Excitatory amino acids in rostral ventrolateral medulla support blood pressure during water deprivation in rats

Virginia L. Brooks, Korrina L. Freeman, and Kathy A. Clow
Department of Physiology and Pharmacology, Oregon Health & Science University, Portland, Oregon 97239

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METHODS

Animals: All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional (Oregon Health and Science University) Animal Care and Use Committee. Experiments were performed with male Sprague-Dawley rats (Sasco, Wilmington, MA) weighing ~250–350 g. All rats were housed in the institutional facility a minimum of 5 days before experimentation. Rats were grouped one to three in a room with a 12:12-h light-dark cycle. All rats had free access to food (Purina 5001). Water-deprived rats were housed singly for either 24 or 48 h without water.

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**Surgery.** Anesthesia was induced with 5% isoflurane in 100% oxygen. A trachea tube was inserted so that the animals could be artificially ventilated, and a surgical plane of anesthesia was maintained with 2% isoflurane in 100% oxygen. Body temperature was maintained throughout the surgery and experiment at 37 ± 1°C with a rectal thermistor and a heating pad.

Femoral arterial and venous catheters were implanted for arterial pressure measurements and infusions, respectively. The rats were then positioned in a stereotaxic device (David Kopf, Tujunga, CA), and a midline incision was made on the back of the head to expose the dorsal surface of the medulla and remove the atlantooccipital membrane by a limited craniotomy. After completion of all surgical manipulations, a blood sample (400 μl) was collected for measurement of plasma osmolality, plasma protein concentration, hematocrit, and plasma concentrations of sodium and chloride. An intravenous infusion of urethane (1.2 g/kg in 1 ml of saline) was then administered over ~30 min; 10 min after the urethane infusion began the gas anesthetic was slowly withdrawn, but the rats were continually artificially ventilated with 100% oxygen throughout the experiment. After completion of the urethane infusion, the rats were allowed to stabilize for ~30–60 min before experimentation. The depth of anesthesia was periodically assessed by confirming the lack of response to tail or paw pinch. Additional urethane (0.2 g/kg) was occasionally administered intravenously when needed.

**RVLM microinjections.** Functional identification of the RVLM was made by observing >20 mmHg pressor responses to L-glutamate (1 nmol/100 nl; Refs. 18, 19, 30). Single-barreled glass micropipettes (20- to 40-μm tip diameter) containing glutamate were positioned as described by Kiely and Gordon (19). Briefly, the tip of the rat’s nose was pushed down until the calamus scriptorius was 2.4 mm posterior to the interaural line. With calamus scriptorius as zero, injections were made 0.5–1.0 mm anterior, 1.8–2.0 mm (usually 1.9) lateral, and 2.7–3.8 mm ventral to the dorsal surface of the medulla. Injections (100 nl) were made over ~3–7 s with either a 1-μl Hamilton syringe held in a micromanipulator or a PicoPump (WPI); the successful microinjection of drugs was verified by watching, through a microscope reticule, the movement of a small bubble in the injection tubing a distance calibrated to be 50 or 100 nl. No more than three penetrations were made per side before the experiment commenced. All drugs [except Kyn, which was first dissolved in 1 part 100 mM sodium bicarbonate and then diluted with 9 parts artificial cerebrospinal fluid (αCSF)] were dissolved in αCSF containing (in mM) 128 NaCl, 2.6 KCl, 1.3 CaCl2, 0.9 MgCl2, 20 NaCO3, and 1.3 Na2HPO4. The pH of all solutions was corrected to 7.4. Before the pipette was filled with a new drug, it was flushed with several volumes of αCSF and then several volumes of the new drug.

At the conclusion of the experiment, ~50 nl of 2.5% Alcian blue in 0.5 M sodium acetate was injected into the RVLM with the same pipette and coordinates used for injections. The brain was removed and placed in 4% formaldehyde in phosphate-buffered saline for at least 48 h. The brain stem was subsequently cut into 50-μm sections with a cryostat; sections were mounted on glass microscope slides and counterstained with neutral red. RVLM injection sites were verified against those previously published, within an area ~500 μm caudal to the caudal end of the facial nucleus and ventral to nucleus ambiguus (Fig. 1).

