Water deprivation increases Fos immunoreactivity in PVN autonomic neurons with projections to the spinal cord and rostral ventrolateral medulla

Sean D. Stocker,1 J. Thomas Cunningham,2 and Glenn M. Toney1

Departments of 1Physiology and 2Pharmacology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229

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Stocker, Sean D., J. Thomas Cunningham, and Glenn M. Toney. Water deprivation increases Fos immunoreactivity in PVN autonomic neurons with projections to the spinal cord and rostral ventrolateral medulla. Am J Physiol Regul Integr Comp Physiol 287: R1172–R1183, 2004. First published July 22, 2004; doi:10.1152/ajpregu.00394.2004.—The present study sought to determine whether water deprivation increases Fos immunoreactivity, a neuronal marker related to synaptic activation, in sympathetic-regulatory neurons of the hypothalamic paraventricular nucleus (PVN). Fluorogold (4%, 50 nl) and cholera toxin subunit B (0.25%, 20–30 nl) were microinjected into the spinal cord (T1–T3) and rostral ventrolateral medulla (RVLM), respectively. Rats were then deprived of water but not food for 48 h. Water deprivation significantly increased the number of Fos-positive nuclei throughout the dorsal, ventrolateral, and lateral paravascular divisions of the PVN (water deprived, 215 ± 23 cells; control, 45 ± 7 cells, P < 0.01). Moreover, a significantly greater number of Fos-positive nuclei were localized in spinally projecting (11 ± 3 vs. 2 ± 1 cells, P < 0.025) and RVLM-projecting (45 ± 7 vs. 7 ± 1 cells, P < 0.025) neurons of the PVN in water-deprived vs. control rats, respectively. The majority of these double-labeled neurons was found in the ventrolateral and lateral paravascular divisions of the ipsilateral PVN. Interestingly, a significantly greater percentage of RVLM-projecting PVN neurons were Fos positive compared with spinally projecting PVN neurons in the ventrolateral (25.8 ± 0.7 vs. 8.0 ± 1.5%, respectively, P < 0.01) and lateral (23.4 ± 2.1 vs. 5.0 ± 0.9%, respectively, P > 0.01) paravascular divisions. In addition, we analyzed spinally projecting neurons of the RVLM and found a significantly greater percentage were Fos positive in water-deprived rats than in control rats (26 ± 3 vs. 3 ± 1%, respectively; P < 0.001). Collectively, the present findings indicate that water deprivation evokes a distinct cellular response in sympathetic-regulatory neurons of the PVN and RVLM.

Water deprivation decreases intravascular volume and increases plasma osmolality (P osmol). In turn, this increases plasma vasopressin and ANG II levels and activates the sympathetic-regulatory system, thereby maintaining arterial blood pressure (ABP). The sympathetic-regulatory activation is reflected by increases in circulating catecholamines (50), elevated heart rate (HR) (17, 40, 41, 44), and an apparent increase in lumbar sympathetic nerve activity (SNA) (40). Moreover, our laboratory recently reported that ganglionic blockade with hexamethonium produced a significantly greater fall in ABP of water-deprived rats than control rats (44). Collectively, these observations suggest that sympathetic outflow is elevated after water deprivation; however, the central neural circuitry and cellular mechanisms supporting the elevated SNA in water-deprived rats are not fully understood.

The hypothalamic paraventricular nucleus (PVN) plays an important role in the regulation of SNA and ABP (1, 51). Parvocellular neurons of the PVN have axonal projections to the intermediolateral cell column (IML) of the spinal cord (34, 37, 42), the location of sympathetic preganglionic neurons. In addition, PVN parvocellular neurons project to the rostral ventrolateral medulla (RVLM) (33, 34, 42), which contains bulbohypothalamic sympathetic premotor neurons that mediate several vasomotor reflexes (for reviews, see Refs. 12, 19). Electrophysiological studies in vivo have also demonstrated that PVN sympathetic-regulatory neurons are responsive to changes in plasma osmolality, circulating ANG II, and blood volume (6, 10, 15, 16, 24, 51). Moreover, changes in SNA and/or ABP during increases in P osmol, ANG II levels, or blood volume depend on neurotransmission in the PVN (9, 16, 18, 20, 25, 56). Taken together, these observations suggest that the PVN is one site in the central nervous system that may contribute to the regulation of ABP and SNA during water deprivation. This notion is supported by recent evidence from our laboratory that demonstrated acute inhibition of the PVN with bilateral microinjection of the GABA_A receptor agonist muscimol decreased renal SNA and ABP to a significantly greater extent in water-deprived rats than control rats (44). These findings suggest that the elevated SNA in water-deprived rats depends, in part, on the tonic activity of PVN neurons.

