Water deprivation-induced sodium appetite and differential expression of encephalic c-Fos immunoreactivity in the spontaneously hypertensive rat

Daniela T. B. Pereira-Derderian,1 Regina C. Vendramini,2 José V. Menani,1 and Laurival A. De Luca Jr.1

1Department of Physiology and Pathology, School of Dentistry and 2Department of Clinical Analysis, School of Pharmaceutical Sciences, São Paulo State University–UNESP, Araraquara, São Paulo, Brazil

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Pereira-Derderian DT, Vendramini RC, Menani JV, De Luca Jr. LA. Water deprivation-induced sodium appetite and differential expression of encephalic c-Fos immunoreactivity in the spontaneously hypertensive rat. Am J Physiol Regul Integr Comp Physiol 298: R1298–R1309, 2010. First published March 3, 2010; doi:10.1152/ajpregu.00359.2009.—The spontaneously hypertensive rat (SHR) has an intense consumption of NaCl solution. Water deprivation (WD) followed by water intake to satiety induces partial rehydration (PR)—the WD-PR protocol—and sodium appetite. In the present work, WD produced similar water intake and no alterations in arterial pressure among spontaneously hypertensive rat (SHR), Wistar-Kyoto, and Holtzman strains. It also increased the number of cells with positive c-Fos immunoreactivity (Fos-IR) in the lamina terminals and in the hypothalamic supraoptic (SON) and paraventricular (parvocellular, PVNp) nucleus in these strains. The WD and WD-PR produced similar alterations in all strains in serum osmolality and protein, plasma renin activity, and sodium balance. The SHR ingested about 10 times more 0.3 M NaCl than normotensive strains in the sodium appetite test that follows WD-PR. After WD-PR, the Fos-IR persisted, elevated in the lamina terminalis of all strains but notably in the subfornical organ of the SHR. The WD-PR reversed Fos-IR in the SON of all strains and in the PVNp of SHR. It induced Fos-IR in the area postrema and in the nucleus of the solitary tract (NTS), dorsal raphe, parabrachial (PBN), pre-locus coeruleus (pre-LC), suprachiasmatic, and central amygdalar nucleus of all strains. This effect was bigger in the caudal-NTS, pre-LC, and medial-PBN of SHRs. The results indicate that WD-PR increases cell activity in the forebrain and hindbrain areas that control sodium appetite in the rat. They also suggest that increased cell activity in facilitatory brain areas precedes the intense 0.3 M NaCl intake of the SHR in the sodium appetite test.

dehydration; thirst; sodium intake; brain mapping; angiotensin

SALT INTAKE IS AN ADAPTIVE behavior to a lack of body water, as shown by the examples of people living in desert environments (24) and by humans and rats exposed to laboratory conditions (6, 30, 34, 36, 43). Salt intake also compensates for volume loss and maintains cardiovascular performance during psychosocial stress or illness, but it is an undesirable behavior when associated with pathological hypertension (13).

Weisunger et al. (43) originally suggested that water deprivation induces sodium appetite. We have extended their findings by showing that water deprivation unequivocally induces sodium appetite in response to the water deprivation-partial repletion (WD-PR) protocol (7, 34). In the WD-PR protocol, the water-deprived rat (WD) is first allowed to drink only water until it satiates its thirst (PR). Then, the PR is immediately followed by access to another bottle containing NaCl solution (sodium appetite test). The ingestion of hypertonic NaCl solution in this context can be considered as an expression of sodium appetite, because it results from negative sodium balance and persistent hypovolemia (19, 34, 43).

Previous studies suggest that sodium appetite results from the predominance of facilitatory over inhibitory signals (35, 26). Interplay between facilitatory and inhibitory signals for the control of sodium appetite may be found in a model that describes sensory information converging from both forebrain and hindbrain to the central amygdala (19, 20).

Facilitatory signals are raised in the lamina terminalis, an encephalic structure fundamental for the regulation of fluid and electrolyte balance of the dehydrated animal (19, 25). The lamina terminalis comprises a triad of circumventricular midline structures aligned in the rostral wall of the third ventricle. Two of them, the subfornical organ (SFO) and the organum vasculosum (OVLT), are located outside the blood-brain barrier and are sensitive to humoral factors, such as circulating ANG II. The third structure, the median preoptic nucleus (MnPO), located within the blood-brain barrier, has reciprocal connections with the two other structures and integrates the humoral sensory information raised in the SFO and OVLT.

The importance of the lamina terminalis for sodium appetite derives from several studies. Damage to its structures impairs the hypertonic NaCl intake induced by procedures that lead to activation of central ANG II receptors (5, 11, 39, 42). ANG II enhances the electrical discharge in neurons of the SFO and OVLT (14) and induces c-Fos expression in the SFO, OVLT, and MnPO in cells that colocalize ANG II type-1 (AT1) receptors (25, 32). Thus, facilitatory mechanisms of sodium appetite may begin with circulating ANG II-activating neurons in the SFO and OVLT. From the SFO and OVLT, perhaps after integration in the MnPO, signals are conveyed from the lamina terminalis to the extended amygdala, a structure that comprises the central amygdala and the bed nucleus of the stria terminalis (19, 20).

The sodium appetite that follows the WD-PR is inhibited by central blockade of the renin-angiotensin system (RAS) (34) and is preceded by intense plasma renin activity (PRA) and activation of c-Fos in cells of the lamina terminalis (7). Therefore, the lamina terminalis may also provide a facilitatory mechanism for the water deprivation-induced sodium appetite.

The density of c-Fos immunoreactivity (Fos-IR), a useful marker of cell activity (1, 22), has been successfully employed in mapping encephalic areas associated with selective dehydration of either the intracellular or the extracellular fluid, and water deprivation-induced double dehydration of both fluids (7, 16, 25, 28, 32, 41).
The spontaneously hypertensive rat (SHR) has been used as an animal model for the study of primary hypertension in humans (13). The SHR ingests more NaCl on a daily basis than normotensive strains do (see Refs. 8 and 10 for review). Its intense sodium intake relies on a hyperactive central RAS rather than an altered fluid-electrolyte balance (8, 9). The SHR also expresses more Fos-IR in the lamina terminalis in response to either central or systemic infusions of ANG II (3, 33). So, one may predict that the SHR has a stronger sodium appetite associated with increased c-Fos expression in facilitatory encephalic areas that control the ingestion of hypertonic NaCl solution.