**Protocols.** After the RVLM was identified functionally, ~30 min was allowed for stabilization and collection of baseline data and then one of the following protocols was performed. 1) To determine whether the EAA drive of the RVLM is increased during water deprivation, bilateral microinjections of Kyn (2.7 nmol in 100 nl) or αCSF were given into the RVLM of water-replete and water-deprived (24 and 48 h) rats. After the first microinjection was completed, the pipette was removed and positioned on the other side and the second injection was made. Both injections were usually completed within 1 min. Some rats were pretreated with an intravenous injection of the vasopressin V1 antagonist [Manning compound: d(CH2)5 1, Tyr(Me)2, Arg8-vasopressin; 5 μg in 100 μl] at least 15 min before injection of Kyn. The responses of animals pretreated with the V1 antagonist were not significantly different from those of untreated rats, so the data were combined. 2) To determine whether the increased EAA drive exhibited by water-deprived rats is due to increased sensitivity of the RVLM to glutamate, varying doses of glutamate (0.1, 0.5, 1, and 5 nmol) were unilaterally microinjected into the RVLM of water-replete and 48-h water-deprived rats. Different doses were administered in random order with the same pipette, but the pipette was removed and refilled with a different concentration of glutamate for each injection. At least 5 min was allowed between injections. While the anterior-posterior and medial-lateral positions were fixed, a total of three or four repetitions of the dose were given as the pipette was lowered in 0.2-mm increments in the region bracketing the functionally identified site. The largest response in each case was chosen for data analysis. 3) To determine whether changes in the glutamate pressor response were due to changes in vascular sensitivity to adrenergic agonists, varying doses of phenylephrine were administered intravenously as a bolus in water-replete and 48-h water-deprived rats. Rats were studied either intact or after intravenous administration of the V1 vasopressin antagonist followed by the ganglionic blocker hexamethonium (30 mg/kg) intravenously when needed.

**Fig. 1.** Coronal section through rat medulla illustrating sites of drug microinjections into the rostral ventrolateral medulla (RVLM) in water-replete, 24-h water-deprived (WD), and 48-h WD rats. Section is ~11.8 mm from bregma; all injections were within ±200 μm from this section.
mg/kg). Doses of phenylephrine (1, 3, 6, 10, and 20 μg/kg) were given in random order; different doses were given after a minimum 5-min interval or after baseline values were achieved. 4 To determine whether water deprivation is also associated with decreased responsiveness of the RVLM to inhibitory neurotransmitters, water-replete and water-deprived (24 and 48 h) rats received unilateral microinjections of GABA (1 and 10 nmol); injections were administered randomly, in duplicate (results were averaged), 15–20 min apart. 5 To determine whether reduced inhibitory input from baroreceptors increases the sensitivity of the RVLM to glutamate, rats underwent cervical vagotomy (n = 4) or vagotomy-sinoaortic denervation (SAD; n = 4) as previously described (14). One rat received SAD alone. Results were similar to those after vagotomy-SAD; therefore, they were combined. After catheterization, but before the deafferentation, the rats were given an intravenous bolus of the V1 antagonist to minimize the hypertension caused by the procedure. Rats were then prepared for RVLM microinjections as described above, and responses to glutamate (1 nmol in 100 nl) were determined.

Chemicals. Kyn, glutamate, hexamethonium, and phenylephrine were all obtained from Sigma, and the V1 antagonist was obtained from Bachem.

Data analysis. Between-group (water replete, 24 h water deprived, 48 h water deprived) differences were determined with ANOVA and the post hoc Bonferroni correction. Differences in changes in arterial pressure with time or in dose-response relationships between phenylephrine and arterial pressure were assessed with two-way ANOVA for repeated measures. Finally, linear least-squares regression analysis was used to determine whether a relationship exists between plasma osmolality and the depressor response to Kyn.

RESULTS

Basal values. Water deprivation increased plasma sodium and chloride concentrations (Table 1) and plasma osmolality (Fig. 2). The rats were also volume depleted, as indicated by increases in hematocrit and plasma protein concentration (Table 1). Nevertheless, mean arterial pressure was not different between groups (Table 1).