Over the last decade, Fos immunoreactivity has been used to identify neurons in the central nervous system involved in cardiovascular regulation and body fluid homeostasis (11, 13). Fos is the protein product of the early intermediate gene c-fos and is part of the AP-1 transcription complex (21, 29). Because Fos production is related to synaptic activation (21), this technique has been used widely as a marker of “neuronal activation.” Although water deprivation has been reported previously to increase Fos immunoreactivity in the PVN (27, 28, 30, 43, 54), these studies were focused primarily on PVN magnocellular neurons. Therefore, we sought to determine whether water deprivation increases Fos immunoreactivity in parvocellular neurons of the PVN with identified projections to the spinal cord and/or RVLM. Because there are conflicting reports regarding the relative number of PVN neurons that project to the spinal cord vs. RVLM (34, 42), we initially examined the distribution of retrogradely labeled neurons in the PVN from these two sites. In addition to the PVN, a recent
report indicates that the RVLM contributes to the maintenance of ABP in water-deprived rats (7). Therefore, a final series of experiments determined whether water deprivation increased Fos immunoreactivity in spinally projecting neurons of the RVLM.

METHODS

Animals

Adult male Sprague-Dawley rats (Charles River Laboratories) weighing 325–375 g were housed in a temperature-controlled room (22–23°C) with a 14:10-h light-dark cycle (lights on at 7 AM). Tap water and laboratory chow (Harlan Teklad LM-485, 0.3% NaCl) were available ad libitum except where noted.

Retrograde Tracing from the Spinal Cord and RVLM

To identify the efferent targets of parvocellular neurons of the PVN, retrograde tracers were injected into the IML and RVLM. In the first series of experiments, rats (n = 12) were anesthetized with pentobarbital sodium (50 mg/kg ip) and received a microinjection of 4% Fluorogold (FG, 50 nl; Fluorochrome, Denver, CO) into the region containing the IML at T1–T3 using the following coordinates (0.7 mm lateral to the midline, 0.7 mm ventral to the dorsal surface). In addition, rats received an injection of a second retrograde tracer, cholera toxin subunit B (CTB, 0.25% in isotonic saline, 20–30 nl; List Biological Laboratories, Campbell, CA) into the RVLM. The RVLM was found by locating the caudal pole of the facial nucleus through the use of field potential recording as described elsewhere (8, 38, 52). Briefly, a left facial nerve was exposed and stimulated electrically with a bipolar stainless steel electrode (0.2-ms pulse duration, 2 mA, 1 Hz). Field potentials were recorded with glass micropipettes filled with 0.9% isotonic saline (tip resistance 1–5 MΩ; tip OD 30–50 μm) connected to an Axoclamp 2B amplifier (Axon Instruments, Union City, CA) in bridge mode. Signals were amplified and displayed on an oscilloscope. Microinjectons were performed approximately 100–300 μm behind the caudal pole of the facial nucleus. This technique has been used previously to reliably locate the area of the RVLM that contains sympathetic premotor neurons that project to the spinal cord (8, 38, 52). Once the RVLM was located, the recording pipette was removed from the brain, emptied, filled with CTB, and lowered back into the RVLM. All microinjections were performed over 1 min with glass micropipettes connected to a pneumatic picopump (WPI). Once the overlying musculature and skin were sutured, each rat was treated with ampicillin (100 mg/kg im) and returned to its home cage.

To determine whether water deprivation increased Fos immunoreactivity in spinally projecting neurons of the RVLM, a second group of rats (n = 10) was microinjected only with 4% FG into the spinal cord at T1–T3 as described above. A second group was used to eliminate any potential influences that the previous injection in group 1 may have on Fos immunoreactivity in the RVLM.

