Dehydration-induced drinking decreases Fos expression in hypothalamic paraventricular neurons expressing vasopressin but not corticotropin-releasing hormone

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Wotus C, Arnhold MM, Engeland WC. Dehydration-induced drinking decreases Fos expression in hypothalamic paraventricular neurons expressing vasopressin but not corticotropin-releasing hormone. Am J Physiol Regul Integr Comp Physiol 292: R1349–R1358, 2007. First published October 26, 2006; doi:10.1152/ajpregu.00304.2006.—Water-restricted (WR) rats exhibit a rapid suppression of plasma corticosterone following drinking. The present study monitored Fos-like immunoreactivity (Fos) to assess the effect of WR-induced drinking on the activity of vasopressin (VP)-positive magnocellular and parvocellular neurons and corticotropin-releasing hormone (CRH)-positive parvocellular neurons in the paraventricular nucleus of the hypothalamus. Adult male rats received water for 30 min (WR) in the post meridiem (PM) each day for 6 days and were killed without receiving water or at 1 h after receiving water for 15 min. In WR rats, Fos increased in VP magnocellular and parvocellular neurons but not CRH neurons. After drinking, Fos was reduced in VP magnocellular and parvocellular neurons but did not change in CRH neurons. To assess the severity of osmotic stress, rats were sampled throughout the final day of WR. Plasma osmolality, hematocrit and plasma VP were increased throughout the day before PM rehydration, and plasma ACTH and corticosterone were elevated at 1230 and 1430, respectively, showing that WR activates hypothalamic-pituitary-adrenal activity during the early PM before the time of rehydration. To determine the effects of WR-induced drinking on CRH neurons activated by acute stress, WR rats underwent restraint. Restraint increased plasma ACTH and corticosterone and Fos in CRH neurons; although rehydration reduced plasma ACTH and Fos expression in VP neurons, Fos in CRH neurons was not affected. These results suggest that inhibition of VP magnocellular and parvocellular neurons, but not CRH parvocellular neurons, contributes to the suppression of corticosterone after WR-induced drinking.

Fos; dehydration; rehydration; magnocellular neurons; parvocellular neurons

The hypothalamic-pituitary-adrenal (HPA) response to stress is initiated by the activation of hypophysiotrophic neurons in the paraventricular nucleus (PVN) of the hypothalamus. Release of the peptides corticotropin-releasing hormone (CRH) and vasopressin (VP) from PVN parvocellular neurons in the hypophyseal portal blood stimulates the release of ACTH (46, 55) from the anterior pituitary, which subsequently stimulates the production and release of glucocorticoids from the adrenal cortex. Suppression of this neuroendocrine response has been shown to occur through hormonal negative feedback, in which glucocorticoids act at the brain and pituitary to limit their own production (10, 32). However, studies in rats have shown that the act of drinking after water restriction results in a rapid decrease in plasma corticosterone, the primary glucocorticoid in the rat (21, 35, 61). These data suggest that signals associated with satiety, or with drive reduction, may have the ability to activate neuronal afferent pathways that result in the inhibition of parvocellular neurons, independent of glucocorticoid negative feedback.

Drinking after water restriction also may reduce HPA activity by inhibiting magnocellular neurons in the PVN and the supraoptic nucleus (SON). Magnocellular-derived VP has been implicated in facilitating release of ACTH in response to stress (17). Whereas dehydration stimulates VP release in the systemic circulation (18), drinking after a period of water deprivation causes a rapid decrease in plasma VP (4, 19, 49, 52). Water restriction-induced drinking results in parallel decreases in plasma VP and corticosterone (63). Furthermore, peripheral administration of VP elevates corticosterone, whereas an injection of VP antibody reduces plasma corticosterone from an elevated state induced by water restriction (63). These data suggest that a decrease in plasma VP may contribute to the decrease in corticosterone after dehydration-induced drinking. However, because immunoneutralization can also block the effect of VP on the pituitary (45), both magnocellular- and parvocellular-derived VP could be involved in HPA responses to rehydration after water restriction. Experiments have not been done to determine whether specific subpopulations of magnocellular and parvocellular neurons in the PVN are affected by water restriction-induced drinking.

Expression of the immediate-early gene c-fos has been extensively used as a marker of neural activation, particularly in neuroendocrine systems (for review, see Ref. 25). Dehydration increases Fos protein expression in both magnocellular and parvocellular PVN neurons in rats (34, 40) and results in elevated VP mRNA in magnocellular (3, 22, 36) and parvocellular neurons (3). In marked contrast, CRH mRNA in parvocellular neurons is reduced by chronic dehydration induced by salt loading (34, 64) or water deprivation (2, 22). These studies suggest that magnocellular and parvocellular neuronal activity in the PVN is differentially activated by chronic dehydration. However, it is not clear how magnocellular and parvocellular PVN neurons respond to water restriction in which rats are repeatedly exposed to dehydration followed by rehydration (63).

