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### Catecholaminergic neurons in the commissural region of the nucleus of the solitary tract modulate hyperosmolality-induced responses

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<sup>2</sup>Department of Physiological Sciences, Federal University of Goiás, Goiânia, Goiás, Brazil; <sup>3</sup>Department of Physiology, Escola Paulista de Medicina, Universidade-Federal de São Paulo, São Paulo, Brazil; <sup>4</sup>Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, University of Bristol, Bristol, United Kingdom; and <sup>5</sup>Department of Physiology, University of Malaya, Kuala Lumpur, Malaysia

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**Freiria-Oliveira AH, Blanch GT, Pedrino GR, Cravo SL, Murphy D, Menani JV, Colombari DS.** Catecholaminergic neurons in the commissural region of the nucleus of the solitary tract modulate hyperosmolality-induced responses. *Am J Physiol Regul Integr Comp Physiol* 309: R1082–R1091, 2015. First published September 2, 2015; doi:10.1152/ajpregu.00432.2014.—Noradrenergic A2 neurons of the nucleus of the solitary tract (NTS) have been suggested to contribute to body fluid homeostasis and cardiovascular regulation. In the present study, we investigated the effects of lesions of A2 neurons of the commissural NTS (cNTS) on the c-Fos expression in neurons of the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei, arterial pressure, water intake, and urinary excretion in rats with plasma hyperosmolality produced by intragastric 2 M NaCl (2 ml/rat). Male Holtzman rats (280–320 g) received an injection of anti-dopamine- $\beta$ -hydroxylase-saporin (12.6 ng/60 nl; cNTS/A2-lesion,  $n = 28$ ) or immunoglobulin G (IgG)-saporin (12.6 ng/60 nl; sham,  $n = 24$ ) into the cNTS. The cNTS/A2 lesions increased the number of neurons expressing c-Fos in the magnocellular PVN in rats treated with hypertonic NaCl ( $90 \pm 13$ , vs. sham:  $47 \pm 20$ ;  $n = 4$ ), without changing the number of neurons expressing c-Fos in the parvocellular PVN or in the SON. Contrary to sham rats, intragastric 2 M NaCl also increased arterial pressure in cNTS/A2-lesioned rats ( $16 \pm 3$ , vs. sham:  $2 \pm 2$  mmHg 60 min after the intragastric load;  $n = 9$ ), an effect blocked by the pretreatment with the vasopressin antagonist Manning compound ( $0 \pm 3$  mmHg;  $n = 10$ ). In addition, cNTS/A2 lesions enhanced hyperosmolality-induced water intake ( $10.5 \pm 1.4$ , vs. sham:  $7.7 \pm 0.8$  ml/60 min;  $n = 8$ –10), without changing renal responses to hyperosmolality. The results suggest that inhibitory mechanisms dependent on cNTS/A2 neurons reduce water intake and vasopressin-dependent pressor response to an acute increase in plasma osmolality.

c-Fos expression; blood pressure; vasopressin; osmoreceptor; thirst

PLASMA OSMOLALITY IS MAINTAINED in a very narrow limit in mammals by homeostatic processes that integrate renal, neuroendocrine, and behavioural responses (2, 32, 39, 52). Increases in sodium plasma concentrations activate central osmoreceptors located in the forebrain areas, such as the

subfornical organ (SFO) and the region surrounding the anteroventral third ventricle (AV3V), signaling for thirst, hormonal (vasopressin and oxytocin release), renal (antidiuresis and natriuresis), and cardiovascular responses (hypertension and increases in sympathetic nerve activity) (6, 7, 30, 31, 37, 52) to restore osmolality to normal levels.

Experimentally, plasma osmolality can be increased by intragastric hypertonic (2 M) NaCl load, which mimics the ingestion of osmotically active substances by food intake, resulting in cellular dehydration (18, 48). Hyperosmolality induced by intragastric 2 M NaCl in rats increases plasma sodium concentration and osmolality by 4% and reduces plasma renin activity, without changing hematocrit and total plasma protein for at least the 60 min after NaCl load (38). Additionally, intragastric 2 M NaCl induces thirst, natriuresis, and increases plasma vasopressin (AVP) and oxytocin (OT), (1, 6, 38).

Recently, we demonstrated that the commissural nucleus of the solitary tract (cNTS), in the brain stem, has an inhibitory effect on the thirst, natriuresis, and vasopressin-dependent pressor response induced by acute hyperosmotic stimuli (6). A population of noradrenergic neurons in the NTS, known as the A2 group, extends from the intermediate NTS (iNTS) at the level of the obex to the caudal pole of the cNTS (14, 43). This neuronal group projects to the lateral parabrachial nucleus (LPBN), the median preoptic nucleus (which is part of the AV3V region), the SFO, and the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus (10, 16, 35, 41, 43, 46). The A2 neurons receive sensory feedback from the cardiovascular, respiratory, and gastrointestinal tract systems (3, 26) and are activated by intraperitoneal or intravenous injection of hypertonic saline (20, 22), gastrointestinal stretch (55), and food intake (42). Thus, it is possible that the inhibitory effect of cNTS electrolytic lesion on hyperosmolality-induced responses (6) is due to damage of the cNTS/A2 neurons.

Therefore, in the present study, we investigated the effects of A2 lesions of the cNTS with anti-D $\beta$ H-saporin on the c-Fos expression in neurons of the PVN and SON and on arterial pressure, water intake, and urinary excretion in rats with increases in plasma osmolality.

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## METHODS

### Animals

Male Holtzman rats weighing 280–320 g were used. A total of 24 rats with sham lesions and 28 rats with cNTS/A2 lesions were used in this study. The animals were housed individually in stainless-steel cages in a room with controlled temperature ( $23 \pm 2^\circ\text{C}$ ) and humidity ( $55 \pm 10\%$ ). Lights were on from 7:00 AM to 7:00 PM. Standard Bio Base rat chow (Águas Frias, SC, Brazil) and tap water were available ad libitum. The Ethics Committee for Animal Care and Use of the School of Dentistry of Araraquara, São Paulo State University, approved the experimental protocols used in the present study (protocol number 09/2007).

### Drugs

Anti-D $\beta$ H-saporin and anti-IgG-saporin (Advanced Targeting Systems, San Diego, CA) were dissolved in saline and injected into the cNTS. Manning compound ([ $\beta$ -Mercapto- $\beta$ , $\beta$ -cyclopentamethylene-propionyl<sup>1</sup>, O-Me-Tyr<sup>2</sup>, Arg<sup>8</sup>]-vasopressin; V<sub>1</sub>-antagonist), vasopressin ([Arg<sup>8</sup>]-vasopressin), phenylephrine, sodium nitroprusside, and potassium cyanide were obtained from Sigma-Aldrich (St. Louis, MO) and were dissolved in saline and injected intravenously.

### Osmotic Stimulus

Intragastric 2 M NaCl (2 ml), which produces 4% elevation of both plasma osmolality and sodium concentration, was used as osmotic stimulus. Concurrent reduction of plasma renin activity and no alteration in plasma volume after 2 M NaCl indicate that this procedure does not induce change in the volume of extracellular compartment (6, 38). Intragastric 0.15 M NaCl was used as an isotonic control. A tube adapted in a 5-ml syringe was advanced into the stomach, and the NaCl solution was delivered as a bolus.