Experimental Procedures

Approximately 2 wk after microinjection of the retrograde tracers, rats were randomly assigned to one of two groups. One group was deprived of water but not food for 48 h, whereas controls had continuous access to both food and water. Then rats were anesthetized with pentobarbital sodium (60 mg/kg ip), and blood (0.4 ml) was collected from the left ventricle into microcentrifuge tubes containing heparin (10 U) by using a 23-gauge needle. Samples were used for determination of hematocrit, Posmol, and plasma protein levels as described below. Immediately after the blood sample was collected, rats were perfused transcardially with heparinized isotonic saline (30 U/ml, 100 ml) followed by 4% paraformaldehyde (4°C, 300 ml) dissolved in 0.1 M PBS. Brains were removed, postfixed overnight in 4% paraformaldehyde at 4°C, and immersed in 30% sucrose dissolved in PBS for at least 2 days. The forebrain and hindbrain were sectioned at 30 μm using a sliding microtome. Sections were collected into five serially adjacent sets and stored in vials containing cryoprotectant (53) at −25°C.

Fos Immunocytochemistry

Sections were brought to room temperature over 30 min, rinsed with PBS several times, and incubated in 0.5% sodium borohydride dissolved in PBS for 30 min. Sections were then rinsed with PBS and incubated with a rabbit polyclonal anti-Fos antibody (1:10,000; Oncogene Research Products, San Diego, CA) at 4°C for 72 h followed by an incubation with biotinylated donkey anti-rabbit IgG for 2 h at room temperature (1:250). Next, tissue was incubated in an avidin-peroxidase conjugate for 2 h at room temperature (ABC Vectastain Kit, Vector Laboratories, Burlingame, CA) and reacted for 5 min in Tris buffer containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma), 2.5% nickel sulfate hexahydrate (Sigma), and 0.006% hydrogen peroxide. The reaction was terminated with several rinses in PBS. All antibody incubations were performed in PBS containing 0.3% Triton X-100 and 1% donkey serum. Donkey serum and all secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

CTB Immunocytochemistry

After Fos immunocytochemical procedures were completed, CTB was visualized by immunofluorescence. Briefly, sections were incubated in a goat anti-choleragenoid antibody (1:3,000; List Biological Laboratories, Campbell, CA) for 48 h at 4°C followed by an overnight incubation in CY3 donkey anti-goat IgG (1:250) at 4°C. CTB injection sites targeted at the RVLM were visualized by similar methods. All sections were mounted on glass slides, dehydrated in graded concentrations of alcohol, cleared in xylene, and coverslipped with Cytoseal 60 (Fisher Scientific).

Determination of Hematocrit, Posmol, and Plasma Protein Concentration

Hematocrit was determined from duplicate capillary tubes using standard methods and measured with a Lancer microhematocrit tube reader (St. Louis, MO). Blood samples were centrifuged (10,000 g, 30 s), and P was determined from duplicate plasma samples analyzed using a vapor pressure osmometer (model 5520, Wescor, Logan, UT). Plasma protein concentration was determined by protein refractometry (National Protometer, National Instruments, Baltimore, MD).

Histological Analysis

Fos-positive neurons in the PVN were examined under light microscopy. The PVN was sampled at three rostral-caudal levels. Level 1 was the most rostral and consisted of the dorsal parvocellular, medial parvocellular, and ventrally located posterior magnocellular subnuclei. Level 2 displayed a prominent posterior magnocellular region and both dorsal and ventrolateral parvocellular divisions. Level 3 was the most caudal and consisted of the medial and lateral parvocellular divisions (Fig. 1). Based on previous anatomic studies (34, 42, 47, 48), these levels of the PVN have efferent projections to the RVLM and IML. The RVLM was located 0–50 μm behind the caudal pole of the facial nucleus with a prominent nucleus ambiguus and a small or barely visible inferior olive. The RVLM was defined as the triangular region ventral to nucleus ambiguus, medial to the spinal trigeminal nucleus, and lateral to the inferior olive or pyramidal tracts (see Fig. 4, right side). Analysis of the PVN and RVLM were performed on one set of tissue (every 5th section). Therefore, counts in the PVN at each level were based on one section, whereas counts in the RVLM were based on the average number of Fos-positive nuclei and retrogradely labeled neurons from one to two sections per animal.