Inhibitory signals produced in forebrain or hindbrain areas potentially counteract facilitatory signals of the lamina terminalis that converge onto the central amygdala (19). Lesion of brain oxytocinergic cells or central oxytocin receptor antagonism increases hypertonic NaCl intake in hypovolemic rats, thus suggesting an inhibitory role for the parvocellular subdivision of the hypothalamic paraventricular nucleus (PVNp), located in the forebrain (2, 35). Blockade of serotoninergic activity, caused by either tissue damage or pharmacological antagonism, in the dorsal raphe nucleus (DRN) or the lateral parabrachial nucleus (LPBN), both areas located in the hindbrain, increases hypertonic NaCl intake in hypovolemic rats (26, 31). In addition, the LPBN may also connect to the area postrema (AP) and the medial nucleus of the solitary tract to form an inhibitory axis (19). Thus, one may also predict that at the end of the WD-PR, the SHR has reduced c-Fos expression in areas associated with the inhibition of sodium appetite, such as LPBN, DRN, AP, and PVNp.

Therefore, the objective of the present work was to investigate whether WD-PR induces an increased sodium appetite and a differential expression of encephalic Fos-IR, in facilitatory (increased) and inhibitory (decreased) areas, in SHR compared with normotensive strains.

**MATERIALS AND METHODS**

**Animals.** Two hundred and thirty-six male rats (290–340 g) of the Holtzman (HTZ; n = 83), Wistar-Kyoto (WKY; n = 79), and SHR (n = 95) strains bred in the animal facility of Universidade Estadual Paulista at Araraquara were 12 wk old at the beginning of the experiments and individually housed in a room with controlled 12:12 h light-dark cycle, 23 ± 2°C, and 55 ± 10% humidity. Only SHRs that had a systolic arterial pressure ranging from 170 to 190 mmHg, as measured by tail plethysmography (Electro-Sphygmomanometer, Nigra Bio-Systems) connected to a computerized system (Codas/ Datag), entered the experiments. Polypropylene bottles (100 ml capacity with divisions to the nearest milliliter) with stainless-steel spouts, one bottle containing filtered water and another 0.3 M NaCl, were available ad libitum to each rat unless otherwise noted (see details of the experiments below). Standard Purina Guabi (Paulínia, SP, Brazil) rodent pellets (0.5–1.0% sodium) were freely available in the areas of interest (see details of the experiments below). Every experiment began at least 3 days after the animals were housed with all fluids available (adaptation period). The experimental tests began between 9 and 11 AM. The protocols were approved by the Institutional Ethical Committee for Animal Care (Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista) and followed the recommendations from the Brazilian College of Animal Experimentation.

**Solutions.** All reagents used to prepare the following solutions, 0.3 M NaCl, 0.1 M PBS, 4% paraformaldehyde (PFA), and 30% sucrose, were made from its respective salt (Labsynth, Diadema, SP, Brazil) dissolved in deionized water. Ethyl alcohol, xylene, 10% hydrogen peroxide, and 10% methanol were also from the same company.

**WD-PR protocol and sodium appetite test.** Animals were deprived of water and 0.3 M NaCl, with free access to food for 24 h (WD). Immediately after, food was removed and a glass burette with 0.1-ml divisions fitted with a stainless-steel spout containing filtered water was offered for 2 h (PR). Water intake was measured at 120 min. Drinking water to satiation during PR leads to dilution of body fluids and a partial recovery of the extracellular volume with sustained high PRA (7). At the end of the PR, a glass burette with 0.1-ml divisions fitted with stainless-steel spout containing 0.3 M NaCl was offered for a sodium appetite test. 0.3 M NaCl and water intake were measured at 15, 30, 60, and 120 min.

**Histology and immunohistochemistry.** Animals for immunohistochemistry entered either WD or WD-PR cycle at least 5 days after the adaptation period. Immediately at the end of a WD or a WD-PR cycle, the animals were deeply anesthetized with an intraperitoneal injection of sodium thiopental at 8 mg/100 g body wt (Cristália, Itapira, SP, Brazil). Hydrated controls (CONT) were anesthetized identically at the same time as the WD and WD-PR groups. Then they were perfused transcardially with 0.1 M PBS, pH 7.4, followed by 300–400 ml of 4% PFA in 0.1 M PBS. The brains were removed, fixed in PFA for 6 or 7 h, and then stored at 4°C in 0.1 M PBS plus 30% sucrose. Coronal sections of the areas of interest were cut into 50-μm slices using a freezing microtome (Leica SM 2000R). Alternate brain sections were taken for Fos-IR from each animal in the following groups: WD, WD-PR, and CONT. Analysis of cell c-Fos expression was performed on every other brain section, and an average of four sections per area of interest was used to count Fos protein. Alternate brain sections were also taken from a single untreated animal for Giemsa staining, which was done to illustrate the coronal section from the middle of the series of slices that were selected for c-Fos analysis. The brain slices were immersed in water containing 10% hydrogen peroxide and 10% methanol for 1 h. Then, the cuts were washed in 0.1 M PBS, and the free-floating sections were incubated under agitation for 22 h at room temperature in primary rabbit anti-Fos antibody raised against the amino acids 210 to 335 in the COOH terminus of human c-Fos (sc-7202, H-125; Santa Cruz Biotechnology, Santa Cruz, CA). The sequence that sc-7202 is raised against has a 91.9% identity to the rat c-fos sequence. The primary antibody had been diluted (1:4,000) in a solution of 0.1 M PBS containing 4% normal goat serum ($1000, Vector Laboratories, Burlingame, CA) and 3% Triton X-100 (Sigma-Aldrich, St. Louis, MO). After incubation with the primary antibody, the brain sections were washed in 0.1 M PBS and then incubated for 1 h at room temperature in 0.1 M PBS with biotinylated anti-rabbit IgG (1:400 dilution) (Vector Laboratories). After the incubation with the secondary antibody, the brain sections were washed in 0.1 M PBS and then incubated for 1 h at room temperature in 0.1 M PBS with avidin-biotin-peroxidase (1:200 dilution) complex (HRP Vectastain, Elite ABC kit, Vector Laboratories). The peroxidase label was detected using diaminobenzidine hydrochloride (D5637; Sigma Chemical, St. Louis, MO, USA), which produces a brown reaction product. Finally, the free-floating sections were mounted on gelatinized slides, air-dried for 3 or 4 days, dehydrated with ethyl alcohol, cleared in xylene, and coverslipped with Permount (SP15-500, Fisher Scientific, Hampton, NH).