Responses to Kyn. Bilateral microinjections of Kyn did not alter arterial pressure in water-replete rats but decreased arterial pressure in 48-h water-deprived rats (Figs. 2 and 3). The depressor response to Kyn in 24-h water-deprived rats was variable (range −2 to −20 mmHg), not significantly different from water-replete animals (Fig. 2), and smaller than the response in 48-h water-deprived rats (P < 0.05). However, when the data from all groups were analyzed together, the decrease in pressure was highly correlated with the plasma osmolality (Fig. 2, inset; P = 0.003; r² = 0.47).

Responses to glutamate. Dose-dependent pressor responses to unilateral glutamate microinjection were observed in both water-replete and water-deprived rats (P < 0.001, ANOVA), but the responses of water-deprived rats were larger (Fig. 4). However, the pressor responses to bolus injections of phenylephrine were smaller in water-deprived rats (Fig. 5A). To assess the pressor effect of phenylephrine in the absence of baroreflexes and vasopressin, a second group of animals was studied after treatment with a V1 antagonist and ganglionic blockade. The V1 antagonist decreased arterial pressure more (P < 0.005) in water-deprived (−20 ± 2 mmHg; n = 6) compared with water replete (−6 ± 3 mmHg; n = 7) rats. However, subsequent hexamethonium injection produced similar depressor responses (−54 ± 9 mmHg, replete; −52 ± 6 mmHg, deprived; P > 0.5). As in intact rats, the pressor responses to phenylephrine were smaller in V1 antagonist- and hexamethonium-pretreated water-deprived rats (Fig. 5B).

Responses to GABA. GABA injections dose-dependently decreased arterial pressure (P < 0.001, ANOVA; Fig. 6). The

Table 1. Basal values

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<th>Water Replete</th>
<th>24-h Water Deprived</th>
<th>48-h Water Deprived</th>
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<tr>
<td>Basal MAP, mmHg</td>
<td>104.7±3.3 (28)</td>
<td>115.7±6.3 (7)</td>
<td>104.9±3.1 (27)</td>
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<td>Na⁺, meq/l</td>
<td>136.2±0.4 (28)</td>
<td>142.2±1.8* (5)</td>
<td>143.1±0.5* (25)</td>
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<td>Cl⁻, meq/l</td>
<td>104.2±0.3 (25)</td>
<td>108.1±1.9* (5)</td>
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<tr>
<td>Hematocrit, %</td>
<td>44.5±0.40 (27)</td>
<td>46.8±0.5* (11)</td>
<td>51.1±0.4* (24)</td>
</tr>
<tr>
<td>Protein, g/dl</td>
<td>6.2±0.1 (28)</td>
<td>6.4±0.1 (11)</td>
<td>7.3±0.1* (23)</td>
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Values are means ± SE for no. of rats in parentheses. MAP, mean arterial pressure. *P < 0.05 compared with water replete by ANOVA post hoc Bonferroni correction.

Fig. 2. Change in mean arterial pressure (ΔMAP) in response to bilateral kynurenate (Kyn) microinjection into the RVLM (top) and plasma osmolality (bottom) in water-replete [WR; open bars (n = 8); control MAP 108 ± 4 mmHg], 24-h WD [hatched bars (n = 6); control MAP 104 ± 8 mmHg], and 48-h WD [filled bar (n = 7); control MAP 107 ± 9 mmHg] rats. Water deprivation increased osmolality and change in MAP (P < 0.001, ANOVA for both). Microinjection of artificial cerebrospinal fluid (aCSF; gray bars; n = 5–6) did not significantly alter MAP. Inset: relationship between ΔMAP (mmHg) and the basal osmolality (Osm, mosmol/kgH₂O) for individual WR (open circles), 24 WD (half-filled circles), and 48 WD (filled circles) rats. *P < 0.05 compared with WR rats, †P < 0.05 compared with 24 WD rats by Bonferroni correction.
Depressor responses were generally larger in water-deprived rats, although there was no difference between 24-h and 48-h water-deprived animals.

Responses to glutamate after baroreceptor deafferentation. Unilateral glutamate microinjection (1 nmol) into the RVLM increased arterial pressure more (P < 0.001, ANOVA, group and interaction) in rats after vagotomy (25 ± 2 mmHg, n = 4) or vagotomy-SAD (27 ± 1 mmHg, n = 5) than in intact rats (22 ± 1 mmHg, n = 10). Nonetheless, it should be noted that these values may underestimate the differences between intact and deafferented animals, because the latter values were obtained on the first injection (e.g., response >20 mmHg) without a systematic search for the most responsive site.