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Although Fos expression has been used primarily to reflect neural activation, recent studies have shown that the reduction in Fos expression can be used as an index of neural inhibition. For example, water deprivation-induced Fos expression in magnocellular SON and PVN neurons is decreased at 2 h after drinking, suggesting that inhibition of neural activity occurs after rehydration (15, 20, 29). Here, we test the hypothesis that water restriction-induced drinking rapidly inhibits both magnocellular and parvocellular neuronal activity in the PVN, reflected by decreases in Fos expression in VP and CRH neurons. Our initial experiment indicated that VP, but not CRH, neurons were activated by 6-day water restriction in the PM and that drinking reversed this response. The finding that CRH neurons were not activated led us to evaluate more thoroughly the ability of 6-day water restriction to chronically stimulate the HPA axis by measuring plasma VP, ACTH, and corticosterone throughout the final day of water restriction. We then assessed the HPA hormonal and PVN response to acute activation by restraint stress and the ability of water restriction-induced drinking to inhibit this response.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (175–200 g; Charles River, Wilmington, MA) were housed two per cage under a 12:12-h light-dark cycle (lights on at 0515) with food and water available ad libitum before initiation of the water restriction schedule. Experiments were initiated at least 2–3 days after arrival. Animals were maintained and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experimental procedures were approved by the University of Minnesota Animal Care and Use Committee.

Experiment 1: Effect of Water Restriction-induced Drinking on Fos Expression in the PVN

To determine if water restriction-induced drinking inhibits the activity of PVN neurons, Fos expression was determined in parvocellular and magnocellular neurons labeled for CRH or VP. Rats were placed on a 6-day water restriction schedule as previously described (62). Rats received water each day for 30 min in the post meridiem (PM) beginning 30 min before the onset of dark (1645, water restricted; WR); control rats received water ad libitum (AL). Rats had access to food at all times. To minimize the association of investigators entering the room with rats receiving water, personnel entered the room several times throughout each day and manipulated cages by opening and closing the lids. After 6 days at the time when WR rats would receive their water in the PM, one group of WR rats (n = 6) that were not given water (WR) and AL rats (n = 4) were killed by decapitation; another group of WR rats (n = 6) were given water to drink for 15 min (WR/Rehydrated) and were killed 60 min later. The time of death after drinking was chosen for the following two reasons: 60 min is within the time period for observing maximal Fos immunoreactivity after the onset of neuronal activation (41), and significant decreases in both VP and corticosterone have been observed 15 min after restriction-induced drinking (62, 63). Brains were rapidly removed and fixed for subsequent immunohistochemical analysis of Fos and VP or CRH immunoreactivity.

Experiment 2: Effect of Water Restriction on HPA Hormones Throughout the Day Before PM Rehydration

To determine whether water restriction activates the HPA axis during the day before PM water administration, rats (n = 6/group) were placed on a 6-day water restriction schedule (WR) or received water ad libitum (AL); food was available at all times. After 6 days, WR and AL rats were killed by decapitation every 2 h starting at 0830 and ending at 1630; at 1630, another group of WR rats was killed 15 min after receiving water (WR/Rehydrated). Trunk blood was collected for the measurement of hematocrit. Plasma was separated into aliquots for assay of osmolality by vapor pressure osmometry and for measurement of VP, ACTH, and corticosterone by RIA.

Experiment 3: Effect of Restriction-induced Drinking on HPA Activation Induced by Acute Stress

Experiment 3a: Effect of restriction-induced drinking on HPA hormones stimulated by acute restraint stress. To determine if water restriction-induced drinking can suppress hormonal responses to an acute stress, rats were placed on the water restriction (WR) schedule for 6 days as described above. Rats were randomly assigned to one of four WR groups (n = 6/group) and killed by decapitation from 1230 to 1330. The WR group was killed without restraint or rehydration; the WR/Restraint group underwent restraint stress by being placed in disposable plastic restrainers (DecapriCones; Braintree Scientific, Braintree, MA) for 15 min before death; the WR/Restraint/Rehydrated group underwent restraint for 15 min and was then returned to home cages with water for 15 min before death; and the WR/Restraint/Rehydrated group underwent restraint for 15 min and was then returned to home cages without water for 15 min before death. At the time of death, trunk blood was collected for hematocrit; plasma was saved for subsequent determination of osmolality and hormone content.

