodic diet induced an increase in sodium fractional excretion, and Los did not affect it (diet factor $F_{(1,27)}$ = 36.83, $p < 0.0001$; treatment factor $F_{(1,27)}$ = 0.24, p = NS; and interaction $F_{(1,27)}$ = 0.99, p = NS). In the RDN group, the hypersodic diet increased sodium fractional excretion in animals administered with Los (diet factor $F_{(1,20)}$ = 19.34, $p < 0.005$; treatment factor $F_{(1,20)}$ = 2.23, p = NS; and interaction $F_{(1,20)}$ = 3.93, p = NS) (Figure 7).

3.5.2. Urinary osmolarity and creatinine excretion

In the sham group, the hypersodic diet induced an increase in osmolarity, and Los administration prevented this response (diet factor $F_{(1,27)}$ = 10.10, $p < 0.005$; treatment factor $F_{(1,27)}$ = 1.65, p = NS; and interaction $F_{(1,27)}$ = 0.49, p = NS). In the RDN group, no differences were found by diet or treatment (diet factor $F_{(1,20)}$ =2.55, p = NS; treatment factor $F_{(1,20)}$ = 0.25, p = NS; and interaction $F_{(1,20)}$ = 2.19, p = NS) (Table 3).

Regarding creatinine excretion, the hypersodic diet induces a decrease that Los administration prevented in the sham group (diet factor $F_{(1,27)}$ = 15.77, $p < 0.001$; treatment factor $F_{(1,27)}$ = 0.67, p = NS; and interaction $F_{(1,27)}$ = 0.77, p = NS) (Table 3). However, in the RDN group, the hypersodic diet decreased creatinine excretion only in the animals administered with Los (diet factor $F_{(1,20)}$ = 4.32, $p < 0.05$; treatment factor $F_{(1,20)}$ = 0.01, p = NS; and interaction $F_{(1,20)}$ = 3.76, p = NS) (Table 3).

3.5.3. Potassium excretion

The differences in potassium excretion in the sham group were not statistically significant (diet factor $F_{(1,27)}$ = 0.15,

Table 3. Urinary parameters

	SHAM				RDN			
	NSD-VEH	HSD-VEH	NSD-Los	HSD-Los	NSD-VEH	HSD-VEH	NSD-Los	HSD-Los
Osmolarity (mOsm/Kg)	1867.7±243.1	1014.0±181.0*	1429.7±236.3	886.6±153.2	1496.3±199.1	1467.7±237.8	1740.3±280.1	971.3±273.9*
Na^+ (mmol/L)	0.6±0.1	5.2±1.2*	0.7±0.1	4.3±1.0*	0.7±0.2	2.5±0.7*	0.5±0.1	3.1±0.7*
Creatinine (mg/dL)	50.8±7.0	18.1±5.6*	39.3±7.9	18.5±2.7	54.2±9.5	52.1±12.7	81.3±24.8	21.5±4.0*
Cl^- (mmol/L)	0.9±0.2	5.8±1.3	1.0±0.2	4.7±1.2	0.9±0.2	3.5±1.1	0.8±0.2	5.6±2.9
K^+ (mmol/L)	1.0±0.2	1.9±0.4	1.4±0.3	1.3±0.1	1.1±0.2	1.2±0.3	1.0±0.2	1.3±0.6
Creatinine clearance	0.50±0.09	0.57±0.07	0.60±0.14	0.61±0.09	0.47±0.11	0.77±0.29	0.93±0.39	0.48±0.13
Osmolar clearance	0.03±0.00	0.08±0.02*	0.04±0.01	0.06±0.0*	0.02±0.00	0.04±0.01	0.03±0.01	0.04±0.01
Free water clearance	-0.02±0.00	-0.05±0.02	-0.03±0.01	-0.04±0.01	-0.02±0.00	-0.03±0.01	-0.02±0.01	-0.01±0.01

Urinary and plasma parameters following an intracerebroventricular injection (ICV) of losartan (Los) or vehicle (VEH; saline, 0.9% NaCl) in adult male rats that underwent renal denervation (RDN) or renal nerve intact (SHAM), and kept in a normal sodium (NSD) or high-sodium (HSD) diet during 5 days. Urine volume (mL), osmolarity (mOsm/Kg). Sodium (Na^+), creatinine (mg/dL), chlorine (Cl^-), potassium (K^+) were recorded. Groups: NSD-VEH, HSD-VEH, NSD-Los, and HSD-Los. Animals with RDN=7 and SHAM=6 in each group. Values are expressed as means±SEM. *p<0.05 with respect to the normal sodium diet.