### Microinjections of anti-D $\beta$ H-Saporin or Anti-IgG-Saporin in the cNTS

Rats were anesthetized with intraperitoneal ketamine (Francotar, Virbac, Jurubatuba, SP, Brazil, 80 mg/kg body wt) combined with xylazine (Xilazine, Syntec, Cotia, SP, Brazil, 7 mg/kg body wt) and adapted to a stereotaxic apparatus (Kopf, Tujunga, CA) with the incisor bar at 11 mm below the interaural line. A partial occipital craniotomy was performed, and the dorsal surface of the brain stem was exposed. In the cNTS/A2-lesioned group, microinjections of anti-D $\beta$ H-saporin (12.6 ng/60 nl) were made at two rostro-caudal levels of the cNTS. In sham rats, anti-IgG-saporin (12.6 ng/60 nl) was injected in the same sites as in the cNTS/A2-lesioned group. Microinjections in the cNTS were performed 0 and 0.5 mm caudal to the calamus scriptorius, at midline, and 0.5 mm ventral to the dorsal surface of the medulla. The microinjections were performed using a glass micropipette adapted to a Picospritzer microinjection system, and the volume microinjected (60 nl) was determined by viewing the movement of the meniscus through a binocular microscope fitted with a precalibrated eyepiece reticule. After the microinjection, the micropipette was left in place for 5 min. A prophylactic dose of antibiotic (benzylpenicillin-80,000 IUs plus streptomycin-33 mg; Pentabiotico Veterinario-Pequeno Porte, Fort Dodge Saude Animal, Campinas, SP, Brazil) and of the anti-inflammatory ketoprofen (1 mg/kg of body wt, sc; Mundo Animal, Sao Paulo, SP, Brazil) were given postsurgery. All of the tests were performed 21–28 days after the microinjections, as described previously (11, 19, 36). The lesion of the A2 neurons of cNTS will be referred as the cNTS/A2-lesion.

### Histology

Animals were deeply anesthetized with sodium thiopental (70 mg/kg body wt, ip). Thereafter, all animals were transcardially perfused with 300 ml of 0.1 M PBS (pH 7.4), followed by 500 ml of 4%

(wt/vol) paraformaldehyde (PFA; Sigma, St. Louis, MO) solution in 0.1 M PBS, pH 7.4. The brains were removed, fixed for 4 h in 4% (wt/vol) PFA solution, and stored at  $4^\circ\text{C}$  in 0.1 M PBS containing 20% (wt/vol) sucrose. For the c-Fos labeling, five sets of coronal sections (30  $\mu\text{m}$ ) of the hypothalamus were sectioned on a cryostat (Leica, CM1800, Wetzlar, Hesse, Germany). The free-floating sections were collected in 24-well tissue culture plates containing PBS. Each fifth hypothalamic section was preincubated for 10 min in 3% (vol/vol) hydrogen peroxide (Sigma, St. Louis, MO) in 0.1 M PBS followed by 15-min incubation in a blocking solution comprising 10% (vol/vol) normal goat serum (NGS; Sigma) and 0.3% (vol/vol) Triton X-100 (Sigma) in 0.1 M PBS followed by rinses in PBS ( $3 \times 10$  min). Sections were then incubated in a polyclonal rabbit anti-c-Fos primary antibody (1:20,000; Ab-5; Calbiochem, San Diego, CA) in PBS containing 1% (vol/vol) NGS and 0.3% (vol/vol) Triton X-100 for 48 h at  $4^\circ\text{C}$ . After the primary antibody incubation, the sections were rinsed in PBS ( $3 \times 10$  min) prior to 1-h incubation with biotinylated goat anti-rabbit IgG (1:500; Vector Laboratories, Burlingame, CA), followed by further rinses in PBS ( $3 \times 10$  min), and incubation with streptavidin HRP (1:500; Vector Laboratories) for 1 h. Sections were rinsed ( $3 \times 10$  min) and diaminobenzidine (DAB) was used to produce a brown nuclear c-Fos reaction product. Sections were rinsed ( $3 \times 5$  min), mounted onto slides in 0.5% (wt/vol) gelatin and allowed to air-dry, dehydrated in a series of alcohols, cleared in xylene, and cover slipped. Cells expressing positive nuclear c-Fos immunoreactivity were counted bilaterally by hand each 150  $\mu\text{m}$ , in matched, representative sections of the tissue.

For the tyrosine hydroxylase (TH) labeling, four sets of coronal sections (40  $\mu\text{m}$ ) of the brain stem were sectioned on a cryostat (Leica, CM1800, Wetzlar, Hesse, Germany). The free-floating sections were collected in 24-well tissue culture plates containing PBS. Each fourth brain stem section was preincubated for 10 min in 3% (vol/vol) hydrogen peroxide (Sigma) in 0.1 M PBS followed by 15 min of incubation in a blocking solution comprising 10% (vol/vol) normal horse serum (NHS; Sigma) and 0.3% (vol/vol) Triton X-100 (Sigma) in 0.1 M PBS followed by rinses in PBS ( $3 \times 10$  min). Sections were then incubated in a mouse monoclonal TH antibody (1:2,000; ImmunoStar, Hudson, WI) in PBS containing 1% (vol/vol) NHS and 0.3% (vol/vol) Triton X-100 for 24 h at  $4^\circ\text{C}$ . After the primary antibody incubation, sections were rinsed in PBS ( $3 \times 10$  min) prior to 1 h of incubation with biotinylated horse anti-mouse IgG (1:500; Vector Laboratories, Burlingame, CA). Following further rinses in PBS ( $3 \times 10$  min) and incubation with streptavidin HRP (1:500; Vector Laboratories) for 1 h. Sections were rinsed ( $3 \times 10$  min), and DAB (Sigma) was used to produce a brown reaction product. Sections were rinsed ( $3 \times 5$  min), mounted onto slides in 0.5% (wt/vol) gelatin, and allowed to air-dry, dehydrated in a series of alcohols, cleared in xylene, and cover slipped. All immunolabeled neuronal perikarya in the ventrolateral medulla (VLM; A1/C1) and NTS (A2/C2) were counted bilaterally by hand each 160  $\mu\text{m}$ , to quantify the extent of the anti-D $\beta$ H-saporin-induced lesion. Neurons were counted at  $\times 200$  magnification with a light microscope (Leica, DM5500 B, Wetzlar, Hesse, Germany).

### Arterial Pressure Recording

Mean arterial pressure (MAP) and heart rate (HR) were recorded in unanesthetized rats with sham or cNTS/A2 lesion. Under ketamine (80 mg/kg body wt) and xylazine (7 mg/kg body wt) anesthesia, polyethylene tubing (PE-10 connected to a PE-50) was inserted into the abdominal aorta through the femoral artery on the day before the experimental procedure. At the same time, PE tubing was also inserted into the femoral vein for drug administration. Arterial and venous catheters were tunneled subcutaneously and exposed on the back of the rat to allow access in unrestrained, freely moving rats. To record pulsatile arterial pressure, MAP, and HR, the arterial catheter was connected to a Stathan Gould (P23 Db) pressure

transducer coupled to a preamplifier (model no. ETH-200 Bridge Bio Amplifier, CScience, Dover, NH) that was connected to a PowerLab computer data acquisition system (model PowerLab 16SP, Dunedin, New Zealand).