The areas of interest to detect expression of the c-Fos protein were defined according to Paxinos and Watson (29): lamina terminalis (OVL’T, SFO, and dorsal and ventral MnPO); supraoptic (SON), supraoptic (SON), magnocellular (m) and parvocellular (p) subdivisions of the PVN hypothalamic nucleus; central nucleus of the amygdala (CEA); lateral (L) and medial (M) parabrachial nucleus (PBN); pre-locus coeruleus (pre-LC); caudal (c), area postrema-adjacent (ap), and intermediate (i) nuclei of the solitary tract (NTS); and area postrema (AP).

The images were examined at ×10 magnification with a microscope (BX 50, Olympus), and photomicrographs were captured.
through a camera (DP10, Olympus) connected to a computer running Image Pro-Plus 4.1.0.0 software. Manual cell counts were performed independently by two persons unaware of the treatment or strain. The density average of four alternate coronal sections was used to determine the number of c-Fos-positive nuclei per 1,000th (10^-3) of a square millimeter (mm²) within a given brain area of interest. The densities were focused on the four largest transversal alternate sections from each area, with a rostral-caudal interval measuring 300 µm. The statistical analysis was performed with the density average data from each animal. Bilateral areas (SCN, SON, PVN, CEA, LPBN, MPBN, pre-SC, apNTS, and iNTS) were counted from both hemispheres, but the representative photomicrographs depict the nuclei in only one hemisphere.

**Blood and urine biochemistry.** Trunk blood was collected immediately after decapitation from 24-h water-deprived (WD), 24-h water-deprived, and 24 h partially rehydrated (WD-PR), and CONT. Samples were collected in tubes containing a separating gel (SST, Becton Dickinson, San Jose, CA), centrifuged at 1,200 g for 10 min at room temperature to obtain serum. Serum sodium and potassium concentrations were measured with an ion-specific electrode (ISE AVL 9180; AVL Scientific, Rosewell, GA). Total serum protein was measured with a refractometer (Atago, Tokyo, Japan) and serum osmolality with an osmometer by freezing-point depression (Digidigm 3D2; Advanced Instruments, Norwood, MA, USA). Samples of the same rats were also collected in prerefrigerated tubes (4°C) containing EDTA (2 mg/ml of blood) and centrifuged at 1,500 g for 30 min at 4°C to obtain plasma for PRA measurement. PRA was determined by radioimmunoassay (Renin-Maia kit; Serono Diagnostics S.A., Coinsins, Switzerland).

Urine was collected immediately after 24 h of WD, 2 h of PR, and 24 h of CONT. Urine samples were collected in 0.1-ml graduated polypropylene tubes, where its volume (UV) was measured. Urine osmolality (UOSmol) and concentration of sodium and potassium were measured as described above. The total amounts of sodium excretions (UNaV) and potassium excretions (UKV) were determined by the product of urine volume (UV) × concentration of each ion.

**Arterial pressure recordings.** The animals were anesthetized with a mixed solution consisting of ketamine and xylazine (União Química, Embu-Guacu, SP, Brazil) at 80 and 7 mg/kg body wt, respectively. The mixed solution was injected at 0.12 ml/100 g body wt per rat to exteriorize at the nape of the neck. Direct mean arterial pressure (MAP) was recorded from the abdominal aorta in unanesthetized, unrestrained rats through the femoral catheter connected to a Narco (P-1000B) pressure transducer coupled to a multichannel recorder (Narcotrace 40, Narco Bio-System). Heart rate (HR) was recorded from the pulse of arterial pressure signal generated in the multichannel recorder.

Two days after the adaptation period, the animals (see experiment 3) were moved from metabolic cages to polypropylene plastic cages for cardiovascular recording in the hydrated state. MAP and respective HR recording were considered for analysis only after a stable baseline was achieved, defined as when MAP remained the same for at least 5 min of continuous recording. The animals were returned to their metabolic cages, where they remained for the next 24 h with only food, without water or 0.3 M NaCl. Immediately after 24 h, each animal was put back in the plastic cage for cardiovascular recording in the water-deprived state.

**Statistical analysis.** Data are reported as means ± SE. One- or two-way repeated- or nonrepeated-measures ANOVA was performed on all data to compare c-Fos expression, noncumulative intake, urine, and blood biochemistry among strains (one-way) or among strains and time or treatment (two-way). Significant interactions were further tested with Student-Newman-Keuls post hoc analysis. Nonpaired t-test was performed between two strains. Significance level was set at \( P < 0.05 \) for all tests.

**Experiment 1. Sodium appetite in response to water deprivation.** One group of each strain (n = 12–19 per strain) entered a WD-PR cycle followed by the sodium appetite test.

**Experiment 2. Fos-IR in HTZ, WKY, and SHR throughout a WD-PR cycle.** Immunohistochemistry for Fos protein was assessed in three groups, hydrated (CONT), water-deprived (WD), and water deprived-partially rehydrated (WD-PR) from all three strains, thus performing a total of nine groups (n = 6–11 per group). The pattern of c-Fos expression was analyzed in the OVLT, SFO, dorsal and ventral MnPO, SCN, SON, PVNm, PVNp, CEA, LPBN, MPBN, pre-SC, cNTS, apNTS, iNTS, and AP for each one of the nine groups.

**Experiment 3. blood and urine biochemistry, and cardiovascular recordings: comparisons among HTZ, WKY, and SHR.** Animals from HTZ, WKY, and SHR (n = 7 per strain) had arterial pressure recorded as previously described, and urine biochemistry determined during the WD period. Another group of each strain (n = 18–27 per strain) were assessed for blood biochemistry determination in three groups (CONT, WD, or WD-PR), thus performing a total of nine groups.