DISCUSSION

The major new findings of the present study are that 1) blockade of EAA receptors in the RVLM by bilateral microinjection of Kyn decreases arterial blood pressure in water-deprived, but not water-replete, rats and 2) the pressor responses evoked by unilateral injection of increasing doses of glutamate are greater in water-deprived than water-replete rats. Collectively, these data suggest that water deprivation is associated with increased EAA drive of RVLM sympathetic premotor neurons, possibly due in part to increased sensitivity of the RVLM to EAA such as glutamate.

Water deprivation causes a decrease in blood volume, as indicated indirectly in the present study by increases in hematocrit and plasma protein concentration. The volume contraction does not lead to hypotension, however, because of homeostatic activation of neurohumoral mechanisms. For example, both vasopressin and angiotensin II levels increase (see, e.g., Ref. 2) and data in the present study confirm that the increased vasopressin levels contribute to pressure maintenance. Although indirect measures of overall sympathetic tone, such as plasma catecholamines (9, 20, 32) or the depressor response to ganglionic blockade (Ref. 11 and present study), are not significantly increased, there is evidence that regional increases in sympathetic activity occur. First, lumbar sympathetic nerve activity (as a function of reflex-induced maximum) is increased (25) and heart rate is either increased or unchanged (2, 11, 25, 26).

Furthermore, the adrenal mRNA levels for tyrosine hydroxylase, the rate-limiting enzyme for norepinephrine production known to be increased by sustained increases in sympathetic activity, are elevated by water deprivation, and this effect is blocked by adrenal denervation (3). On the other hand, renal sympathetic activity (as a percentage of baroreflex maximum) is unchanged (26).

Thus basal sympathetic activity to some vascular beds is increased, but the mechanisms of altered brain regulation of sympathetic tone have not been previously investigated. The present study tested the hypothesis that increased EAA input to the RVLM may contribute to regional increases in sympathetic tone, and the finding that bilateral microinjection of Kyn into...
the RVLM lowers arterial pressure in 48-h water-deprived, but not water-replete, rats supports this hypothesis. Interestingly, Kyn did not produce a consistent or statistically significant depressor response in 24-h water-deprived rats, despite the existence of elevated sodium and chloride levels and volume contraction. Thus it may be that a larger or more prolonged stimulus is required before brain regulation of sympathetic tone is significantly altered. In support of this, we found that the magnitude of the depressor response to Kyn is correlated with the basal level of osmolality. Furthermore, a previous study investigating brain regions activated during water deprivation, as assessed by expression of the inducible transcription factor c-Fos, indicates that activation of some sites, such as the subfornical organ, is greater after 48 h than after 24 h of water deprivation (22).

The nature of the EAA input to the RVLM is complex and incompletely understood. As observed in the present and previous studies, in normal rats, bilateral blockade of EAA receptors generally does not significantly alter arterial pressure (for reviews, see Refs. 6, 27). One interpretation of this finding is that the EAA drive is zero in unstressed rats. However, Lipski et al. (21) and Ito and Sved (18) have offered an alternative explanation, that EAA excitatory input is offset by EAA drive of inhibitory input. In support of this model, Ito and Sved (18) showed that, after blockade of the inhibitory influence from the CVLM or the NTS, injection of Kyn into the RVLM produces a profound decrease in arterial pressure. Moreover, they propose that the EAA drive of the RVLM can increase if the normal balance of inhibition and excitation shifts toward excitation. Increased excitation could occur secondary to increased excitatory EAA input or increased sensitivity of the RVLM to EAA. Our test of the latter possibility indicated that water deprivation increases the responsiveness of the RVLM to glutamate injections. Further experiments demonstrated that the increased pressor response was not due to enhanced vascular reactivity to α-adrenergic agonists, because these responses were reduced. Decreased baroreflex sensitivity also cannot explain the larger glutamate pressor responses, because baroreflex gain appears to be increased during water deprivation (2, 24) and reduced phenylephrine pressor responses were also observed in ganglionic blocked water-deprived rats, which effectively removes the baroreflexes. Thus we conclude that the greater falls in pressure in water-deprived rats after Kyn microinjection suggest an increased EAA drive of RVLM neurons, due in part to increased sensitivity of the RVLM to EAA.