#### Water Intake and Urinary Excretion

Water and urine samples were collected in metabolic cages. Water was offered in graduated (0.1 ml) glass burettes fitted with metal spouts. Urine samples were collected in graduated tubes by gravity and urinary  $\text{Na}^+$  and  $\text{K}^+$  concentrations were measured by ion-specific electrode (NOVA 1; Nova Biomedical, Waltham, MA). Total  $\text{Na}^+$  and  $\text{K}^+$  excretions were determined by the product of urine volume and the concentrations of each ion in the urine.

#### Statistical Analysis

All data are expressed as the means  $\pm$  SE. One- or two-way (as appropriate) ANOVA followed by Student-Newman-Keuls post hoc or Student's *t*-test were used for comparisons. Differences were taken as significant at  $P < 0.05$ .

#### Experimental Protocols

*c-Fos expression in neurons of the PVN and SON in sham or cNTS/A2-lesioned rats treated with intragastric 2 M NaCl load.* Immunohistochemistry for c-Fos protein expression in the parvocellular and magnocellular regions of the PVN (pPVN and mPVN, respectively) and in the SON were performed in sham and cNTS/A2-lesioned rats treated with intragastric gavage of 2 M or 0.15 M NaCl (2 ml). To minimize any possible c-Fos expression related to stress, in *day 1*, rats received an intragastric gavage of 2 ml of 2 M NaCl, and in the subsequent 6 days, an intragastric gavage of 2 ml of 0.15 M NaCl. In the day of the experiment (*day 8*), water and food were removed from the cages, and all rats (sham and cNTS/A2-lesioned rats,  $n = 3$  or 4/group) received an intragastric gavage of 2 ml of 0.15 M NaCl or 2 M NaCl randomly. Two hours after the gavage, rats were deeply anesthetized with sodium thiopental (70 mg/kg body wt, ip), perfused with 4% (wt/vol) PFA, and the brains were processed for immunohistochemistry.

*Cardiovascular changes in sham or cNTS/A2-lesioned rats treated with intragastric 2 M NaCl load.* MAP and HR were recorded in conscious chronic sham or cNTS/A2-lesioned rats. Tests started at least 20 min after connecting the arterial cannula to the recording system. Sham ( $n = 5$ ) and cNTS/A2-lesioned rats ( $n = 9$ ) received an intragastric gavage of isotonic saline (0.15 M NaCl, 2 ml), and MAP and HR were continuously recorded for 60 min, followed by an intragastric gavage of 2 M NaCl (2 ml) and an additional 60-min recording of MAP and HR. In other groups of sham ( $n = 14$ ) or cNTS/A2-lesioned rats ( $n = 16$ ), the role of vasopressin in the cardiovascular responses to intragastric 2 M NaCl (2 ml) was tested. Manning compound ( $[\beta\text{-Mercapto-}\beta\text{-cyclopentamethylenepropionyl}^1, \text{O-Me-Tyr}^2, \text{Arg}^8]\text{-vasopressin}$ ; Sigma,  $\text{V}_1$  antagonist; 10  $\mu\text{g/kg}$  body wt, iv) was injected 10 min before intragastric 2 M NaCl. The changes in MAP and HR after intragastric 2 M NaCl were recorded for an additional 60 min. A pressor dose of vasopressin ( $[\text{Arg}^8]\text{-vasopressin}$ , Sigma; 12.5 ng/0.1 ml/rat) was injected intravenously before and 5 and 70 min after the Manning compound to test the efficacy of vasopressin  $\text{V}_1$  receptor blockade.

*Water intake and urinary excretion in sham or cNTS/A2-lesioned rats treated with intragastric 2 M NaCl load.* Water intake and urine output were measured after the gavages in sham ( $n = 5$ ) or cNTS/A2-lesioned rats ( $n = 9$ ). On the day of the experiments, animals were placed in metabolic cages in the morning and after a 30-min period of acclimation, food and water were removed from the cages, and the rats received an intragastric 0.15 M NaCl load (2 ml). Urine was collected for 60 min after the intragastric 0.15 M NaCl load in graduated tubes commencing right after the gavages. At the end of the

urine collection, water was returned to the cages and monitored for the next 120 min, with recordings made at 30-min intervals. At the end of the last water intake recording, water was removed again from the cage, and the animals received an 2 M NaCl ig (2 ml) load. Urine was collected for the next 60 min, and at the end of the urine collection, water was returned to the cages and monitored for the next 120 min, with recordings made at 30-min intervals.

## RESULTS

### *TH-Positive Cells in the cNTS and VLM in Rats Treated with Anti-D $\beta$ H-Saporin*

Data from different groups of rats treated with anti-D $\beta$ H-saporin were similar and, therefore, they were pooled and analyzed together. TH-positive cells were found within the NTS and the VLM from  $\sim 2$  mm rostral to 2 mm caudal from the obex (Fig. 1). The number of TH-positive cells in the cNTS was reduced in anti-D $\beta$ H-saporin-treated rats (cNTS/A2-lesioned group;  $n = 28$ ) compared with the treatment with anti-IgG-saporin (sham group;  $n = 24$ ), [Fig. 1, A–C;  $F(1,971) = 77.66$ ;  $P < 0.05$ ]. Compared with sham animals, the number of TH-positive cells in the NTS was reduced by around 76% (ranging from 61% to 86%) in the area that extended from 1.3 mm caudal to 0.3 mm rostral to the obex in the cNTS/A2-lesioned rats. This reduction in the noradrenergic cell number after anti-D $\beta$ H-saporin injection is in accordance with previous studies that used the same treatment (19, 36). Injections of anti-D $\beta$ H-saporin within the NTS did not reduce the number of TH-immunopositive cells in the region of the ventrolateral medulla located between 2.0 mm caudal and 1.9 mm rostral to the obex (Fig. 1A), the region encompassing the A1 (Fig. 1, D and E) and C1 cell group (Fig. 1, F and G).

### *c-Fos Expression in the PVN and SON in cNTS/A2-Lesioned Rats Treated with Intragastric 2 M NaCl*

Compared to sham rats, the number of c-Fos-positive cells increased in the mPVN of cNTS/A2-lesioned rats treated with intragastric 2 M NaCl ( $90 \pm 13$ , vs. sham:  $47 \pm 20$  cells/section, each 150  $\mu\text{m}$ ) [ $F(3,10) = 8.69$ ;  $P < 0.05$ ] (Fig. 2). c-Fos expression in sham and cNTS/A2-lesioned rats was similar in the pPVN (cNTS/A2:  $37 \pm 6$ , vs. sham:  $22 \pm 9$  cells/section, each 150  $\mu\text{m}$ ;  $P > 0.05$ ) and in the SON (cNTS/A2:  $88 \pm 7$ , vs. sham:  $105 \pm 27$  cells/section, each 150  $\mu\text{m}$ ;  $P > 0.05$ ) (Fig. 2).