**Experiment 4. feeding and sodium balance: comparisons between HTZ and SHR.** This experiment was performed to have the sodium balance evaluated prior to the sodium appetite test. Detailed analysis of daily fluid and food intake, and of sodium balance, in response to WD-PR, was performed for SHR. Because of the similarities between the two normotensive strains, HTZ and WKY, in the sodium appetite test, blood and urine biochemistry, and cardiovascular recordings, only one of them entered this experiment as control to the SHR. We have chosen the HTZ to do so because this is the strain most used in our laboratory.

Animals from HTZ (n = 7) and SHR (n = 7) strains were housed in metabolic cages, and measurements of daily fluid intake (0.3 M NaCl and water), powdered regular chow, and urine biochemistry were done for 3 days after the adaptation period. On the 3rd day, a suprapubic massage to induce urine void was performed immediately prior to water deprivation in all animals of both strains (WD). After 24 h, the suprapubic massage was performed again, overnight urine was collected for biochemistry determination, and food was removed from the cage for intake measurement, thus allowing them to begin the 2-h PR drinking test. Water intake and urine volume were measured, and urine biochemistry was determined in both strains at the end of the test. Sodium balance was calculated in CONT, WD, and WD-PR for both HTZ and SHR.

**RESULTS**

**Experiment 1. Sodium appetite of HTZ, WKY, and SHR, in response to water deprivation.** The body weight after 24 h of WD was 303 ± 6, 343 ± 8, and 298 ± 7 g for HTZ, WKY, and SHR, respectively. The SHR weighed similarly to the HTZ, but both strains weighed less than WKY (\( F_{2,45} = 11.6, P < 0.05 \)). All three strains ingested similar amounts of water during the PR, which preceded the sodium appetite test (HTZ: 13.0 ± 0.9, WKY: 12.0 ± 0.5, and SHR: 11.5 ± 0.7 ml/240 min, \( n = 12–19 \) per strain).

The noncumulative 0.3 M NaCl intake of SHR was different from the normotensives throughout the sodium appetite test (\( F_{2,184} = 106.3; P < 0.05 \)) (Fig. 1, top left). Cumulative intakes suggest that the SHR ingested about 10 times more 0.3 M NaCl than HTZ and WKY (Fig. 1, top right). The noncumulative water intake during the sodium appetite test was higher in SHR than the other strains only at 60 and 120 min (\( F_{2,184} = 43.6; P < 0.05 \)) (Fig. 1, bottom left). Cumulative intakes suggest that the SHR also ingested about 5 times more water than HTZ and WKY (Fig. 1, bottom right).
Experiment 2. Fos-IR in HTZ, WKY, and SHR throughout a WD-PR cycle. Water deprivation produced a general increase in Fos-IR in the facilitatory area lamina terminalis of all strains compared with the hydrated state or control (CONT) (OVLT: $F_{2,69} = 83.7$; MnPO: $F_{2,66} = 53.4$; SFO: $F_{2,72} = 37.2$, $P < 0.05$) (Fig. 2, top left and right, and bottom left). Because there was not any difference between ventral and dorsal MnPO, the data of this nucleus are represented as the average of both.

The WD-PR induced greater Fos-IR in the SHR compared with WKY or HTZ (SFO: $F_{2,80} = 4.4$, $P < 0.05$), and WD-PR further enhanced Fos-IR in the SFO of all strains compared with WD, with SHR showing the most enhancement (Fig. 2, top left). After WD-PR, Fos-IR remained the same in the OVLT of SHR and HTZ and in the MnPO of all strains compared with WD of each strain (Fig. 2, top right and bottom left). However, WD-PR enhanced Fos-IR in the OVLT of WKY compared with WD of the same strain (Fig. 2, top right).

In the facilitatory area CEA, water deprivation did not induce Fos-IR in all strains compared with CONT of each strain ($F_{2,68} = 28.3$, $P < 0.05$), but Fos-IR was greater in SHR compared with HTZ and WKY after WD ($F_{2,76} = 6.2$, $P < 0.05$) (Fig. 2, bottom right). WD-PR induced a marked increase in Fos-IR in the CEA of all strains compared with the CONT, but there was not any difference between WD-PR and WD in SHR (Fig. 2, bottom right).

A different pattern of Fos-IR was found in the forebrain compared with the hindbrain concerning inhibitory areas. In the forebrain, water deprivation induced Fos-IR in the PVNp of
only HTZ and SHR compared with CONT (strain, $F_{2,78} = 7.1$, group, $F_{2,70} = 6.8$, strain vs. group, $F_{4,70} = 3.6$, $P < 0.05$) (Fig. 3, top left). There was a complete reversal to control level in SHR after the WD-PR. There was an apparent reversal in HTZ, but the Fos-IR after WD-PR in this strain was not significantly different from either CONT or WD. A slight increase in Fos-IR was induced by only WD-PR in the PVNp of WKY (Fig. 3, top left). In the hindbrain, WD-PR, but not water deprivation, induced Fos-IR in the LPBN and DRN (Fig. 3, top right and bottom). WD-PR induced an increase in Fos-IR in the LPBN of all strains compared with WD and control of each strain ($F_{2,71} = 22.7$, $P < 0.05$) (Fig. 3, top right). WD-PR also induced Fos-IR in DRN of all strains compared with WD and control of each strain, except in the DRN of SHR, which was different than only WD ($F_{2,64} = 12.9$, $P < 0.05$) (Fig. 3, bottom).

Water deprivation induced similar Fos-IR in the PVNm of HTZ and SHR (CONT: $1.5 \pm 0.5$ and WD: $10.8 \pm 2.0$ c-Fos-positive nuclei/10$^{-3}$ mm$^2$) ($F_{2,70} = 11.3$, $P < 0.05$). In the PVNm, the WD-induced Fos-IR of both HTZ and SHR was greater than Fos-IR of WKY after WD ($0.1 \pm 0.1$ c-Fos-positive nuclei/10$^{-3}$ mm$^2$) ($F_{2,78} = 9.7$, $P < 0.05$). The Fos-IR in the PVNm was completely reversed to control levels by WD-PR in HTZ and SHR (2.8 $\pm$ 1.5 c-Fos-positive nuclei/10$^{-3}$ mm$^2$). WD-PR, as well as WD, did not induce Fos-IR in the PVNm of WKY (CONT: $0.2 \pm 0.2$ and WD-PR: $1.9 \pm 1.1$ c-Fos-positive nuclei/10$^{-3}$ mm$^2$).