Experiment 3b: Effect of restriction-induced drinking on Fos expression in PVN neurons after acute restraint stress. To determine if water restriction-induced drinking inhibits the activity of PVN neurons induced by acute stress, Fos expression was determined in rats exposed to restraint stress followed by drinking. All rats were placed on the water restriction (WR) schedule for 6 days as described above. Rats were randomly assigned to one of five WR groups (n = 5/group) and killed by decapitation from 1130 to 1330. The first two groups were left undisturbed in their home cages and did not receive water; the WR/Pre group was killed within 6 min of entering the animal room, whereas the WR/No Water group was killed during the same period required for brain collection from the WR/Rehydrated group. The WR/Rehydrated group received water for 30 min and was killed 45 min later (60 min after drinking for 15 min). Two groups of WR rats underwent restraint stress for 15 min and were then returned to their home cages; one group (WR/Restraint/Rehydrated) received water for 30 min and was killed 90 min after the initiation of the stress (60 min after drinking for 15 min), and the other group (WR/Restraint/No Rehydration) was killed 90 min after the initiation of restraint stress without receiving water. Brains were rapidly removed and fixed for subsequent immunohistochemical analysis of Fos and VP or CRH immunoreactivity.

Determination of Plasma Hormones

Plasma ACTH was determined by RIA with 125I-labeled ACTH (DiaSorin, Stillwater, MN) as described previously (28). Plasma corticosterone was determined by an RIA kit (ICN Biochemical, Costa Mesa, CA). The intra-assay and interassay coefficients of variation (CVs) for corticosterone were 7.6 and 13.3%, respectively. Plasma VP was extracted with acetone and petroleum ether (47) and then determined by RIA with a VP antibody provided by Dr. Alan Robinson (University of California Los Angeles; see Ref. 56). 125I-labeled VP (Perkin-Elmer, Boston, MA), and synthetic VP (Bachem, Torrance, CA). The intra-assay and interassay CVs for VP were 13.3 and 14.4%, respectively.

Processing of Neural Tissue

Brains were fixed by 10% formalin. After 2 weeks, brains were dehydrated and paraffin-embedded. Paraffin sections were mounted on coated slides and processed for immunohistochemistry.

Figure 1. (A) Representative photomicrographs of PVN showing Fos expression in CRH neurons (B) and VP neurons (C). (D) Comparison of Fos expression in the PVN after restraint stress between control and experimental groups (n = 10/group). Fos expression was significantly reduced in PVN neurons of rats exposed to restraint stress followed by drinking (P < 0.05 vs. control).*
paraformaldehyde solution buffered with sodium acetate (0.1 M, pH 6.0), incubated while being shaken for 7 h at 4°C and then transferred to a 4% paraformaldehyde solution buffered with sodium borate (0.1 M, pH 9.5), and incubated for 48 h at 4°C. Brains were then transferred to 20% glycerol in phosphate buffer (0.1 M) and incubated at 4°C for 48 h, after which time they were cut into segments containing the hypothalamus and frozen in optimum cutting temperature mounting media (Miles, Elkhart, IN) until sectioning. Brain sections containing the PVN defined using a rat stereotaxic atlas (44) were cut at 40 μm, washed, free floating, in PBS (0.1 M), and stored in cryoprotectant (30% ethylene glycol and 20% glycerol in 0.05 M PBS) at −20°C until the time of labeling.

**Immunohistochemistry**

For each animal, serial brain sections containing the medial parvocellular (mp) and posterior magnocellular (pm) divisions of the PVN were thawed and then blocked for 10 min in 3% H2O2 in 10% methanol. After being rinsed with PBS, sections were blocked for 1 h in 20% normal goat serum (NGS; Vector Laboratories, Burlingame, CA) at room temperature, and then incubated for 48 h at 4°C with rabbit anti-Fos primary antibody (Ab-5, directed against the NH2-terminal 4–17 of human Fos; Oncogene, San Diego, CA) diluted 1:160,000 in 0.3% Triton X-100 in 0.1 M PBS with 2% NGS. With the use of a Vectastain ABC kit (Vector Laboratories), sections were then incubated for 1 h at room temperature with goat anti-rabbit biotinylated secondary antibody followed by a 1-h incubation with the avidin-biotin complex. Fos labeling was visualized using diaminobenzidine (DAB) with nickel (Vector Laboratories), resulting in a black/purple nuclear staining. Alternate sections from each brain were then double labeled for either VP or CRH immunoreactivity using the same procedure described for Fos labeling. Primary rabbit anti-VP (1:80,000; Chemicon, Temecula, CA) and rabbit anti-CRH (1:160,000; generously provided by Dr. Wylie Vale, The Salk Institute, La Jolla, CA) antibodies were visualized using DAB alone, resulting in brown cytoplasmic staining. Sections were washed, mounted, and dried on glass slides and dehydrated and covered with a cover slip in DPX media (Sigma Chemical, St. Louis, MO).