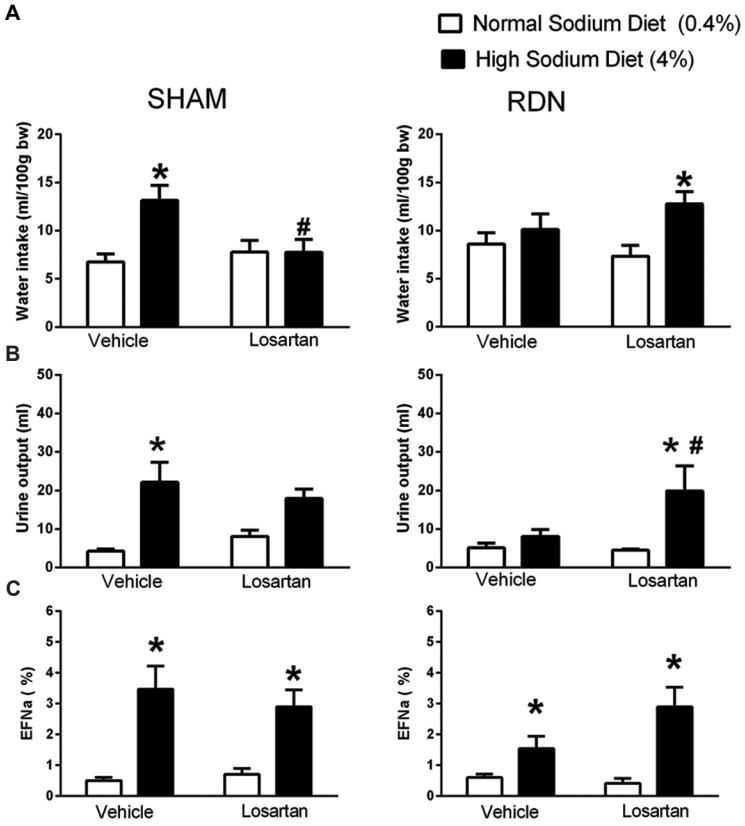

Figure 7. Water intake, urinary volume, and fractional sodium excretion. (A) Water intake (mL) per 100 g of body weight. B) Urinary volume (mL) and fractional excretion of sodium EFNa (%) in rats treated with a normosodic diet (0.4%) and with a hypersodium diet (4%) and injected with vehicle or losartan (4 µg/µL). *$p < 0.05$ compared with the normosodic diet. #$p < 0.05$ compared with the animals injected with vehicle, in SHAM control animals ($n = 7$) and RDN denervated animals ($n = 6$). Mean ± SEM.

p = NS; treatment factor $F_{(1,27)} = 0.74$, p = NS; and interaction $F_{(1,27)} = 2.85$, p = NS) (Table 3). In the RDN group, no differences were found by diet or treatment (Table 3).

3.5.4. Urinary creatinine, osmolar, and free water clearance

No differences in creatinine clearance by diet or treatment were found in either the sham or RDN group (Table 3). In sham group, the results were found as follows: Diet factor $F_{(1,27)} = 0.13$, p = NS; treatment factor $F_{(1,27)} = 0.29$, p = NS; and interaction $F_{(1,27)} = 0.06$, p = NS (Table 3). In RDN group, the results were found as follows: diet factor $F_{(1,20)} = 0.10$, p = NS; treatment factor $F_{(1,20)} = 0.09$, p = NS; and interaction $F_{(1,20)} = 2.14$, p = NS (Table 3).

Regarding osmolar clearance in the sham group, the hypersodic diet induced an increase that the Los administration blunted (diet factor $F_{(1,27)} = 11.52$, $p < 0.005$; treatment factor $F_{(1,27)} = 0.05$, p = NS; and interaction $F_{(1,27)} = 1.01$, p = NS) (Table 3). In the RDN group, no differences were found in creatinine clearance by diet or Los administration (diet factor $F_{(1,20)} = 3.42$, p = NS; treatment factor $F_{(1,20)} = 0.04$, p = NS; and interaction $F_{(1,20)} = 0.17$, p = NS) (Table 3).

In the sham group, free water clearance did not show statistical differences in response to diet or Los administration (diet factor $F_{(1,27)} = 2.83$, p = NS; treatment factor $F_{(1,27)} = 0.07$, p = NS; and interaction $F_{(1,27)} = 0.19$, p = NS) (Table 3). Similar results were observed in the RDN group (diet factor $F_{(1,20)} = 0.16$, p = NS; treatment factor $F_{(1,20)} = 1.48$, p = NS; and interaction $F_{(1,20)} = 4.12$, p = NS) (Table 3).