Water deprivation induced Fos-IR in the SON of all strains ($F_{2,68} = 45.1$, $P < 0.05$), more in HTZ and SHR (11.7 $\pm$ 2.1 c-Fos-positive nuclei/10$^{-3}$ mm$^2$) than WKY (2.1 $\pm$ 0.7 c-Fos-positive nuclei/10$^{-3}$ mm$^2$) ($F_{2,76} = 8.7$, $P < 0.05$). Fos-IR in the SON was then reversed to control levels by WD-PR in all strains (WD-PR: $0.5 \pm 0.3$ and CONT: $0.3 \pm 0.2$ c-Fos-positive nuclei/10$^{-3}$ mm$^2$).

Water deprivation did not induce Fos-IR in other areas of interest, such as pre-LC, cNTS, and MPBN of any strain compared with CONT (Fig. 4). However, the WD-PR induced Fos-IR in pre-LC of all strains compared with WD and CONT of each strain ($F_{2,71} = 21.6$, $P < 0.05$) (Fig. 4, top left). The WD-PR increased Fos-IR in the cNTS of HTZ and SHR compared to CONT and WD ($F_{2,71} = 16.9$, $P < 0.05$) (Fig. 4, top right). Similar to what happened in the SFO, the WD-PR induced more Fos-IR in the pre-LC ($F_{2,79} = 5.4$, $P < 0.05$) and cNTS ($F_{2,79} = 4.6$, group, $P < 0.05$) of SHR compared with...
HTZ and WKY (Fig. 4, top). WD-PR also increased Fos-IR in the MPBN of SHR compared with CONT ($F_{2,71} = 6.4, P < 0.05$) (Fig. 4, bottom).

As illustrated by Figs. 2 and 4, differential expression of Fos-IR among strains occurred at the end of the WD-PR cycle. SHR showed greater Fos-IR than WKY or HTZ in the SFO, pre-LC, and cNTS. Representative photomicrographs of immunohistochemistry of these nuclei are depicted at Fig. 5.

The WD-PR, but not water deprivation, induced Fos-IR in still other areas of interest, such as SCN ($F_{2,58} = 28.5, P < 0.05$), AP ($F_{2,56} = 39.4, P < 0.05$), apNTS ($F_{2,58} = 28.9, P < 0.05$), and iNTS ($F_{2,58} = 43.9, P < 0.05$) of all strains compared with CONT and WD (Table 1). Control SHR also had more Fos-IR in iNTS than WKY ($F_{2,64} = 4.2, P < 0.05$) (Table 1).

The effects of WD and WD-PR in the Fos-IR of all strains are summarized at Table 2.

Experiment 3. Blood and urine biochemistry, and cardiovascular recordings: comparisons among HTZ, WKY, and SHR.

Blood biochemistry. In all strains, water deprivation induced hypernatremia ($F_{2,69} = 101.8, P < 0.05$), hyperproteine-"nemia ($F_{2,69} = 58.2, P < 0.05$), and hyperosmolality ($F_{2,81} = 48.8, P < 0.05$), while rehydration produced hyponatremia and hypoproteinemia, but maintained hyperproteine"mnia (Table 3). Hyperosmolality was higher in SHR and HTZ than WKY, and hypoprot"molarity was lower in SHR than HTZ ($F_{2,88} = 2.9, P < 0.05$) (Table 3). Total protein was higher for all treatments in WKY compared with the other two strains ($F_{2,77} = 28.4, P < 0.05$) (Table 3).

Water deprivation also increased PRA in all strains ($F_{2,36} = 54.9, P < 0.05$), and rehydration further increased PRA in HTZ and WKY (Table 3). Water deprivation increased PRA more in SHR compared with HTZ and rehydration increased PRA more in WKY compared with SHR and HTZ ($F_{2,62} = 6.0, P < 0.05$) (Table 3). Kalemia was not altered by WD or WD-PR treatment.

A slight hyperkalemia was found in SHR compared with HTZ in the CONT ($F_{2,77} = 5.8, P < 0.05$) (Table 3).

Urine biochemistry and cardiovascular recordings. All strains had similar UV (HTZ: 7.8 ± 1.3, WKY: 6.9 ± 0.9, and SHR: 6.0 ± 0.9 ml), UOsmol (HTZ: 1.918 ± 80, WKY: 1.615 ± 183, and SHR: 1.754 ± 87 mOsmol/kg), and UNaV (HTZ: 720 ± 112, WKY: 597 ± 140, and SHR: 499 ± 111 μeq) during the 24 h of water deprivation. However, WKY (278 ± 27 μeq) and SHR (232 ± 35 μeq) had reduced UKV

Fig. 3. Inhibitory areas: immunohistochemistry for c-Fos expression in cells from the parvocellular subdivision of the paraventricular nucleus (PVN), lateral parabrachial nucleus (LPBN), and dorsal raphe nucleus (DRN) of CONT, WD, and WD-PR HTZ, WKY, and SHR rats.
compared with HTZ (413 ± 57 μeq) ($F_{2,20} = 6.8; P < 0.05$).

There were no differences in MAP or HR between the hydrated and dehydrated states (Table 4). In both the hydrated and dehydrated states, MAP was higher in SHR than in both normotensive strains ($F_{2,18} = 51.1, P < 0.05$), and HR was lower in WKY than HTZ and SHR ($F_{2,18} = 4.3, P < 0.05$).

Experiment 4. Feeding and sodium balance: comparisons between HTZ and SHR. Daily 0.3 M NaCl was about 5 times higher in SHR than HTZ, while daily water and food intake was slightly lower in SHR (nonpaired t-test; $P < 0.05$) (Table 5). UV, UNaV, and UKV were also higher, while UOsmol was lower in SHR (nonpaired t-test; $P < 0.05$) (Table 5). Sodium balance (sodium loss in urine minus the sum of sodium ingested in the solution and/or food) in the hydrated state was more positive in the SHR than HTZ (nonpaired t-test; $P < 0.05$) (Table 5).