### *Changes in Arterial Pressure and Heart Rate in cNTS/A2-Lesioned Rats Treated with Intragastric 2 M NaCl*

The cNTS/A2 lesions did not change baseline values of MAP or HR ( $107 \pm 3$  mmHg and  $335 \pm 11$  bpm, respectively) compared with sham rats ( $113 \pm 2$  mmHg and  $346 \pm 14$  bpm, respectively). Intragastric 0.15 M NaCl did not significantly affect MAP in cNTS/A2-lesioned rats (Fig. 3). However, compared with sham, the intragastric 2 M NaCl promoted a pressor response in cNTS/A2-lesioned rats (Fig. 3A), commencing 10 min after intragastric 2 M NaCl and remaining above baseline values until the end of the recording period ( $16 \pm 3$ , vs. sham:  $2 \pm 2$  mmHg, at 60 min after intragastric 2 M NaCl), [ $F(1,168) = 38.55$ ;  $P < 0.05$ ]. The variability of the HR was high in sham and cNTS/A2-lesioned rats, with significant, but inconsistent, increases in



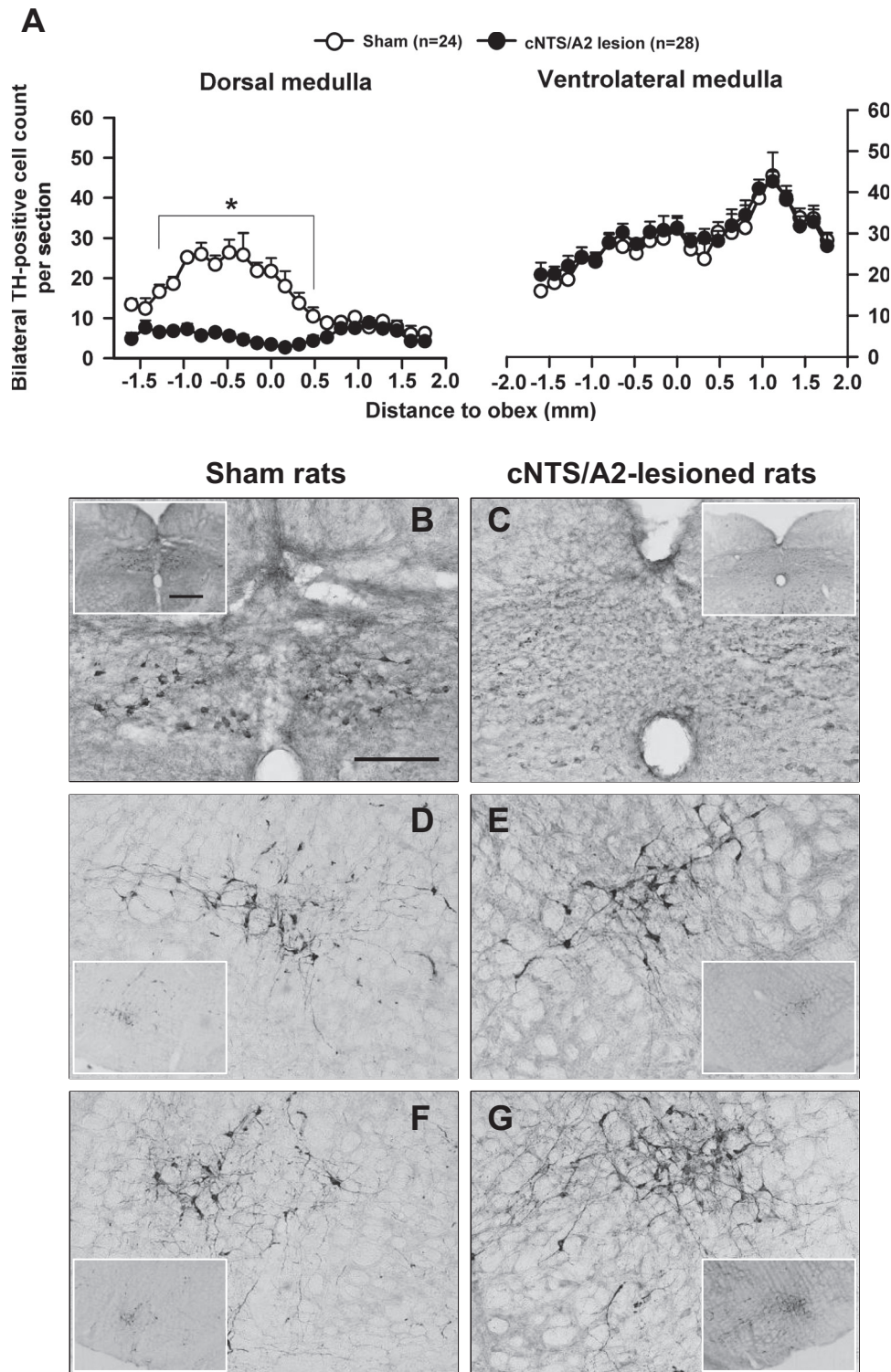


Fig. 1. A: number of tyrosine hydroxylase (TH)-positive cells in 40- $\mu$ m-thick sections from the dorsal and ventrolateral medulla of rats treated with injections of anti-D $\beta$ H-saporin (cNTS/A2 lesion) or anti-IgG-saporin (sham) in cNTS. \*Significantly different from cNTS/A2 lesion (one-way ANOVA, followed by Student-Newman-Keuls test,  $P < 0.05$ ). The results are expressed as means  $\pm$  SE;  $n$  = number of rats. B–G: photomicrographs of three levels of the medulla (640  $\mu$ m caudal, at the obex and 640  $\mu$ m rostral to the obex) showing TH-immunoreactive cells in the cNTS (A2 area; B and C), CVLM (A1 area; D and E) and RVLM (C1 area; F and G) of rats that received bilateral injections of anti-D $\beta$ H-saporin (cNTS/A2 lesion) or IgG-saporin (sham) into cNTS. Scale bar = 500  $\mu$ m.

HR in cNTS/A2-lesioned rats during the experimental period (Fig. 3B).

*Changes in Arterial Pressure and Heart Rate in cNTS/A2-Lesioned Rats Treated with Intragastric 2 M NaCl Combined with Intravenous Vasopressin Antagonist*

The blockade of vasopressin V<sub>1</sub> receptor with intravenous Manning compound (10  $\mu$ g/kg body wt) prevented the pressor

response to 2 M NaCl ig in cNTS/A2-lesioned rats [ $F(3,208) = 20.99$ ;  $P < 0.05$ ], (Fig. 4A). The intragastric 2 M NaCl produced no significant change in MAP in sham rats previously treated with intravenous saline or V<sub>1</sub>-antagonist, (Fig. 4A). There were no major changes in HR in sham or cNTS/A2-lesioned rats treated previously with intravenous saline or V<sub>1</sub>-antagonist, only a transient increase in the first 20 min in cNTS/A2-lesioned rats compared with the lesioned group treated