Labeling for each of the antibodies was eliminated when the primary antibodies were omitted or preabsorbed with their cognate peptides (50 μg/ml, 4°C, overnight). Additionally, we performed the double-labeling protocol omitting the first or the second primary antibody. When the primary against Fos was omitted, only brown cytoplasmic labeling for CRH or for VP remained; conversely, when the primary against CRH or for VP was omitted, only black/purple, nuclear labeling for Fos was observed.

**Quantification of Fos labeling.** In each animal, Fos-positive nuclei were counted in two serial sections through the midrostrocaudal level of the PVN that included the pmPVN and mpPVN divisions as defined previously (50). In adjacent sections, neurons double labeled for Fos and VP or for Fos and CRH were counted in the PVN. The parvocellular VP-positive neurons were distinguished from the magnocellular neurons based on size and location. Counts were determined by two independent observers blind to the treatment conditions and were averaged for each section. The total number of double-labeled (Fos-positive and VP-positive or Fos-positive and CRH-positive) cells counted within the PVN from each animal was then expressed as a percentage of the total number of VP-positive or CRH-positive cells. Optical images were collected using a digital camera and computer processed using Adobe Photoshop 5.0.

**Statistical Analysis**

Data are presented as means ± SE. Before analysis, nonhomogeneous data were subjected to square-root transformation. In experiment 1, differences between groups were determined by one-way ANOVA for each neuronal phenotype. In experiment 2, differences between groups over time were assessed by two-way ANOVA; differences between WR and WR/Rehydrated groups at 1630 were assessed by t-test. In experiment 3, differences between groups 1 (Pre) and 2 (no restraint, no WR) were assessed by t-test; differences between groups 2 and 4 were assessed by two-way ANOVA. When ANOVA showed a significant difference within an experiment, Fisher’s post hoc analysis was used to identify differences between groups. Differences were considered statistically significant when the test yielded a P < 0.05.

**RESULTS**

**Experiment 1: Effect of Drinking on Water Restriction-induced Fos Expression in VP and CRH Neurons in the PVN**

Changes in neural activity were assessed by monitoring Fos expression in magnocellular VP (pmPVN), parvocellular VP (mpPVN), and parvocellular CRH (mpPVN) neurons after water restriction and subsequent drinking (Fig. 1). Rats restricted to 30 min of water in the PM for 6 days (WR) showed an increased percentage of Fos-positive magnocellular and parvocellular VP neurons but not parvocellular CRH neurons compared with rats given water ad libitum (AL).

Rats receiving water for 30 min (WR/Rehydrated) exhibited a marked reduction in the percentage of Fos-positive VP magnocellular and parvocellular neurons (Figs. 1 and 2). The changes in Fos expression are not because of differences in the number of VP neurons counted, since the total number of VP-positive magnocellular and parvocellular neurons did not vary across treatment groups (Table 1). However, the total number of CRH-positive neurons was lower in the WR group compared with the other groups (Table 1).

**Experiment 2: Plasma Osmolality and HPA Hormones on the Last Day of Water Restriction**

To better establish the changes in HPA activity resulting from repeated water restriction, plasma osmolality, hematocrit, and plasma hormones were measured throughout the 6th day before PM rehydration. Control AL rats showed no change in plasma osmolality or hematocrit over the 0830 to 1630 time period (Table 2). In contrast, WR rats had elevated plasma osmolality and hematocrit compared with AL rats over the entire sampling period; in addition, plasma osmolality increased in WR rats between 1030 and 1230. Plasma VP did not vary between 0830 and 1630 in AL rats, whereas plasma VP was elevated in WR rats throughout the day (Fig. 3A). Plasma ACTH did not exhibit a clear diurnal rhythm in AL rats, although the highest values occurred at 1630 (Fig. 3B, C). In contrast, WR rats showed a reduction in plasma ACTH in AL and WR rats at 1030 but no corresponding elevation in corticosterone. Plasma ACTH in AL rats returned to ante meridiem (AM) levels by 1230, whereas plasma ACTH in WR rats remained elevated (Fig. 3B). Plasma corticosterone exhibited a rhythm in AL and WR rats; however, the peak occurred earlier in WR rats (1430) compared with AL rats (1630; Fig. 3C). As expected, WR rats receiving water for 15 min at 1630 showed a reduction in plasma osmolality (WR: 298.5 ± 1.3 mosmol/kgH2O vs. WR/Rehydrated: 290.3 ± 2.4 mosmol/kgH2O; P < 0.05), VP (Fig. 3A), ACTH (Fig. 3B), and corticosterone (Fig. 3C) compared with WR rats that did not receive water at that time of day.
Experiment 3: Effect of Water Restriction-induced Drinking on HPA Responses to Acute Restraint Stress