Water deprivation reduced food intake by 41% in HTZ and 57% in SHR ($F_{1,14} = 424.0, P < 0.05$), and this reduction was 27% greater in SHR than HTZ ($F_{1,14} = 14.8, P < 0.05$) (Table 5). In both strains, water deprivation also reduced UV ($F_{1,14} = 42.6, P < 0.05$), UNaV ($F_{1,14} = 5.7, P < 0.05$), and UKV ($F_{1,14} = 58.7, P < 0.05$), and it enhanced the UOsmol ($F_{1,14} = 149.3, P < 0.05$) (Table 5). The UV, UOsmol, and UNaV were similar between the two strains. However, SHR had reduced UKV compared with HTZ, a similar result from experiment 3 (urine biochemistry) (nonpaired t-test; $P < 0.05$). Sodium balance at the end of 24 h of water deprivation was similar between strains.

Water intake, UV, UOsmol, UNaV, and UKV were similar between the two strains after rehydration (Table 5). Sodium balance was similar between HTZ and SHR at the end of WD-PR, right before the time for sodium appetite test (Table 5). The 0.3 M NaCl intake was again higher in SHR (14.8 ± 0.8 ml/120 min) than in HTZ (3.7 ± 1.5 ml/120 min) in the sodium appetite test (nonpaired t-test; $P < 0.05$).

**DISCUSSION**

The results show that SHR have a stronger sodium appetite than WKY or HTZ and a different pattern of encephalic c-Fos expression in response to WD-PR.

Water deprivation induced similar amounts of water intake in HTZ, WKY, and SHR. The WD also induced a similar increase in serum sodium and osmotic concentration, total serum protein, and PRA between SHR and normotensives. It also induced similar urinary volume and sodium output. The cycle of water deprivation and partial rehydration reversed the...
high serum sodium and osmotic concentration, but it only partially reduced the total serum protein in all strains. The elevated PRA persisted after WD-PR in all strains. Animals of all strains ingested 0.3 M NaCl in the sodium appetite test that followed the WD-PR, but the SHR ingested about 10 times more than normotensive strains. The WD activated Fos-IR compared with hydrated control in the lamina terminalis, SON, and PVNm of all strains and in the PVNp of only HTZ and SHR. The WD-PR further increased Fos-IR in the SFO of all strains and in the OVLt of WKY. The WD-PR did not alter the Fos-IR activated by WD in the OVLt of HTZ and SHR and in the MnPO of all strains. The WD-PR reversed Fos-IR to control in the SON of all strains, PVNp of SHR, and PVNm of HTZ and SHR. The WD-PR activated Fos-IR compared with control in the CEA, SCN, LPBN, DRN, pre-LC, and NTS of all strains, except cNTS of WKY. The WD-PR activated more cells in the SFO, pre-LC, MPBN, and cNTS of SHR than HTZ and WKY.

The stronger sodium appetite of SHR may not be surprising, but there are a few examples of studies with this strain of rats showing that they increase hypertonic NaCl intake in response to dehydration associated with negative sodium balance, thus characterizing a true sodium appetite (12, 23). Water deprivation induces an increased ingestion and a slower decline in lick rate of 0.3 M NaCl in a one-bottle test in SHR compared with WKY, in spite of their similar urinary output (12).

![Fig. 5. Representative photomicrographs from brains of HTZ (left), WKY (middle), and SHR (right) corresponding to results from Figs. 2 and 4, showing c-Fos expression in the subfornical organ (SFO, top), pre-locus coeruleus (pre-LC, middle), and caudal nucleus of the solitary tract (cNTS; bottom) at the end of a WD-PR cycle. Far right: each box indicates the area stained with Giemsa that corresponds to the coronal section from the middle of the series of slices that were selected for c-Fos analysis.](image)

Table 1. **Number of Fos-positive nuclei/10⁻³ mm² in other encephalic areas of interest of hydrated controls and rats that responded to a cycle of water-deprivation or water deprivation-partial rehydration**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>SCN</th>
<th>AP</th>
<th>apNTS</th>
<th>iNTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTZ</td>
<td>CONT</td>
<td>4.3 ± 0.6</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>WD</td>
<td>4.5 ± 1.9</td>
<td>0.2 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>WD-PR</td>
<td>20.3 ± 5.3*†</td>
<td>3.2 ± 0.8*†</td>
<td>5.7 ± 1.8*†</td>
<td>8.3 ± 1.9*†</td>
</tr>
<tr>
<td>WKY</td>
<td>CONT</td>
<td>4.2 ± 1.1</td>
<td>0.0 ± 0.0</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>WD</td>
<td>5.7 ± 1.4</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>WD-PR</td>
<td>20.6 ± 3.0*†</td>
<td>2.6 ± 0.6*†</td>
<td>3.0 ± 0.6*†</td>
<td>6.3 ± 0.9*†</td>
</tr>
<tr>
<td>SHR</td>
<td>CONT</td>
<td>3.8 ± 1.2</td>
<td>0.4 ± 0.2</td>
<td>1.0 ± 0.7</td>
<td>3.5 ± 1.2‡</td>
</tr>
<tr>
<td></td>
<td>WD</td>
<td>7.7 ± 0.8</td>
<td>1.5 ± 0.5</td>
<td>1.6 ± 0.5</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>WD-PR</td>
<td>16.0 ± 3.5*†</td>
<td>3.2 ± 0.4*†</td>
<td>4.1 ± 0.8*†</td>
<td>7.7 ± 1.6*†</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE, n = 6–11 per strain per group. HTZ, Holtzman; WKY, Wistar Kyoto; SHR, spontaneously hypertensive rats; CONT, hydrated control; WD, water deprivation; WD-PR, water deprivation-partial rehydration; SCN, suprachiasmatic nucleus; AP, area postrema; apNTS, postrema-adjacent nucleus of the solitary tract; iNTS, intermediate nucleus of the solitary tract. *P < 0.05 vs. CONT, †P < 0.05 vs. WD, and ‡P < 0.05 vs. WKY CONT.