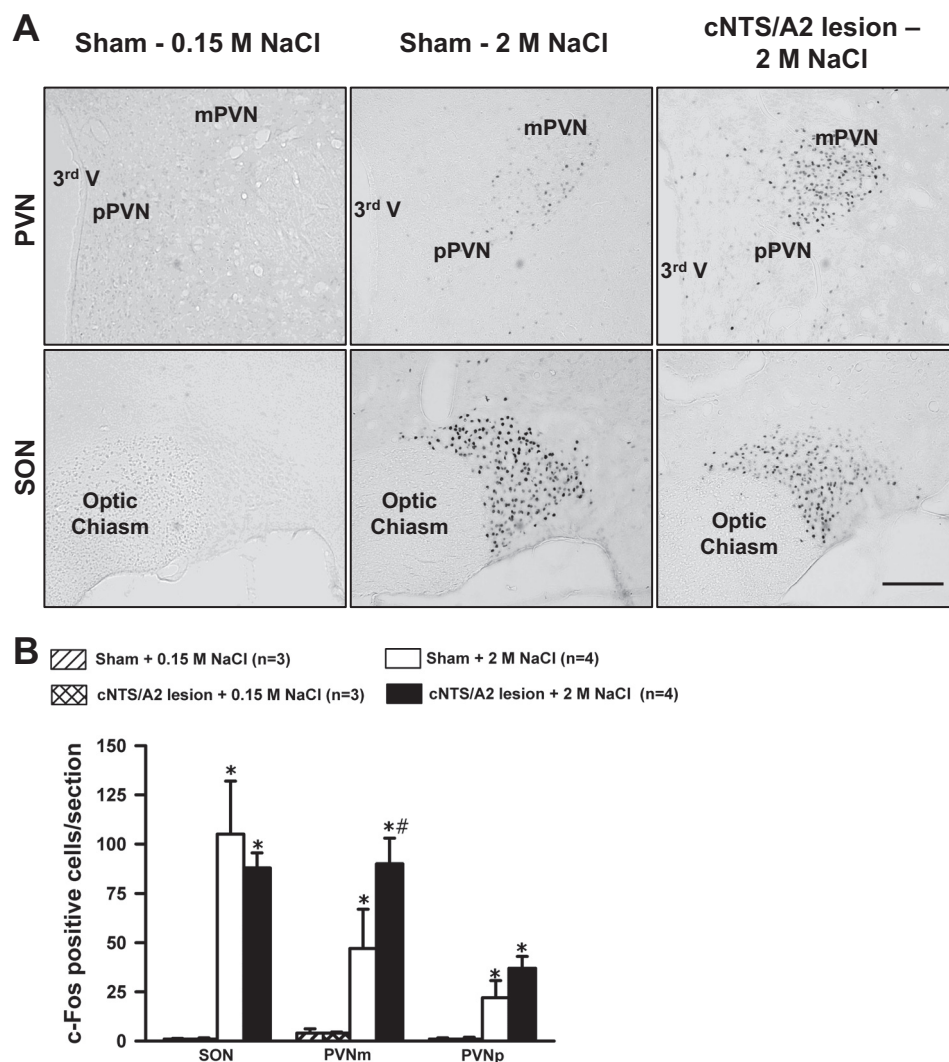


Fig. 2. *A*: photomicrographs of coronal brain sections showing typical c-Fos expression in the paraventricular nucleus (PVN) and in the supraoptic nucleus (SON) of the hypothalamus in a sham or cNTS/A2-lesioned rat representative of the groups tested with intragastric 0.15 M NaCl or 2 M NaCl. Scale bar = 200  $\mu$ m. 3<sup>rd</sup> V, third ventricle; mPVN, magnocellular PVN; pPVN, parvocellular PVN. *B*: number of c-Fos-positive cells/section in the SON, mPVN, and pPVN in sham or cNTS/A2-lesioned rats treated with intragastric 0.15 M NaCl or 2 M NaCl. \*Significantly different from 0.15 M NaCl,  $P < 0.05$ . #Significantly different from sham + 2 M NaCl (one-way ANOVA, followed by Student-Newman-Keuls test,  $P < 0.05$ ). The results are expressed as means  $\pm$  SE;  $n$  = number of rats.

with  $V_1$ -antagonist [ $F(3,208) = 6.17$ ;  $P < 0.05$ ] (Fig. 4*B*). Representative MAP and HR recordings are shown in Fig. 4*C*.

The pressor response to intravenous AVP (12.5 ng/0.1 ml for each rat) was abolished at least until 70 min after the treatment with  $V_1$  antagonist in cNTS/A2-lesioned rats ( $1 \pm 1$ , vs. control:  $21 \pm 2$  mmHg) or sham ( $-1 \pm 1$ , vs. control:  $26 \pm 3$  mmHg) [ $F(3,29) = 2.69$ ;  $P < 0.05$ ].

#### Water Intake and Urinary Excretion in cNTS/A2-Lesioned Rats Treated with Intragastric 2 M NaCl

Compared to sham, water intake induced by 2 M NaCl ig was enhanced in cNTS/A2-lesioned rats during the first 60 min of the experimental recording ( $10.5 \pm 1.4$ , vs.  $7.7 \pm 0.8$  ml/60 min), [ $F(3,112) = 69.18$ ;  $P < 0.001$ ] (Fig. 5*A*). The diuresis [ $F(7,48) = 11.66$ ;  $P < 0.05$ ], natriuresis [ $F(7,48) = 18.81$ ;  $P < 0.05$ ], and kaliuresis [ $F(7,48) = 16.88$ ;  $P < 0.05$ ] (Fig. 5, *B–D*, respectively) to intragastric 2 M NaCl were comparable in cNTS/A2-lesioned and sham rats.

The increase in body weight of cNTS/A2-lesioned rats was comparable to sham rats [ $F(1,10) = 0.26$ ,  $P = 0.3545$ ] (Table 1). In addition, daily water intake was not different among groups [ $F(1,18) = 3.281$ ,  $P = 0.100$ ]. Water intake was  $32.9 \pm 2.4$  and  $30.2 \pm 2.0$  ml/24 h, respectively, in the control

period presurgery, in rats signed to be sham and cNTS/A2 lesioned and  $38 \pm 5$  and  $33.3 \pm 2.9$  ml/24 h in sham and cNTS/A2-lesioned rats, respectively, 18 days after lesions.

#### DISCUSSION

The present data show that the cNTS/A2 lesions increase intragastric 2 M NaCl-induced water intake, pressor response, and the number of c-Fos labeling in the magnocellular neurons of the PVN. The increased pressor response to intragastric 2 M NaCl was abolished by pretreating rats with vasopressin antagonist intravenously, which suggests that this response is dependent on increased vasopressin secretion. The cNTS/A2 lesions produced no change in the number of c-Fos-labeled neurons in the parvocellular PVN or in the SON and on natriuresis, kaliuresis, and diuresis to intragastric 2 M. The injections of anti-D $\beta$ H-saporin into the cNTS destroyed around 76% of the A2 neurons in this region, without changing the number of catecholaminergic neurons in other medullary sites, such as in the A1 and C1 regions located in the ventral surface of medulla, as previously demonstrated (19, 36).