4. Discussion

The main finding of this study is the ANG II's main role, through AT_1-R, in the complex interaction system between CNS and SNS over renal function control. There is considerable evidence supporting that RAS components are present in the tissue and plasma, and they can act independently or linked through regulatory pathways[35-38].

The SNS involvement in the renal sodium equilibrium under normal and pathological conditions is modulated by ANG II actions over AVP. Moreover, it has been shown that Los reduces RAS and SNS activity. Renal denervation is a delicate and specific surgical approach. It is very useful as it can recreate conditions similar to renal transplanted individuals.

In the present study, we perform the experiments 6 days after renal denervation, based on the fact that in this period, the anatomical-physiological renal nerve interruption persists, allowing the renal function evaluation without sympathetic influence[27-30]. The applied experimental protocol using renal denervation and central Los administration evidenced the ANG II role at the brain level, independently from its functions through SNS modulation.

4.1. Hypersodic diet induced-neuronal activation

Evidence shows that brain ANG II -through AT_1-R- is involved in blood pressure control, SNS stimulation, AVP release, and water and sodium intake[36]. The presence of AT_1-R in PVN, SON, OVLT, and SFO brain areas involved in arterial blood pressure control and hydroelectrolytic homeostasis has been described[36,39].

The marked increase in the number of c-Fos-IR neurons in the SON from intact animals was blunted by Los administration, suggesting that AT_1-R mediates the neuronal activation induced by a moderated hypersodic diet[40]. The renal denervation avoided the described increase in neuronal activation induced by the hypersodic diet, evidencing the close interaction between SON vasopressinergic neurons and SNS activity. These constitute the essential circuit loop SON-SNS-kidney, the principal components involved in the sodium overload neuroregulation in this experimental paradigm[12,41].

The PVN physiological relevance appears to be lower than SON since the neuronal activity was not affected by the hypersodic diet. It is important to highlight the great cell heterogeneity in PVN compared to SON. In this sense, PVN is a highly complex nucleus when CRH, GNRH, AVP, and oxytocin synthesis take place and receive inputs from several brain regions[42,43]. Meanwhile, SON presents less complexity when the neurons synthesizing oxytocin and AVP are bigger and easily identified[44].

The SNS activity interruption induced a marked decrease in SFO neuronal activity, indicating a basal stimulatory tone in this nucleus that is not observed in OVLT. Nevertheless, in both nuclei, the sodium overload alters the neuronal activation under renal denervation conditions, evidencing a differential regulatory role from SNS on the neural components of these nuclei[12]. SFO and OVLT exert key functions in the extracellular sodium concentrations since they are related to sodium appetite and are sensitive to ANG II[12,14,34,45].

4.2. Renal effects: Water and sodium balance

The variations in sodium excretion affect the extracellular volume activating complex response mechanisms that re-establish the hydrosaline homeostasis.

It is known that AVP and oxytocin play a key role in osmoregulation through natriuresis and diuresis[46]. The anatomic-physiological evidence shows that the sympathetic nerves regulate changes in the urinary sodium and water excretion through the tubular reabsorption at the nephron level. AVP targets the collector tubules, and its release is under brain ANG II regulation through AT_1-R[9]. In this sense, renal denervation allows us to unmask the central actions of these receptors and their interaction with SNS in renal sodium regulation.

Our results show that the increase in water intake induced by a hypersodic diet is mediated by brain AT_1-R since this increase was prevented by Los administration. Moreover, this regulation involves the SNS considering that renal denervation was able to avoid the increase in water intake induced by the hypersodic diet, and Los administration re-established this response[47]. A similar phenomenon was observed in the urinary volume increase induced by hypersodic diet in intact animals. This increase was abolished by renal denervation and re-established by the AT_1-R blockade. These results not only confirm the existence of the SNS-brain ANG II circuit loop but also its critical regulatory function.

Regarding daily food intake, we did not observe differences between diets, meaning sodium did not modify palatability. In the same way, renal denervation or Los administration did not affect food intake. However, water intake was increased by a hypersodic diet, as expected[33,47,48]. Our experimental design allowed independent evaluation of water and sodium intake, representing an advantage over protocols where the sodium is administered through drinking water.

In accordance with other authors, we found that 6 days of hypersodic diet (4%) did not affect plasmatic sodium and creatinine[49,50]. Moreover, it has been described that this experimental protocol did not increase blood pressure[28,49,51]. Although renal denervation diminished sodium excretion modifying the sodium balance, the water balance remained unaffected. Concerning potassium, differences in excretion and balance were not observed.