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Retrograde Labeling in the PVN from the Spinal Cord and RVLM

Previous studies have reported conflicting results regarding the relative number of PVN neurons that project to the spinal cord vs. the RVLM (34, 42). Therefore, we initially examined the distribution of neurons retrogradely labeled from these two regions at three different rostral-caudal levels of the PVN described previously in Fig. 1. Because there were no significant differences in the retrograde labeling between water-deprived and control rats (Table 1; \( P > 0.3 \) from overall ANOVAs), the results have been combined.

Microinjection of FG in the spinal cord and CTB in the RVLM produced similar patterns of labeling throughout the rostral-caudal extent of the ipsilateral PVN (Figs. 2 and 3). The majority of retrogradely labeled neurons from either site was present in the dorsal parvocellular (Fig. 2, A, B, and D), ventrolateral parvocellular (Fig. 2, B and E), and lateral parvocellular divisions (Fig. 2, C and F). However, levels 2 and 3 had a significantly greater number of neurons retrogradely labeled from the spinal cord or RVLM than level 1 (Table 1, Fig. 3). Furthermore, a significantly greater number of retrogradely labeled neurons were present in the ventrolateral than the dorsal parvocellular division at level 2. The location of double-labeled neurons, FG + CTB, followed a similar rostral-caudal distribution (Fig. 3), and the majority was located in the ventrolateral and lateral parvocellular PVN (Figs. 2 and 3). Moreover, there were no differences between the percentage of spinally projecting neurons also projecting to the RVLM vs. RVLM-projecting neurons also projecting to the spinal cord at any level or division of the PVN (Table 1). This overall pattern of dual retrograde labeling from the spinal cord and RVLM was similar in the contralateral PVN; however, the absolute number of retrogradely neurons was significantly less in the contralateral vs. ipsilateral side (Fig. 3).

Table 1. Number of retrogradely labeled neurons in the ipsilateral hypothalamic PVN after microinjection of FG in the spinal cord (T1-T3) and CTB in the RVLM of rats

<table>
<thead>
<tr>
<th>Level</th>
<th>FG (Spinally Projecting)</th>
<th>CTB (RVLM Projecting)</th>
<th>Double Labeled</th>
<th>%FG with CTB</th>
<th>%CTB with FG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>Dorsal parvocellular</td>
<td>22±3</td>
<td>13±3</td>
<td>2±1</td>
<td>14±3</td>
</tr>
<tr>
<td>Level 2</td>
<td>Dorsal parvocellular</td>
<td>36±5</td>
<td>20±2</td>
<td>4±1</td>
<td>12±4</td>
</tr>
<tr>
<td></td>
<td>Ventrolateral parvocellular</td>
<td>57±8†</td>
<td>58±8†</td>
<td>9±2†</td>
<td>16±2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>93±12*</td>
<td>78±11*</td>
<td>13±2*</td>
<td>14±2</td>
</tr>
<tr>
<td>Level 3</td>
<td>Lateral parvocellular</td>
<td>112±8*</td>
<td>106±12*</td>
<td>17±2*</td>
<td>15±2</td>
</tr>
<tr>
<td>Overall total in PVN</td>
<td>227±14*</td>
<td>197±15*</td>
<td>32±3*</td>
<td>14±1</td>
<td>17±1</td>
</tr>
</tbody>
</table>

Values are mean±SE; \( n = 12 \) rats. The results from water-deprived (\( n = 6 \)) and control (\( n = 6 \)) were combined because there were no differences between groups. Additionally, there were no differences between the number of fluorogold (FG)- vs. cholera toxin subunit B-labeled neurons in any subnucleus or level of the paraventricular nucleus (PVN) (\( P > 0.1 \) from overall ANOVAs). RVLM, rostral ventrolateral medulla. †Significant difference from level 1-dorsal parvocellular (\( P < 0.05 \)). *Significant difference in level 2 between dorsal and ventrolateral parvocellular (\( P < 0.05 \)).
Figure 4 illustrates the CTB injection site in the RVLM. The injection site did not spread laterally to the spinal trigeminal nucleus, medially to the inferior olive or pyramidal tract, or dorsal to nucleus ambiguus.