The cNTS electrolytic or D $\beta$ H-saporin lesions restricted to cNTS produce no change in baseline MAP and HR values or arterial pressure lability (5, 6, 12, 19, 36). Others studies have



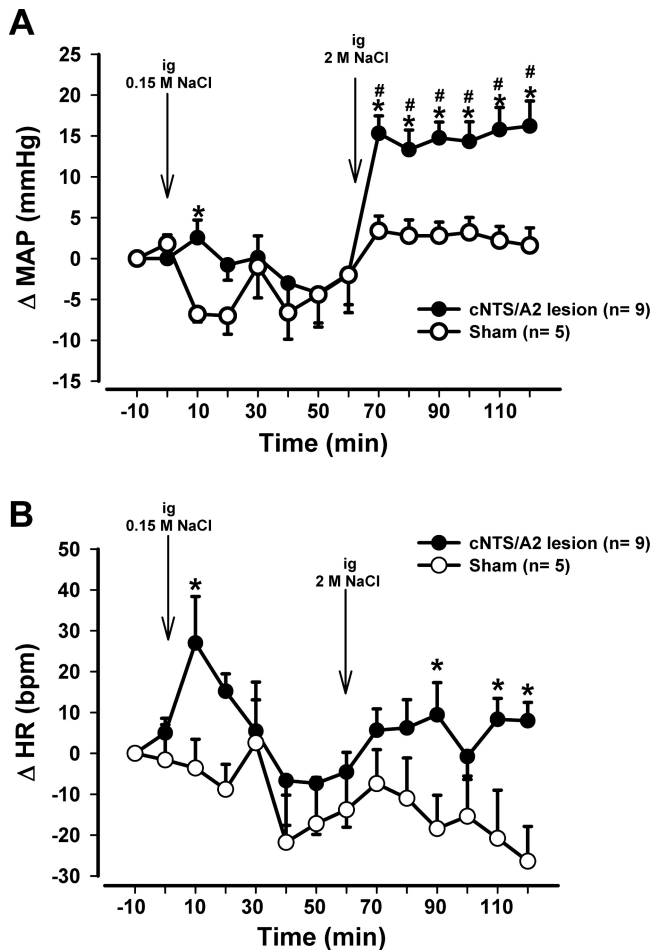


Fig. 3. Changes in mean arterial pressure ( $\Delta$  MAP) (A) and heart rate ( $\Delta$  HR) (B) to intragastric 0.15 M NaCl or 2 M NaCl (2 ml/rat, arrows) in sham or cNTS/A2-lesioned rats. \*Significantly different from sham rats,  $P < 0.05$ . #Significantly different from baseline ( $-10$  min); two-way ANOVA, followed by Student-Newman-Keuls test,  $P < 0.05$ . The results are expressed as means  $\pm$  SE;  $n$  = number of rats.

found an increase in arterial pressure or arterial pressure lability using electrolytic, chemical, or neuronal inactivation with potassium channel overexpression in the A2 region. The main difference between the studies is the large extension of the lesion along the A2 region, particularly at the rostro-caudal levels, in those studies compared with the present study (15, 17, 47, 49, 50). In those studies, the lesions or the inactivation of A2 neurons most exclusively comprised the iNTS, which is an area involved with baroreflex pathways (9). In the present study, the lesion of the A2 neurons was restricted to the commissural levels of the NTS, which is an area more related with chemoreflex pathways (9).

In unanesthetized rats, intragastric 2 M NaCl increased MAP in cNTS/A2-lesioned rats, whereas in anesthetized rats, the pressor response to intravenous 3 M NaCl (1.8 ml/kg body wt) infusion was not modified by the same lesion (37). Probably, the main difference between the present and the previous study (36) is the use of urethane-anesthetized rats in one study and conscious rats in the other. However, the route of administration of hypertonic NaCl was also different. The intragastric 2 M NaCl in intact rats usually produces nonsignificant changes in extracellular fluid volume and MAP (6), whereas intrave-

nous hypertonic NaCl increases extracellular fluid volume and MAP (36, 37, 54). Osmoreceptors, and probably baroreceptors, are activated in both cases; however, the volume receptors are activated by intravenous hypertonic NaCl, but not by intragastric 2 M NaCl.

The increase in MAP produced by intragastric 2 M NaCl in cNTS/A2-lesioned rats was blunted by the pretreatment with intravenous vasopressin antagonist, which suggests that the increase in MAP in this case is dependent on vasopressin. These results are similar to those produced by intragastric 2 M NaCl in rats with electrolytic lesion of the cNTS (6). In addition, the number of neurons of the mPVN expressing c-Fos increased in cNTS/A2-lesioned rats treated with intragastric 2 M NaCl. Considering that vasopressin antagonist prevented the increase in MAP in cNTS/A2-lesioned rats during hyperosmolality, it is possible that the neurons activated in the mPVN are vasopressinergic. Therefore, previous (6) and the present data suggest that hyperosmolality induces an increase in vasopressin secretion and consequently increases MAP in cNTS/A2-lesioned rats, suggesting that part of the inhibitory drive from the cNTS to the mPVN depends on A2 noradrenergic cells. Contrary to the previous study with electrolytic lesion of the cNTS (6), intragastric 2 M NaCl produced no increased activation of the SON in cNTS/A2-lesioned rats. One possible explanation for this is that the neurons of the cNTS that project to SON and inhibit AVP secretion during hyperosmolality are not catecholaminergic. Although peripheral vasopressin is suggested to increase baroreflex sensitivity (53), no major changes in HR were observed after intragastric 2 M NaCl in the present or in the previous study (6). It has been shown that baroreflex sensitivity was either decreased (4) or not affected by acute hyperosmolality, even though the animals had an increased plasma AVP level (44).

The present study demonstrated that cNTS/A2 lesions increase water intake induced by 2 M NaCl ig, similarly to a previous study using electrolytic lesion of the cNTS (6). The cNTS/A2 neurons project to forebrain and hindbrain areas related with the control of water intake like the median preoptic nucleus (which is part of the AV3V region), the SFO, and the LPBN (10, 35, 45, 51). Plasma hyperosmolality activates central osmoreceptors located in the SFO and in the organum vasculosum of the lamina terminalis, which is also part of the AV3V region (7, 25). Inhibitory signals may arise from the cNTS/A2 neurons to these forebrain areas involved in the control of water intake, and a connection may occur between cNTS/A2 and forebrain through the LPBN, an important inhibitory area for the control of water intake that projects to the forebrain (28, 33). Therefore, the present results extend the conclusion of the previous one (6) and suggest that the A2 neurons in the cNTS are also part of the hindbrain circuitry that exert an inhibitory influence on forebrain mechanisms that regulate water intake during hyperosmolality, avoiding hypervolemia or blood volume expansion during this situation. In addition, the increase in body weight gain and daily water intake was not different between groups, suggesting a complete recovery of these behaviors in cNTS/A2-lesioned rats. A decrease in the body weight gain was observed in animals with electrolytic lesion of the cNTS (6, 34) or the area postrema/medial NTS (24). Although NTS may regulate food intake, the A2 neurons of the commissural NTS seem not to be involved.