The finding that drinking in WR rats decreased pituitary-adrenal hormones (Fig. 3) in parallel with decreases in Fos expression in VP neurons (Figs. 1 and 2) suggests that the inhibitory response to drinking is selective for VP neurons. To assess whether the inhibitory effect of rehydration could affect CRH neurons activated by an acute stress, WR rats underwent restraint stress to stimulate pituitary-adrenal hormones and Fos in CRH neurons and then the response to drinking was determined.

Experiment 3a: Effect of drinking on hormonal responses to restraint stress. In WR rats, restraint stress for 15 min increased plasma ACTH and corticosterone but decreased plasma VP without affecting plasma osmolality or hematocrit (WR vs. WR-Restraint; Table 3). After restraint, drinking for 15 min reduced plasma osmolality, VP, and ACTH but not corticosterone [WR/Restraint/Rehydrated vs. WR/Restraint/(-)Rehydrated; Table 3].

Experiment 3b: Effect of drinking on Fos responses to restraint stress. The time for sample collection was extended compared with experiment 1, since rats in some groups underwent restraint stress followed by drinking. To account for changes in Fos expression that could occur in WR rats over the 2-h collection period, brains were collected from rats that were killed at the time of entering the animal room (Pre group) and rats killed up to 2 h later (no restraint, no water, WR group). The percentage of Fos-positive VP neurons and CRH neurons in the PVN did not differ between these groups; thus, data from these groups were averaged and served as the WR group for all comparisons. Similar to the results of experiment 1, drinking for 15 min resulted in a decreased percentage of Fos-positive VP parvocellular and magnocellular neurons, but not CRH parvocellular neurons in the PVN (WR vs. WR/Rehydrated; Figs. 4 and 5). Restraint stress for 15 min did not change the percentage of Fos-positive VP neurons in WR rats (WR vs. WR/Restraint; Figs. 4 and 5), yet drinking after restraint reduced Fos in VP parvocellular and magnocellular neurons.
dependent on demonstrating elevated Fos expression in PVN to the changes in plasma ACTH and corticosterone observed. Of VP neurons in the PVN and that these VP neurons contribute to the changes in plasma ACTH and corticosterone observed. The ability to observe neuronal inhibition in this study was dependent on demonstrating elevated Fos expression in PVN neurons before drinking. Our finding that Fos was increased in magnocellular and parvocellular PVN neurons in water-restricted animals is consistent with previous studies showing that water deprivation-induced dehydration increases Fos expression in PVN and SON neurons compared with hydrated controls (15, 20, 29, 40, 43, 48). However, these studies did not assess the phenotype of PVN neurons expressing Fos. By double labeling for Fos and VP or Fos and CRH, our data show clearly that neural activation is limited to VP magnocellular and parvocellular neurons in the PVN. Water restriction did not affect the total number of VP-labeled magnocellular or parvo- cellular neurons but resulted in a small decrease in the total number of CRH-labeled neurons. This observation is consistent with previous work showing that chronic dehydration produced by water deprivation or salt loading results in decreased CRH expression reflected by reduced CRH mRNA (2, 34, 60, 64) and protein (31) in the mpPVN. Water restriction differs from these other models of dehydration in that animals are rehydrated daily, receiving water for 30 min each day over the course of 6 days. To better assess the severity of the osmotic stress, rats were sampled throughout the final day of water restriction. The elevation in plasma osmolality, hematocrit, and plasma VP observed from 0830 to 1630 on the 6th day of restriction indicates that signals associated with dehydration are activating neural pathways regulating water balance throughout the day before PM rehydration. In addition, plasma ACTH and corticosterone were elevated in WR rats at 1230 and 1430, respectively. Previous studies in which sampling was limited to early AM (21) and/or to the PM immediately before rehydration (21, 62) concluded that PM water restriction did not alter basal HPA activity. By sampling throughout the day, the present study shows that water restriction activates HPA activity during the early PM before the time of rehydration. It is not clear whether the increase in HPA activity results from osmotic stress that increases in intensity throughout the day before PM rehydration or reflects a shift in the diurnal rhythm. The increase in plasma ACTH at 1230 coincided with an increase in plasma osmolality in WR rats, suggesting that the pituitary response at this time of day may be because of osmotic stress. However, plasma corticosterone is not elevated in WR rats until 1430. Although there is no clear explanation for the temporal dissociation between plasma ACTH and corticosterone, increased corticosterone without a concomitant increase in ACTH could result from increased adrenal sensitivity to ACTH. Because increases in adrenal sensitivity have been observed previously after water deprivation (2, 54) and under nonstress conditions at the peak of the diurnal rhythm

\[ \text{DISCUSSION} \]