**Effect of 48-h Water Deprivation on Fos Immunoreactivity in the PVN**

A major goal of the present study was to determine whether 48-h water deprivation increased Fos immunoreactivity in autonomic regions of the PVN. As expected (27, 28, 30, 43, 54), water deprivation increased Fos immunoreactivity in both magnocellular and parvocellular divisions of the PVN (Fig. 5). To determine whether water deprivation increased Fos immunoreactivity in autonomic regions of the PVN, we used the retrograde labeling from both the spinal cord and RVLM to provide the anatomic boundaries of the dorsal, ventrolateral, and lateral PVN at the respective level. The number of Fos-positive nuclei was significantly higher in 48-h water-deprived rats in each of these subdivisions and at every level of the PVN (Figs. 5 and 6A). Similar to the distribution of retrogradely labeled neurons, a significantly greater number of Fos-positive nuclei were present in levels 2 and 3 compared with level 1 (Fig. 6A). With regard to level 2, the ventrolateral parvocellular division had significantly more Fos-positive nuclei than the dorsal parvocellular division (Fig. 6A). Not surprisingly, a similar pattern of Fos immunoreactivity was present throughout the contralateral PVN (data not shown).

**Effect of Water Deprivation on Fos Immunoreactivity in PVN Neurons With Projections to the Spinal Cord and RVLM**

Spinally projecting PVN neurons. Because water deprivation increased Fos immunoreactivity in autonomic regions of the PVN, we determined whether these Fos-positive nuclei were present in spinally projecting (FG) and/or RVLM-projecting (CTB) neurons. Among spinally projecting (FG labeled) neu-
rons of the ipsilateral PVN, 48-h water deprivation significantly increased Fos immunoreactivity (Figs. 6B and 7). In fact, a greater percentage of spinally projecting PVN neurons were Fos positive in water-deprived rats compared with control rats (21.9 ± 2.2 vs. 3.9 ± 1.0%, respectively; P < 0.01). Again, the majority of these neurons was found in the ventrolateral and lateral parvocellular divisions of the PVN, whereas there was no difference in the number of these double-labeled neurons (CTB + Fos) between control and water-deprived rats in the dorsal parvocellular division at levels 1 and 2 (Fig. 6C). Moreover, there were no differences in the number of RVLM-projecting neurons that were Fos positive between control and water-deprived rats throughout the entire contralateral PVN (1.0 ± 0.4 vs. 4.2 ± 1.3 cells, respectively).

Spinally vs. RVLM-projecting PVN neurons. The proportion of PVN neurons that project to the RVLM and were Fos positive after water deprivation was greater than the proportion of spinally projecting neurons that were Fos positive. This was the case for the ventrolateral (25.8 ± 0.7 vs. 8.0 ± 1.5%, respectively; P < 0.01) and lateral (23.4 ± 2.1 vs. 5.0 ± 0.9%,
respectively; $P < 0.01$) parvocellular divisions on the ipsilateral side. No differences were observed in the dorsal parvocellular PVN at level 1 (4.2 ± 4.0 vs. 1.7 ± 1.0%, respectively) or level 2 (12.3 ± 6.1 vs. 1.7 ± 0.8%, respectively).

Effect of Water Deprivation on Fos Immunoreactivity in PVN Neurons with Projections to Both the Spinal Cord and the RVLM

With regard to PVN neurons that project ipsilaterally to both the spinal cord and RVLM (FG + CTB), a significantly greater number of neurons were triple labeled (Fos + FG + CTB) in water-deprived rats than control rats in the lateral parvocellular division (2.4 ± 0.3 vs. 0.2 ± 0.2 cells, respectively; $P < 0.01$) but not the ventrolateral division (1.5 ± 0.7 vs. 0.2 ± 0.2 cells) of the PVN. No triple-labeled neurons were observed in either water-deprived or control rats in the dorsal parvocellular division at level 1 or 2.