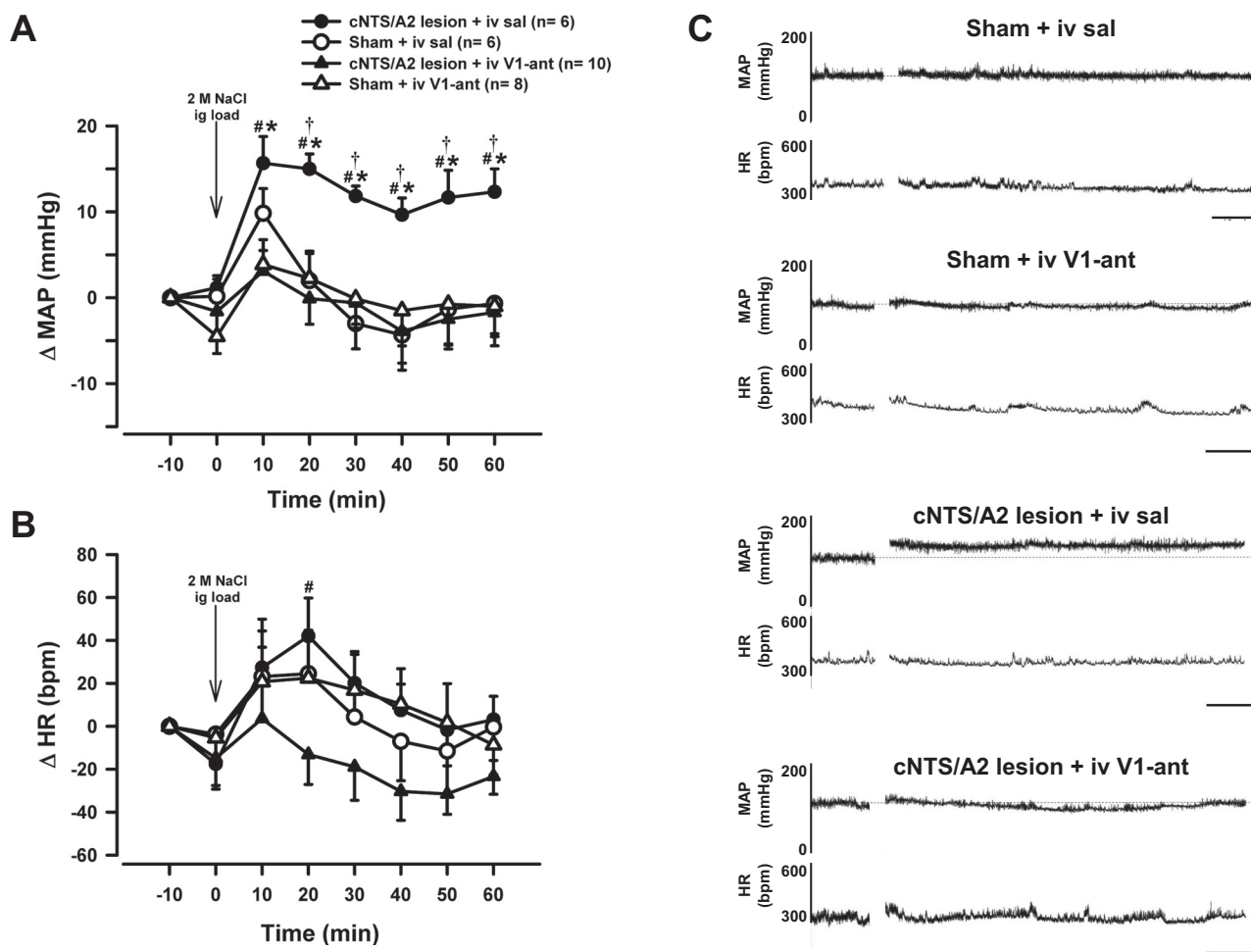


Fig. 4. Changes in mean arterial pressure ( $\Delta$  MAP) (A), heart rate ( $\Delta$  HR) (B) to intragastric 2 M NaCl (2 ml/rat; arrow) in sham or cNTS/A2-lesioned rats pretreated with intravenous vasopressin  $V_1$  receptor antagonist ( $V_1$ -ant; Manning compound, 10  $\mu$ g/kg body wt) and MAP and HR recordings (C) in sham or cNTS/A2-lesioned rats pretreated with intravenous vasopressin  $V_1$  receptor antagonist ( $V_1$ -ant; Manning compound, 10  $\mu$ g/kg body wt) before and after 2 M NaCl (2 ml/rat ig; detached part of the recording). \*Significantly different from baseline ( $-10$  min). #Significantly different from intravenous  $V_1$ -ant treated groups. †Significantly different from sham + iv saline (two-way ANOVA, followed by Student-Newman-Keuls,  $P < 0.05$ ). The results are expressed as means  $\pm$  SE;  $n$  = number of rats. Scale bar = 10 min.

The natriuresis, kaliuresis, and diuresis in rats treated with intragastric 2 M NaCl were not modified by cNTS/A2 lesions. An increase in filtered sodium and reduced sodium reabsorption due to a direct renal tubular effect of OT are mechanisms involved on hyperosmolality-induced natriuresis (1, 6, 13, 23). Although OT levels were not measured in the present study, it is possible that OT plasma levels increased similarly in sham and lesioned rats, as observed previously (6), resulting in similar natriuresis. Although the blockade of the pressor response by an intravenous vasopressin antagonist suggests that 2 M NaCl ig increases plasma vasopressin in cNTS/A2-lesioned rats, these rats did not display reduced diuresis when treated with 2 M NaCl ig. No change in the diuresis to 2 M NaCl ig in cNTS/A2-lesioned rats may result from two opposing mechanisms. The pressor response to intragastric 2 M NaCl in cNTS/A2-lesioned rats probably increases renal filtration, whereas, on the other hand, increased plasma levels of AVP probably increases water reabsorption opposing to the increased filtration, resulting in a net unchanged diuresis in cNTS/A2-lesioned rats. Since diuresis, natriuresis, and kaliure-

sis to hypertonic sodium load were not modified by cNTS/A2 lesion, the increased water intake to 2 M NaCl ig in cNTS/A2-lesioned rats is not secondary to changes in diuresis. In the present study, the pressor response to intragastric 2 M NaCl in lesioned rats was blocked by vasopressin antagonist, suggesting that sympathetic system has only a minor contribution to the increase of arterial pressure, if it has some. On the other hand, a possible sympathoinhibition (36) might also explain the sodium excretion produced by intragastric 2 M NaCl in the present study. However, the renal responses to intragastric 2 M NaCl were not modified by A2 lesion, suggesting that A2 neurons do not modulate the activity of sympathetic system to the control of renal or cardiovascular function in this situation.

Although immunostaining for other neuron phenotypes was not performed in the present study, evidence for neurochemical lesion specificity has been addressed by different studies that have shown that anti-D $\beta$ H-saporin (5 to 42 ng) in the NTS reduced TH and D $\beta$ H immunoreactivity in this site, but spared NK1, 11 $\beta$ HSD2, cholinergic, glucagon-like peptide-1-positive neurons (15, 40, 49), suggesting a neurochemical specificity of