The decreased urinary osmolarity and creatinine excretion concomitant with increased urinary volume

induced by a hypersodic diet revealed the AVP role over renal function. This is also supported by the effects induced by renal denervation. Moreover, the ANG II role over AVP becomes evident since the AT_1-R antagonist restores the AVP actions at the renal level. Considering all together, it is possible to suggest that the hypersodic diet could promote AVP release as a consequence of ANG II acting on AT_1-R (Figure 8).

4.3. Limitations

The present study lacks AVP quantification in serum or urine since a time course is necessary to find out the best period to perform the determination. The conclusions are based on evidence collected from brain immunostaining and urine parameters.

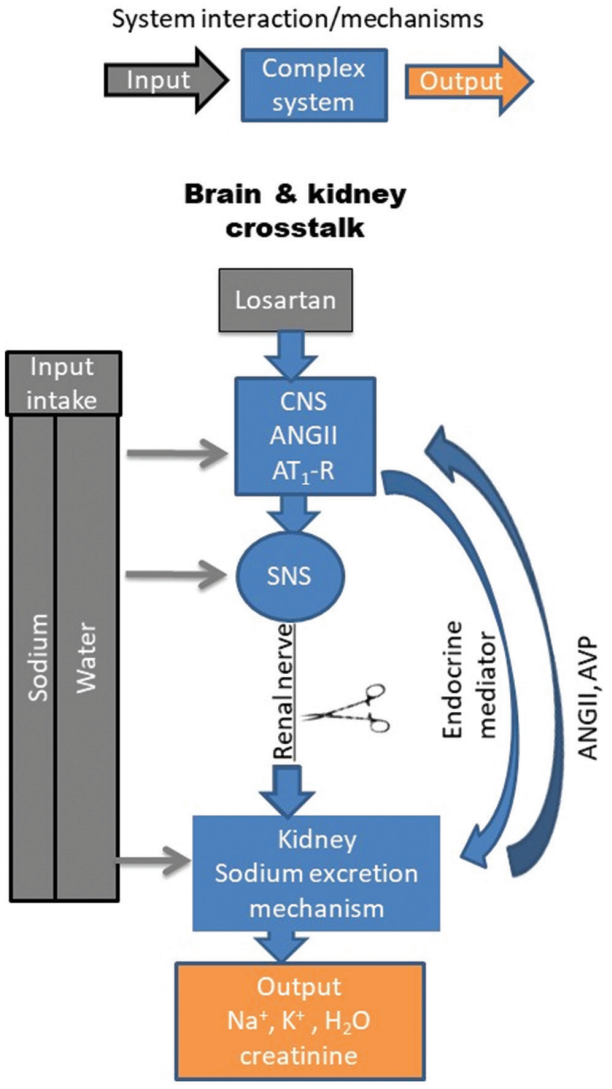

Figure 8. The scheme shows the involvement of AT1 receptors in the brain and kidney crosstalk mediating the interactions between the central nervous system, the sympathetic nervous system and the renal function. Angiotensin II, through its AT_1 receptors, has a critical role in the brain and kidney crosstalk mediating the interactions between the central nervous system, the sympathetic nervous system, and the renal function.

5. Conclusions

The sedentary lifestyle of modern society and diets rich in glucose, lipids, and sodium has detrimental consequences for human health. Preclinical and epidemiological studies have associated metabolic syndrome with the development of neurodegenerative pathologies. Since brain ANG II through AT_1-R exerts a close interaction with SNS in the renal sodium equilibrium in normal and pathological conditions, this opens the possibility of new interventions with ANG II inhibitors as sympatholytic agent. Considering that most evidence is from male rats, it is important to develop studies including both sexes and to analyze the role of sex hormones.

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Conflict of interest

The authors have no conflicts of interest to report nor any involvements to disclose, financial, or otherwise.

Author contributions

Conceptualization: Celia Ruberto, Claudia Bregonzio, Gustavo Baiardi
Formal analysis: Claudia Bregonzio and Gustavo Baiardi
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Project Administration: Claudia Bregonzio, Gustavo Baiardi
Supervision: Claudia Bregonzio, Gustavo Baiardi
Writing – original draft: Celia Ruberto, Victoria Belén Occhieppo, Claudia Bregonzio
Writing – review & editing: Celia Ruberto, Victoria Belén Occhieppo, Claudia Bregonzio.

Ethics approval and consent to participate

All procedures were carried out following the Guide for the Care and Use of Laboratory Animals as adopted and

promulgated by the National Institutes of Health and the EU (Eighth Edition, 2011) and approved by the Animal Care and Use Committee, School of Chemical Sciences (Res HCD no. 46/15 and 270/18), National University of Cordoba. Efforts were made to minimize the number of animals used and their suffering.

Consent for publication

Not applicable.

Availability of data

The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are included in the paper.

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