Fig. 5. Representative photomicrographs from brains of HTZ (left), WKY (middle), and SHR (right) corresponding to results from Figs. 2 and 4, showing c-Fos expression in the subfornical organ (SFO, top), pre-locus coeruleus (pre-LC, middle), and caudal nucleus of the solitary tract (cNTS; bottom) at the end of a WD-PR cycle. Far right: each box indicates the area stained with Giemsa that corresponds to the coronal section from the middle of the series of slices that were selected for c-Fos analysis.
consistent with the similar peripheral responses among strains found in the present work. Thus, it seems that the mechanisms of the stronger sodium appetite of the SHR are a feature of how the central nervous system works in this strain. Flynn et al. (12) also suggest that the reduced decline in lick rate may result from attenuation of postigestive negative feedback because lick rate declines slowly only after an initial similar rate between SHR and WKY (12). This reduced negative feedback could at least, in part, result from a predominant activity of facilitative areas vs. inhibitory areas that control sodium appetite.

The increased number of cells expressing Fos-IR in the SFO, OVLT, MnPO, and CEA—correlated with high PRA at the end of the WD-PR in the three strains—is consistent with the lamina terminalis sensory model of sodium appetite mentioned in the introduction (19, 20). It also confirms what was found previously in the lamina terminalis of Sprague-Dawley rats (7). Moreover, one may predict that enhanced activity in these structures correlates with enhanced sodium intake. It is known that ANG II increases neuronal firing rate in the circumventricular organs, including OVLT and SFO (14), but it is also possible that an increased number of cells expressing c-Fos in these structures corresponds to an increased number of cells that are activated to encode the intensity of a stimulus, similarly to what other sensory systems do (21). For example, the number of neurons expressing Fos-IR in the trigeminal sensory nucleus and in neurons of the olfactory bulb is positively correlated with the intensity of the respective adequate stimulus (18, 37). A possibility to be tested is whether the number of cells activated by a stimulus relates to the total number of cells in particular regions of particular strains, like what we may find in some structures of the medulla (44).

The SHRs have a hyperactive RAS and increased density of ANG II binding sites in several brain areas, including the lamina terminalis (27, 40). This is consistent with the enhanced Fos-IR in the forebrain of SHR compared with normotensive rats in response to exogenous ANG II (Ref. 3, but see Ref. 33). Because the present work shows that peripheral signals are similar among strains, it is possible that the enhanced number of cells expressing c-Fos that we found in the facilitatory areas of the brain of SHR, particularly in the SFO, reflects an enhanced central sensitivity to facilitatory signals, for example, ANG II.

The pattern of Fos-IR that we found in the PVNp would, in principle, correlate with an inhibitory area that counteracts the activity of the lamina terminalis. The SHR was the only strain in which the Fos-IR was induced by WD and reversed to the hydrated state by PR. Although double labeling was not performed in the present work, this reversal of Fos-IR is consistent with deactivation of cells that produce oxytocin. Recall that the PVNp is a source of central oxytocin, an inhibitory peptide of sodium appetite, and that peptidergic neurons of the PVNp are activated by hyperosmolality (17, 35). Sodium appetite is inhibited by central injections of oxytocin and is enhanced when central oxytocin neurons are killed by intracerebroventricular injections of oxytocin conjugated to the potent toxin ricin (2, 35). The oxytocin inhibitory hypothesis of sodium appetite states that ANG II and hyperosmolality activate oxytocin release from the PVNp, which is suppressed when the animal drinks water. This suppression allows the facilitatory action of ANG II (2, 35).

The relative importance of facilitatory vs. inhibitory areas, here suggested by the different Fos-IR in the lamina terminalis—and perhaps in the pre-LC, cNTS, CEA, and MPBN—vs. the PVNp of SHR, provides an overview, even if framed in a single snapshot, of how the activity in those areas is associated with the beginning of sodium intake in the sodium appetite test. Also recall that the NTS, MPBN, and pre-LC might belong to a hindbrain-forebrain axis with intermingled inhibitory and excitatory areas that are modulated in different ways by different hormones and neurotransmitters.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>SFO</th>
<th>OVLT</th>
<th>MnPO</th>
<th>CEA</th>
<th>LPBN</th>
<th>DRN</th>
<th>PVNp</th>
<th>PVNm</th>
<th>SON</th>
<th>SCN</th>
<th>pre-LC</th>
<th>aNTS</th>
<th>cNTS</th>
<th>iNTS</th>
<th>AP</th>
<th>MPBN</th>
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<tbody>
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<td>WD</td>
<td>+</td>
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<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td></td>
<td>WD-PR</td>
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<tr>
<td></td>
<td>WD-PR</td>
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</tr>
</tbody>
</table>

Values are 0, no activation; +, activation; ++, further activation; and −, deactivation; n = 6–11 per strain per group. For further details, see Figs. 2–6, Table 1, and the RESULTS.
facilitatory circuits of sodium appetite (15), and these areas could somehow participate in the interaction between these circuits.

However, there are some caveats about the inhibitory areas in the hindbrain that must be considered when we try to build a meaningful picture of the Fos-IR as a potential marker of circuits that operate to produce a sodium appetite, in general, and in the SHR, in particular.

The LPBN apparently protects the animal against hypertonicity because it restrains the ingestion of NaCl in hypertonic concentration but not in isotonic (4), and serotonin, a neurotransmitter that induces satiety, is released in the LPBN when the rat starts to ingest hypertonic NaCl (38). An enhanced ingestion of hypertonic NaCl may be unmasked, for example, by injecting a serotoninergic antagonist into the LPBN (4, 26) or by damage to another source of serotonin, the DRN (31). In addition, water deprivation induces both hyperosmolality and increased levels of ANG II in the blood. The increase in osmolality induces Fos-IR in the LPBN and DRN (17, 46) and inhibits sodium intake (2, 35). ANG II induces Fos-IR also in the LPBN (45), like it does in the PVNp (35), perhaps emulating the oxytocin inhibitory mechanism. This suggests that the LPBN, DRN, and PVN might belong to a similar inhibitory circuit.

However, water deprivation did not alter the number of cells expressing Fos-IR in DRN or LPBN compared with control. Moreover, we predicted a low Fos-IR in response to PR, but we found exactly the opposite; water intake increased Fos-IR in these nuclei, which was similar between the SHR compared with the other strains. The increase in Fos-IR produced by water intake in these two nuclei is similar to what has been observed in previous works, in which either water or 0.15 M NaCl intake produced Fos-IR in the PBN of water-deprived animals (16).