The rapid decreases in plasma VP and corticosterone that occur after dehydration-induced drinking have been well documented (19, 21, 26, 35, 49, 52, 62, 63). The present study was designed to test the hypothesis that drinking after a period of chronic water restriction inhibits the activity of hypothalamic magnocellular and specific subpopulations of parvocellular neurons in the PVN that control pituitary-adrenal activity. We have shown that water restriction for 6 days results in increased Fos expression in VP magnocellular and parvocellular neurons but not CRH parvocellular neurons. Because rehydration results in reduced Fos expression in the VP neurons, our results support the hypothesis that water restriction-induced drinking decreases pituitary-adrenal activity by inhibiting neural activity in VP magnocellular and parvocellular neurons. In addition, rehydration in water-restricted rats that have also undergone acute restraint stress reduces plasma ACTH and Fos in VP magnocellular and parvocellular neurons. Because restraint stress-induced increases in Fos in CRH neurons are not affected by rehydration, our data implicate inhibition of VP neurons in drinking-induced suppression of HPA responses to acute stress. These results suggest that during water restriction, repeated dehydration, and rehydration targets subpopulations of VP neurons in the PVN and that these VP neurons contribute to the changes in plasma ACTH and corticosterone observed.

The ability to observe neuronal inhibition in this study was dependent on demonstrating elevated Fos expression in PVN neurons before drinking. Our finding that Fos was increased in magnocellular and parvocellular PVN neurons in water-restricted animals is consistent with previous studies showing that water deprivation-induced dehydration increases Fos expression in PVN and SON neurons compared with hydrated controls (15, 20, 29, 40, 43, 48). However, these studies did not assess the phenotype of PVN neurons expressing Fos. By double labeling for Fos and VP or Fos and CRH, our data show clearly that neural activation is limited to VP magnocellular and parvocellular neurons in the PVN. Water restriction did not affect the total number of VP-labeled magnocellular or parvo- cellular neurons but resulted in a small decrease in the total number of CRH-labeled neurons. This observation is consistent with previous work showing that chronic dehydration produced by water deprivation or salt loading results in decreased CRH expression reflected by reduced CRH mRNA (2, 34, 60, 64) and protein (31) in the mpPVN. Water restriction differs from these other models of dehydration in that animals are rehydrated daily, receiving water for 30 min each day over the course of 6 days. To better assess the severity of the osmotic stress, rats were sampled throughout the final day of water restriction. The elevation in plasma osmolality, hematocrit, and plasma VP observed from 0830 to 1630 on the 6th day of restriction indicates that signals associated with dehydration are activating neural pathways regulating water balance throughout the day before PM rehydration. In addition, plasma ACTH and corticosterone were elevated in WR rats at 1230 and 1430, respectively. Previous studies in which sampling was limited to early AM (21) and/or to the PM immediately before rehydration (21, 62) concluded that PM water restriction did not alter basal HPA activity. By sampling throughout the day, the present study shows that water restriction activates HPA activity during the early PM before the time of rehydration. It is not clear whether the increase in HPA activity results from osmotic stress that increases in intensity throughout the day before PM rehydration or reflects a shift in the diurnal rhythm. The increase in plasma ACTH at 1230 coincided with an increase in plasma osmolality in WR rats, suggesting that the pituitary response at this time of day may be because of osmotic stress. However, plasma corticosterone is not elevated in WR rats until 1430. Although there is no clear explanation for the temporal dissociation between plasma ACTH and corticosterone, increased corticosterone without a concomitant increase in ACTH could result from increased adrenal sensitivity to ACTH. Because increases in adrenal sensitivity have been observed previously after water deprivation (2, 54) and under nonstress conditions at the peak of the diurnal rhythm

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**Table 1. No. of hypothalamic neurons staining for VP or CRH in each treatment condition in experiment 1: AL, WR, and WR/Rehydrated animals**