Effect of Water Deprivation on Fos Immunoreactivity in RVLM Neurons With Projections to the Spinal Cord

A recent report indicates that the RVLM contributes to the maintenance of ABP in water-deprived rats (7). Therefore, we determined whether water deprivation increased Fos immunoreactivity in spinally projecting neurons of the RVLM. Results from animals receiving only FG in the spinal cord were not different from those of animals that received both FG in the spinal cord plus CTB in the RVLM. Therefore, the data were combined. Water deprivation significantly increased the number of Fos-positive nuclei in the RVLM (Table 2, Fig. 9). In addition, a greater number of retrogradely labeled neurons in the RVLM were Fos positive in water-deprived vs. control rats (Table 2, Fig. 9), even though the absolute number of RVLM neurons retrogradely labeled was not different between groups. Therefore, a significantly greater percentage of spinally projecting neurons in the RVLM were Fos positive in water-deprived than in control rats (Table 2).

Effect of Water Deprivation on $P_{\text{osmol}}$, Hematocrit, and Plasma Protein Concentration

As expected (7, 44), 48-h water deprivation significantly increased $P_{\text{osmol}}$ (water deprived 313 ± 1 vs. control 299 ± 1 mosmol/kgH$_2$O, $P < 0.001$), hematocrit (water deprived 49.9 ± 0.6 vs. control 40.5 ± 0.5%, $P < 0.001$), and plasma protein concentration (water deprived 6.3 ± 0.1 vs. control 5.0 ± 0.2 g/dl, $P < 0.001$).

DISCUSSION

Several observations indicate that sympathetic outflow is elevated during water deprivation (17, 40, 41, 44, 50); however, the central neural circuitry and cellular mechanisms...
WATER DEPRIVATION EVOKES FOS IN PVN AUTONOMIC NEURONS

Fig. 6. Average number (per section) of Fos-positive nuclei (A), Fos + FG (spinally projecting) neurons (B), and Fos + CTB (RVLM projecting) neurons (C) in the dorsal, ventrolateral, and lateral parvocellular divisions at 3 different rostral-caudal levels of the ipsilateral PVN in water-deprived and control rats (see Fig. 1 for a description of rostral-caudal levels). The respective level is indicated in parentheses. In addition, the total across all levels and subdivisions of the PVN is included. A: water deprivation significantly increased Fos immunoreactivity in every PVN subdivision examined. The number of Fos-positive nuclei was significantly greater at levels 2 and 3 vs. level 1 (P < 0.05). B and C: in addition, a significantly greater number of spinally projecting and RVLM-projecting neurons were Fos positive in water-deprived rats than control rats. The greatest number of double-labeled neurons was present at levels 2 and 3. Within level 2, a significantly greater number of Fos-positive nuclei and double-labeled (Fos + FG and Fos + CTB) neurons were present in the ventrolateral than the dorsal subnucleus. Values are means ± SE. *Significant difference from control rats (P < 0.025). †Significant difference between dorsal and ventrolateral parvocellular subnuclei at level 2 (P < 0.05).

Supporting this elevated SNA are not fully understood. Because inhibition of the PVN with bilateral microinjection of the GABA\textsubscript{A} receptor agonist muscimol has been shown to significantly decrease renal SNA and ABP in 48-h water-deprived rats (44), we hypothesized that water deprivation would increase Fos immunoreactivity in sympathetic regulatory neurons of the PVN. The present findings support this hypothesis as water deprivation increased the number of Fos-positive nuclei in parvocellular PVN neurons with identified projections to the spinal cord and/or RVLM.

Previous studies have demonstrated that parvocellular neurons of the PVN terminate in the IML of the spinal cord and RVLM, and these projections are predominantly ipsilateral (33, 34, 37, 42, 47). The present findings confirm those observations. However, there are conflicting reports regarding the relative number of PVN neurons that project to these two regions (34, 42). Shafton and colleagues (42) reported that a greater number of PVN neurons are retrogradely labeled from the RVLM than the spinal cord, whereas Pyner and Coote (34) suggest the contrary. Although these differences may be explained by the use of different retrograde tracers or the location of injection sites in the spinal cord (upper thoracic vs. lumbar), the present findings suggest that a similar number of PVN neurons project to the upper thoracic spinal cord and to the RVLM, and ~15–20% of these neurons have collateral projections to both sites. These observations are in agreement with preliminary findings from another laboratory (46).