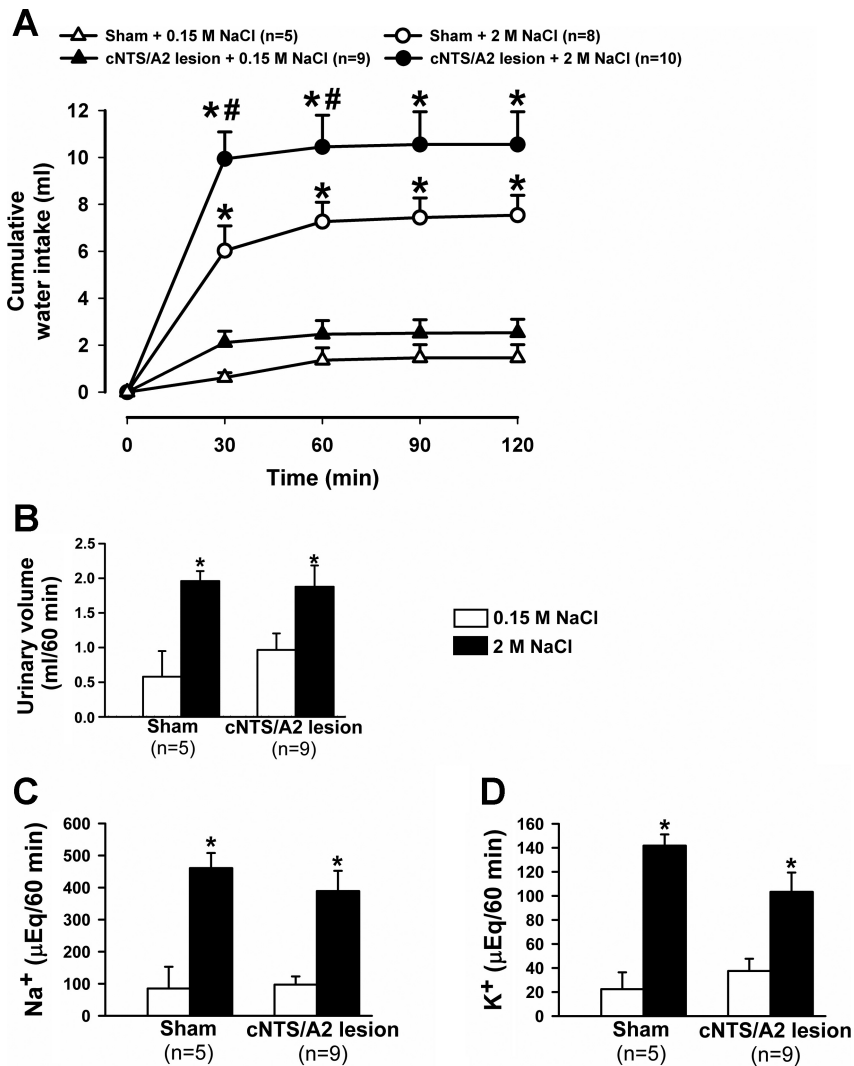


Fig. 5. Cumulative water intake (A), urinary volume (B), urinary sodium excretion (C), and urinary potassium excretion (D) after intragastric administration of 0.15 M NaCl or 2 M NaCl (2 ml/rat) in sham or cNTS/A2-lesioned rats. \*Significantly different from NaCl 0.15 M,  $P < 0.05$ . #Significantly different from sham treated with 2 M NaCl (two-way ANOVA, followed by Student-Newman-Keuls test,  $P < 0.05$ ). The results are expressed as means  $\pm$  SE;  $n$  = number of rats.

anti-D $\beta$ H-saporin. In the present and previous study (19), the number of A1/C1 neurons was not significantly modified after anti-DBH-saporin microinjection into the cNTS. In addition, anti-D $\beta$ H-saporin microinjected into the NTS in a concentration similar to that used in the present study did not significantly change D $\beta$ H-positive neurons in the rostral ventrolateral medulla (C1 neurons) or A6 neurons, but decreased in about 30% the D $\beta$ H-positive A5 neurons, an area not analyzed in the present study. Although possible, it is not clear how a partial lesion of the A5 neurons might produce the effects described in the present study. There is no evidence that A5 neurons project to PVN and SON (8, 46), and the activation of A5 neurons increases sympathetic nerve activity (27), which suggests that lesions of this area might impair and not potentiate MAP

increases. Moreover, electrolytic lesion of A5 induces hyperdipsia (56), a response not seen in the present study. The effects of cNTS/A2 lesions are also similar to those produced by electrolytic lesions of this area, which does not destroy A5 neurons. Thus, it is conceivable that the effects seen in the present study are due to cNTS/A2 lesions and not to lesions in other catecholaminergic areas. Anti-D $\beta$ H-SAP and unconjugated saporin have been suggested to decrease the immunoreactivity to astrocytes in the NTS affecting cardiovascular reflexes (29). Even if lesion in the glial cells by anti-D $\beta$ H-saporin or unconjugated saporin occurred in the present study, it was already demonstrated that cNTS/A2 lesion with anti-D $\beta$ H-SAP produces no change in cardiovascular reflexes or in arterial pressure lability (19).

Table 1. Body weight of sham and cNTS/A2-lesioned rats

Group	n	Presurgery	Days After Surgery								
			2	4	6	8	10	12	14	16	18
Sham	7	311 $\pm$ 6	295 $\pm$ 5	293 $\pm$ 6	303 $\pm$ 8	317 $\pm$ 6	329 $\pm$ 9	339 $\pm$ 8	351 $\pm$ 9	359 $\pm$ 8	363 $\pm$ 9
Lesion	9	306 $\pm$ 4	286 $\pm$ 3	300 $\pm$ 5	306 $\pm$ 5	319 $\pm$ 6	334 $\pm$ 7	344 $\pm$ 7	355 $\pm$ 8	363 $\pm$ 8	372 $\pm$ 8

Values are expressed as means  $\pm$  SE. Two-way ANOVA, followed by Student-Newman-Keuls;  $P > 0.05$ ;  $n$  = number of rats.



In summary, the results extend previous data (6), suggesting that noradrenergic A2 cells from the cNTS inhibit water intake and vasopressin-dependent pressor response during increases in plasma osmolality.

### Perspectives and Significance

The present results suggest that a potent inhibitory mechanism involving the cNTS/A2 neurons acts to limit the activity of magnocellular neurons in the PVN, possibly vasopressinergic, and the pressor and dipsogenic responses to an acute increase in plasma osmolality. These findings extend those from a previous study (6) that suggested the existence of cNTS inhibitory mechanisms that act to suppress vasopressin release and overdrinking. Therefore, an imbalance in the mechanisms dependent on cNTS/A2 neurons may affect body fluid homeostasis, causing hemodynamic changes, which may result in cardiovascular diseases like hypertension. In addition to the facilitation of cardiovascular responses to plasma hyperosmolality, lesions of the cNTS/A2 noradrenergic neurons also facilitate the activation of angiotensinergic mechanisms in response to hemorrhage, which again suggests that cNTS/A2 inhibitory mechanisms oppose pressor mechanisms (19). In future studies, it would be interesting to investigate the importance of the cNTS/A2 inhibitory mechanisms in the responses to the activation of different pressor mechanisms and if vasopressin secretion is the only mechanism involved, or if angiotensinergic mechanisms are also involved. The A2 neurons receive sensory feedback from the cardiovascular, respiratory, and gastrointestinal tract systems (3, 26); thus, it is also interesting to investigate whether signals from these afferents are those that activate cNTS/A2 neurons promoting inhibition of pressor mechanisms and affecting the control of fluid electrolyte balance. Finally, the mRNA encoding cAMP-responsive element-binding protein-3 like-1 (CREB3L1), a transcription factor of the CREB/activating transcription factor family, increases in parallel to the increase in AVP expression in the SON and PVN during increases in plasma osmolality (21). Future studies might investigate whether CREB3L1 expression is modulated by cNTS/A2 neurons during plasma hyperosmolality.

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### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

### AUTHOR CONTRIBUTIONS

Author contributions: A.H.F.-O. and G.T.B. performed experiments; A.H.F.-O., G.T.B., and D.S.A.C. analyzed data; A.H.F.-O., G.T.B., D.M., J.V.M., and D.S.A.C. interpreted results of experiments; A.H.F.-O. prepared

figures; A.H.F.-O., G.R.P., S.L.C., J.V.M., and D.S.A.C. drafted manuscript; A.H.F.-O., G.T.B., G.R.P., S.L.C., D.M., J.V.M., and D.S.A.C. approved final version of manuscript; G.R.P., S.L.C., J.V.M., and D.S.A.C. conception and design of research; G.R.P., S.L.C., D.M., J.V.M., and D.S.A.C. edited and revised manuscript.

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