Increased net EAA input to the RVLM induced by water deprivation could also be secondary to reduced inhibitory influences on RVLM neurons. However, the present results reveal that GABA elicits a larger decrease in blood pressure in water-deprived rats, indicating that the increased EAA excitatory drive is not due to decreased responsiveness of the RVLM to GABA.

Fig. 5. Effect of bolus intravenous phenylephrine injections on MAP in intact (A) and V1 antagonist-pretreated/hexamethonium-pretreated (B) WR and WD rats. MAP (in mmHg) just before injections (phenylephrine doses in μg/kg in parentheses) in intact WR rats was 103 ± 3 (1), 108 ± 2 (3), 110 ± 3 (6), 104 ± 2 (10), and 102 ± 2 (20), and in intact WD rats was 109 ± 8 (1), 111 ± 6 (3), 110 ± 6 (6), 111 ± 7 (10), and 107 ± 6 (20). MAP (in mmHg) just before injections (phenylephrine doses in parentheses) in rats pretreated with the V1 antagonist and hexamethonium was 51 ± 3 (1), 47 ± 4 (3), 50 ± 3 (6), 50 ± 3 (10), and 54 ± 4 (20) and in WD rats was 41 ± 2 (1), 36 ± 2 (3), 38 ± 2 (6), 40 ± 2 (10), and 41 ± 2 (20). Pressor responses were smaller in both untreated and pretreated WD rats (P < 0.01, ANOVA, dose, group, and interaction).

Fig. 6. Effect of unilateral microinjection of GABA on MAP in WR [control MAP 104 ± 7 (1 nmol) and 106 ± 6 (10 nmol) mmHg], 24 WD [control MAP 105 ± 7 (1 nmol) and 103 ± 5 (10 nmol) mmHg] and 48 WD [control MAP 88 ± 7 (1 nmol) and 92 ± 6 (10 nmol) mmHg] rats. *P < 0.05 compared with WR by Bonferroni correction.
The mechanism by which water deprivation increases net EAA excitatory input to the RVLM or sensitivity of the RVLM to glutamate was not directly investigated, but two possibilities can be considered. First, it may be secondary to the increased osmolality. Indeed, we observed a strong relationship between basal osmolality and the depressor response to Kyn (Fig. 2). Increased glutamate pressor responses have also been observed in normal Sprague-Dawley and Dahl salt-resistant rats placed on a high-salt diet (15, 17, 23), and both water deprivation and a high-salt diet (8, 13) increase body fluid osmolality. In conscious water-deprived rats, acute normalization of the elevated plasma osmolality decreases lumbar sympathetic nerve activity (25), suggesting that the hypertonicity contributes to sympathetic tone. The brain neurocircuitry by which increased osmolality activates the sympathetic nervous system has not been completely delineated (31). However, because acute increases in osmolality activate PVN neurons that project to the RVLM (31), the hypertonicity may contribute to the increased EAA drive of the RVLM observed in the present studies.

Second, water deprivation decreases blood volume and therefore presumably reduces inhibitory baroreceptor influences in the RVLM. The present experiments demonstrate that RVLM responsiveness to glutamate increases after decreases in baroreceptor input following acute deafferentation, similar to the increased responses reported after blockade of the CVLM (18) and similar to the enhanced glutamate pressor responses observed in water-deprived rats. The enhanced pressor responses could occur secondary to reduced convergent GABAergic inhibition, and they suggest that the increased EAA drive exhibited by water-deprived rats could be produced without a change in EAA input. Alternatively, the volume depletion, when sensed by the renal baroreceptor, could lead to increases in renin secretion and elevated plasma levels of angiotensin II, which could act on the brain to ultimately increase EAA input to the RVLM (10). Thus these findings are also consistent with the possibility that the volume depletion, via reduced baroreceptor input or increased circulating angiotensin II levels, also contributes to the increased responsiveness to glutamate and the increased EAA drive in the RVLM.

In summary, the present data indicate that during water deprivation, arterial pressure is supported by increased EAA drive of the RVLM. Because the pressor responsiveness of the RVLM to glutamate is also increased, the increased EAA drive may be due in part to enhanced sensitivity to EAA.

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