<table>
<thead>
<tr>
<th>Condition</th>
<th>AL</th>
<th>WR</th>
<th>WR/Rehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmPVN VP</td>
<td>79.4 ± 10.5</td>
<td>74.3 ± 2.9</td>
<td>83.9 ± 4.2</td>
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<tr>
<td>mpPVN VP</td>
<td>17.8 ± 3.9</td>
<td>16.8 ± 1.5</td>
<td>16.9 ± 0.7</td>
</tr>
<tr>
<td>mpPVN CRH</td>
<td>71.0 ± 9.9</td>
<td>53.6 ± 4.0*</td>
<td>74.9 ± 3.4</td>
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</tbody>
</table>

Values represent means ± SE. \( n = 4 \) rats/group. VP, vasopressin; CRH, corticotropin-releasing hormone; AL, water ad libitum; WR, water restricted; WR/Rehydrated, WR rats given water for 15 min and killed 60 min later; pm, posterior magnocellular; mp, medial parvocellular; PVN, paraventricular nucleus. *\( P < 0.05 \) vs. AL and WR/Rehydrated.
Table 2. Plasma osmolality and hematocrit in AL and WR rats during the last (6th) day of water restriction, before rehydration

<table>
<thead>
<tr>
<th>Time of Day</th>
<th>AL pOsm, mosmol/kgH2O</th>
<th>WR pOsm, mosmol/kgH2O</th>
<th>AL Hematocrit</th>
<th>WR Hematocrit</th>
</tr>
</thead>
<tbody>
<tr>
<td>0830</td>
<td>288.3 ± 1.0</td>
<td>296.7 ± 0.9*</td>
<td>38.6 ± 0.3</td>
<td>43.2 ± 0.6*</td>
</tr>
<tr>
<td>1030</td>
<td>290.2 ± 1.5</td>
<td>295.3 ± 0.5*</td>
<td>38.1 ± 0.6</td>
<td>44.8 ± 0.3*</td>
</tr>
<tr>
<td>1230</td>
<td>292.2 ± 1.1</td>
<td>302.0 ± 0.9*†</td>
<td>38.6 ± 0.5</td>
<td>43.0 ± 1.0*</td>
</tr>
<tr>
<td>1430</td>
<td>289.7 ± 1.5</td>
<td>300.3 ± 1.5*</td>
<td>37.4 ± 0.6</td>
<td>43.6 ± 1.0*</td>
</tr>
<tr>
<td>1630</td>
<td>287.8 ± 1.5</td>
<td>298.5 ± 1.3*</td>
<td>38.4 ± 0.8</td>
<td>42.8 ± 0.8*</td>
</tr>
</tbody>
</table>

Values represent means ± SE; n = 6 rats. pOsm, plasma osmolality. P < 0.05 vs. AL at the same time point (*) and vs. preceding time point (†).

Table 3. Plasma osmolality, hematocrit, and plasma hormones in 6-day WR, WR/Restraint, WR/Restraint/Rehydrated, and WR/Restraint/(−) Rehydrated rats

<table>
<thead>
<tr>
<th></th>
<th>WR</th>
<th>WR/Restraint</th>
<th>WR/Restraint/Rehydrated</th>
<th>WR/Restraint/(−) Rehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>pOsm</td>
<td>298.3 ± 0.5</td>
<td>297.2 ± 0.7</td>
<td>287.5 ± 1.6*</td>
<td>295 ± 1.8</td>
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<tr>
<td>Hematocrit</td>
<td>44.0 ± 0.7</td>
<td>44.6 ± 0.1</td>
<td>43.6 ± 0.4</td>
<td>43.2 ± 0.5</td>
</tr>
<tr>
<td>Plasma VP, pg/ml</td>
<td>2.8 ± 0.3</td>
<td>1.7 ± 0.2†</td>
<td>0.6 ± 0.2*†</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>Plasma ACTH, pg/ml</td>
<td>28.2±2</td>
<td>58 ± 7†</td>
<td>35 ± 3*</td>
<td>61 ± 13†</td>
</tr>
<tr>
<td>Plasma Corticosterone, ng/ml</td>
<td>195±59</td>
<td>480 ± 38†</td>
<td>418 ± 72†</td>
<td>575 ± 57†</td>
</tr>
</tbody>
</table>

Values represent means ± SE; n = 6 rats/group. WR, water-restricted rats before restraint stress; WR/Restraint, WR rats, after 15 min of restraint; WR/Restraint/Rehydrated, WR rats, after 15 min of restraint and 15 min of rehydration; WR/Restraint/(−) Rehydrated, WR rats after 15 min of restraint and 15 min without rehydration. P < 0.05 vs. WR (†) and vs. WR/Restraint/(−) Rehydrated (‡).
CRH to VP parvocellular neuronal activity. Chronic osmotic stress induced by 7-day salt loading increases VP mRNA and VP immunoreactivity in parvocellular neurons (3), but 48 h water deprivation results in no change in VP heteronuclear RNA and mRNA (22). Additionally, after 48 h water deprivation, PVN parvocellular neurons show reduced VP heteronuclear RNA and mRNA responses to acute heterotypical stress, suggesting that chronic osmotic stress decreases VP parvocellular neuronal activity (22). It is possible that the duration or intensity of osmotic stress dictates whether VP parvocellular neurons show an enhanced or a reduced response. With the use of Fos expression as an indicator of neuronal activity, our data suggest that water restriction results in upregulation of VP parvocellular neuronal activity to compensate for the reduction in CRH neuronal activity.