Water deprivation has been reported previously to increase Fos immunoreactivity in magnocellular neurons of the PVN (27, 28, 30, 43, 54). The present findings confirm and extend these observations by demonstrating that 48-h water deprivation also increases Fos immunoreactivity in the dorsolateral, ventrolateral, and lateral parvocellular divisions of the PVN. More importantly, a subset of these Fos-positive neurons had identified projections to the spinal cord and/or RVLM. Taken together, these findings suggest that the increased contribution of PVN neuronal activity to ABP and the elevated SNA in water-deprived rats may be due to activation of PVN pathways descending to both the spinal cord and RVLM (44).

Because water deprivation decreases intravascular volume and increases Posmol and circulating ANG II, one or a combination of these signals could contribute to the increase in Fos immunoreactivity in PVN sympathetic-regulatory neurons during water deprivation. In this regard, changes in intravascular volume, Posmol, and circulating ANG II have been reported to independently increase Fos immunoreactivity in parvocellular neurons of the PVN (2–5, 22, 32, 35, 36, 39). However, only a limited number of studies have examined whether these stimuli increase Fos immunoreactivity in parvocellular PVN neurons with identified projections to the spinal cord or RVLM (4, 5, 22). Recently, Kantzides and Badoer (22) reported that intravenous infusion of hypertonic saline did not increase Fos immunoreactivity in spinally projecting PVN neurons. These data raise the possibility that increases in Posmol may not contribute to the increase in Fos immunoreactivity observed in PVN sympathetic-regulatory neurons during water deprivation. Although this possibility cannot be excluded, it is noteworthy that an intravenous infusion of hypertonic saline likely increases intravascular volume due to the infusion and to osmotic movement of water into the intravascular space. Thus blood volume expansion could mask an increase in Fos immunoreactivity by elevated Posmol as volume expansion has been reported to inhibit the activity of spinally projecting PVN neurons (24). This would not be expected to occur during water deprivation as intravascular volume is reduced. In addition, an intravenous infusion of hypertonic saline reduces circulating ANG II levels, whereas plasma ANG II levels increase in response to 48-h water deprivation (45). Last, the acute infusion of hypertonic saline rapidly increases Posmol, whereas
water deprivation progressively increases $P_{\text{osmol}}$ and ANG II levels and decreases intravascular volume. Thus several factors preclude a direct comparison between the present findings and those reported previously with an infusion of hypertonic saline (22). Nevertheless, in vivo electrophysiological recordings have demonstrated that PVN neurons with axonal projections to the spinal cord and/or hindbrain are responsive to changes in circulating ANG II, intravascular volume, and $P_{\text{osmol}}$ (6, 10, 15, 16, 24, 51). Moreover, changes in ABP and SNA in response to intracarotid injections of hyperosmotic solutions or changes in intravascular volume are dependent on neurotransmission within the PVN (9, 20, 25, 56). Collectively, these observations suggest that one or a combination of the aforementioned signals may increase Fos immunoreactivity in parvocellular PVN neurons and may also underlie the increased contribution of PVN neuronal activity to the elevated SNA and maintenance of ABP in water-deprived rats.

In addition to the PVN, water deprivation increased Fos immunoreactivity in bulbospinal RVLM neurons and thereby suggests that the RVLM may play a role in the elevated SNA and maintenance of ABP in water-deprived rats. A myriad of studies have demonstrated that the RVLM plays a pivotal role in the regulation of SNA and ABP (for reviews, see Refs. 12, 19). This region contains spinally projecting, barosensitive neurons that mediate several vasomotor reflexes. Interestingly, bilateral blockade of excitatory amino acid receptors with kynurenic acid in the RVLM has been reported to decrease ABP in water-deprived rats (7). The decrease in ABP correlated with $P_{\text{osmol}}$ when data were analyzed across control, 24-h, and 48-h water-deprived rats (7). This observation indicates that the increase in $P_{\text{osmol}}$ could contribute to the greater dependence of ABP on excitatory amino acid transmission in the RVLM and might also underlie the increase in Fos immunoreactivity observed in bulbospinal RVLM neurons during water deprivation. Additional studies are needed to investigate these possibilities.