The increase in LPBN, DRN, and AP (plus the adjacent aNNTS) activity, as shown in the present work, is not compatible with their inhibitory function of sodium intake. This is more relevant when we recall that serotoninergic antagonism in the LPBN also enhances hypertonic NaCl intake induced by water deprivation (26), and the AP may act with the LPBN to inhibit sodium intake (19). Also recall that it is not any fluid intake that increases Fos-IR in these nuclei. For example, whereas increased hypertonic or isotonic NaCl intake enhances Fos-IR in serotoninergic neurons of DRN, only hypertonic NaCl intake enhances Fos-IR in cells of the LPBN of rats depleted of sodium by intraperitoneal dialysis (17). Therefore, it is possible that the increase in Fos-IR in these nuclei occurs when water-deprived animals engage in their first bout of fluid intake, no matter which kind; water (in the present work) or water or isotonic NaCl [in a study by Gottlieb et al. (16)]. The combination of these results raises the possibility that the increased activity in LPBN, DRN, and AP relates not only to the inhibition of sodium intake, but also to the control of another function. Their response to fluid intake that follows water deprivation could relate, for example, with the satiation of thirst.

**Perspectives and Significance**

The Fos-IR mapping in the forebrain as shown here allows a picture of similar activity in several areas that belong to the model proposed by Johnson (19) about the control of sodium appetite induced by selective extracellular dehydration. This picture is partly consistent with the model, at least in the forebrain. However, the results with the hindbrain strongly warn us to exercise caution when interpreting Fos-IR, particularly in terms of a causal relationship between cell activity and behavior. Moreover, it would be of utmost importance to know the dynamics of the circuit, particularly from the moment the animal is rehydrated to the end of the sodium appetite test to understand the causal mechanisms of sodium appetite. The correlation between increased Fos-IR and the stronger sodium appetite of SHR also point to the necessity to increase the knowledge of how the activity in facilitatory areas, particularly in the sensory lamina terminalis, encodes the humoral stimuli that signal sodium deficit or hypovolemia. This would require

### Table 4. Mean arterial pressure and heart rate of hydrated and water-deprived HTZ, WKY, and SHR rats

<table>
<thead>
<tr>
<th>Strain</th>
<th>MAPCONT</th>
<th>MAPWD</th>
<th>ΔMAPWD−CONT</th>
<th>HRCONT</th>
<th>HRWD</th>
<th>ΔHRWD−CONT</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTZ</td>
<td>120 ± 3</td>
<td>125 ± 3</td>
<td>3 ± 4</td>
<td>428 ± 138†</td>
<td>438 ± 14†</td>
<td>3 ± 10</td>
</tr>
<tr>
<td>WKY</td>
<td>103 ± 2</td>
<td>115 ± 5</td>
<td>4 ± 7</td>
<td>371 ± 8</td>
<td>378 ± 17</td>
<td>5 ± 13</td>
</tr>
<tr>
<td>SHR</td>
<td>188 ± 10†</td>
<td>170 ± 8†</td>
<td>−13 ± 4</td>
<td>420 ± 21†</td>
<td>444 ± 14†</td>
<td>18 ± 16</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = 7 per strain. MAP, mean arterial pressure; HR, heart rate. *P < 0.05 vs. HTZ and †P < 0.05 vs. WKY.

### Table 5. Fluid and food intake, and urinary biochemistry of 24-h hydrated and water-deprived rats, and of 24-h water-deprived rats that had a partial rehydration period of 2 h

<table>
<thead>
<tr>
<th>Intake</th>
<th>Strain</th>
<th>Treatment</th>
<th>Water, ml</th>
<th>0.3 M NaCl, ml</th>
<th>Food, g</th>
<th>UV, ml</th>
<th>UNaV, μEq</th>
<th>UKV, μEq</th>
<th>UOsmol, mOsmol/kg</th>
<th>Sodium balance, μEq</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HTZ</td>
<td>CONT</td>
<td>35 ± 1</td>
<td>7 ± 2</td>
<td>24.2 ± 0.6</td>
<td>8.7 ± 1.5</td>
<td>1888 ± 35</td>
<td>691 ± 116</td>
<td>2756 ± 252</td>
<td>2178 ± 482</td>
</tr>
<tr>
<td></td>
<td>WD</td>
<td></td>
<td></td>
<td></td>
<td>13.5 ± 0.6†</td>
<td>4.3 ± 0.4†</td>
<td>1173 ± 85†</td>
<td>341 ± 37†</td>
<td>3783 ± 224†</td>
<td>−18 ± 100</td>
</tr>
<tr>
<td></td>
<td>WD-PR</td>
<td></td>
<td>12.5 ± 0.9</td>
<td></td>
<td>−</td>
<td>1.8 ± 0.5</td>
<td>25 ± 14</td>
<td>86 ± 27</td>
<td>658 ± 136</td>
<td>−44 ± 93</td>
</tr>
<tr>
<td>SHR</td>
<td>CONT</td>
<td>22 ± 1*</td>
<td>35 ± 4*</td>
<td>21.3 ± 0.9*</td>
<td>22.5 ± 3.4*</td>
<td>8666 ± 1063*</td>
<td>1624 ± 207*</td>
<td>1801 ± 113*</td>
<td>3944 ± 626*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WD</td>
<td></td>
<td>9.8 ± 0.5†</td>
<td></td>
<td>3.3 ± 0.5†</td>
<td>1002 ± 136†</td>
<td>216 ± 23†</td>
<td>3739 ± 196†</td>
<td>−163 ± 114</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WD-PR</td>
<td></td>
<td>9.4 ± 1.2</td>
<td></td>
<td>1.2 ± 0.3</td>
<td>13 ± 2</td>
<td>50 ± 9</td>
<td>819 ± 133</td>
<td>−117 ± 115</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = 7 per strain. Urine (UV) and osmolality (UOsmol) volume, total amount of sodium (UNaV), and potassium (UKV) excreted, and sodium balance. *P < 0.05 vs. HTZ same group. †P < 0.05 vs. CONT same strain.

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more studies with Fos or other cell markers perhaps combined with tracing methods and recording of cell activity in vitro or in conscious animals.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES

No references are provided in the document.