Decreases in plasma VP within minutes of dehydration-induced drinking have been well documented in several species, including humans (4, 26, 49, 52, 63). It is believed that drinking-induced decreases in plasma VP result from a decrease in magnocellular neuronal activity primarily because of signals associated with the act of drinking per se (4, 51, 52) and removal of stimulatory input from peripheral (7–9) and, sub-
individual neurons is not feasible, one would predict that the rapid suppression of firing in osmosensitive neurons after drinking (59) would be reflected by decreased expression of Fos in hypothalamic neurons activated by dehydration. By using decreases in Fos to infer decreases in neural activity, our data show that both VP magnocellular and parvocellular PVN neurons are inhibited by drinking.

These results are consistent with the hypothesis that rapid drinking-induced decreases in ACTH and corticosterone result from decreases in VP secretion from magnocellular and parvocellular neurons. Our previous finding that plasma corticosterone is reduced in water-restricted rats following peripheral administration of VP antibody (63) implicates both VP secreted systemically from magnocellular neurons and VP released in the median eminence from parvocellular neurons in the response. Magnocellular-derived VP could act directly on the adrenal to contribute to the plasma corticosterone response (63) or, with parvocellular-derived VP, be delivered to the median eminence to facilitate pituitary corticotroph secretion of ACTH (17). As discussed previously, changes in plasma corticosterone can occur without concomitant changes in ACTH (16, 61, 62); thus, extra-ACTH mechanisms most likely are required to reduce plasma corticosterone. In addition to putative direct adrenal effects of VP (63), increases in corticosterone clearance (16, 62) and changes in adrenal sensitivity to ACTH mediated by adrenal neural input (61) have been implicated in the response. It is likely that each of these factors acts in concert to rapidly reduce plasma corticosterone during restriction-induced drinking.

The rapid suppression of ACTH and corticosterone observed after administering nutrient to water- or food-restricted rats has led other investigators to assess whether HPA hormonal responses to acute stress can be inhibited by nutrient consumption. When water intake was restricted to a short period of time in the PM, the magnitude of the plasma corticosterone response to ether stress was diminished by drinking, although drinking did not affect the stress response in rats that were restricted to water intake in the AM (21). However, in response to handling stress, rats restricted to food and water intake in the AM showed a diminished ACTH and corticosterone response when administered nutrient (24). These experiments show that nutrient consumption in restricted rats can inhibit hormonal responses to acute stress, but they do not address potential hypothalamic mechanisms that mediate the response. The present experiments assessed whether water restriction-induced drinking would inhibit the HPA hormonal response and the Fos response of PVN neurons to restraint stress. Restraint stress was used to activate PVN neurons, since it has been shown to increase Fos expression and gene transcription in VP neurons.
and CRH parvocellular neurons in the PVN (12, 23, 27, 37, 57, 58). Consistent with previous results, acute restraint stress in WR rats increased plasma ACTH and corticosterone in parallel with increased Fos expression in CRH neurons (58). In contrast, neither plasma VP nor Fos expression in VP magnocellular neurons was increased by restraint stress, confirming previous work showing that magnocellular VP neurons are not responsive to restraint stress in hydrated (1) or dehydrated (22) rats. Drinking for 15 min after restraint was effective in decreasing plasma ACTH and VP, but not corticosterone; a more prolonged sampling period after drinking may be required to observe decreased corticosterone in response to the change in ACTH. Although rehydration reduced the ACTH response to restraint stress, the Fos response in CRH neurons was not affected. In contrast, although restraint stress did not increase Fos expression in VP parvocellular or magnocellular neurons above that which was induced by water restriction alone, Fos expression in both populations of VP neurons and plasma VP was reduced by drinking. Thus it is possible that decreases in VP secretion from parvocellular or magnocellular neurons contribute to the rehydration-induced inhibition of the ACTH response to restraint stress. Evaluation of the effects of drinking on the HPA response to other forms of stress may determine if this hypothesis is valid.

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REFERENCES

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