Collectively, these observations raise the prospect that water deprivation activates a pathway from the PVN to RVLM that increases SNA and maintains ABP. In this regard, the changes in SNA and ABP after disinhibition of the PVN with the GABA$_A$ receptor antagonist bicuculline are attenuated by bilateral blockade of ANG II AT$_1$ receptors in the RVLM (49), whereas blockade of excitatory amino acid receptors in the RVLM has been reported to have no effect on the hemodynamic responses to PVN stimulation (23, 49). Moreover, excitatory amino acid stimulation of the PVN has been reported to increase the discharge of bulbospinal RVLM neurons (55, 57); however, this increase in discharge was attenuated by iontophoretic application of excitatory amino acid receptor antagonist kynurenic acid or a vasopressin (type 1) receptor antagonist (55). Taken together, it appears that the RVLM likely contributes to PVN-evoked changes in SNA and ABP, but the neurotransmitters and/or cellular mechanisms mediating PVN inputs to the RVLM remain unclear. The discrepan-

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Fig. 7. Examples of FG-labeling from the spinal cord (T1–T3) and Fos immunoreactivity at level 2 (A) and level 3 (B) of the PVN in a water-deprived rat. Areas outlined in A and B are shown in C–E. White arrows denote double-labeled neurons. Summary counts are presented in Fig. 6B.
cies regarding the role of ANG II AT1 receptors and excitatory amino acid receptors in the RVLM in mediating the response to PVN disinhibition vs. direct excitation may be explained by the possibility that these stimuli recruit different populations of PVN neurons. Surprisingly, there is no direct evidence detailing the neurochemical phenotype of PVN neurons that project to the RVLM. Future experiments are needed to fully establish whether activation of a mono- or polysynaptic pathway from the PVN to the RVLM contributes to the elevated SNA and maintenance of ABP in water-deprived rats and to determine the neurotransmitters utilized by this pathway.

In summary, the present findings demonstrate that water deprivation increases Fos immunoreactivity in parvocellular PVN neurons with identified projections to the spinal cord and RVLM and in bulbospinal neurons of the RVLM. While these observations are consistent with the hypothesis that water deprivation activates PVN sympathetic-regulatory neurons, it cannot be definitively stated that these neurons have an increased discharge frequency. That is, an increase in Fos immunoreactivity indicates an increase in transcription but may not equate to an increase in electrical activity (26). Moreover, a significant number of PVN and RVLM neurons were not immunoreactive for Fos after water deprivation. This may suggest that only a small population of PVN and RVLM neurons is activated by water deprivation. Alternatively, it should be mentioned that not all neurons express Fos (14), and Fos immunoreactivity is typically an indicator of neuronal activation over a short time frame. Therefore, it is not clear whether the Fos immunoreactivity in PVN neurons observed in the present study is due to prolonged activation of the c-fos gene as stimulus intensity increases over time or whether different PVN neurons transiently express an immunodetectable level of Fos during the 48-h period of water deprivation. Consequently, it is possible that the present findings may underestimate the number of sympathetic-regulatory neurons.

Table 2. Number of Fos-positive nuclei and spinally projecting (FG positive) neurons in the RVLM of control and water-deprived rats

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 11)</th>
<th>Water Deprivation (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Fos positive nuclei</td>
<td>6.6±1.0</td>
<td>34.8±2.1*</td>
</tr>
<tr>
<td>Number of FG neurons</td>
<td>21.9±1.6</td>
<td>19.1±1.5</td>
</tr>
<tr>
<td>Number of double-labeled neurons (Fos and FG)</td>
<td>0.6±0.2</td>
<td>4.3±0.4*</td>
</tr>
<tr>
<td>Percentage of FG neurons positive for Fos</td>
<td>2.9±1.2</td>
<td>25.6±2.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Neurons were retrogradely labeled from the spinal cord by microinjecting 4% FG (100 nl) at T1–T3 levels ~14 days before experiments. Results from animals receiving FG only in the spinal cord or FG in the spinal cord plus CTB in the RVLM were not different and therefore were combined. The RVLM was analyzed ipsilateral to the spinal cord injection. *Significant difference from control rats (P < 0.001).
affected by water deprivation. Because water deprivation produces a slow and progressive change in the variables that may underlie Fos immunoreactivity in these neurons, other inducible transcription factors such as Fos B may serve as a more accurate index of neuronal activation under these conditions (11, 21). Future experiments are needed to investigate this possibility. Nevertheless, the present findings strongly indicate that water deprivation evokes a distinct cellular response in sympathetic-regulatory neurons of the PVN and RVLM.

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