Sodium depletion activates the aldosterone-sensitive neurons in the NTS independently of thirst

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Geerling JC, Loewy AD. Sodium depletion activates the aldosterone-sensitive neurons in the NTS independently of thirst. Am J Physiol Regul Integr Comp Physiol 292: R1338–R1348, 2007. First published October 26, 2006; doi:10.1152/ajpregu.00391.2006.—Thirst and sodium appetite are both critical for restoring blood volume. Because these two behavioral drives can arise under similar physiological conditions, some of the brain sensory sites that stimulate thirst may also drive sodium appetite. However, the physiological and temporal dynamics of these two appetites exhibit clear differences, suggesting that they involve separate brain circuits. Unlike thirst-associated sensory neurons in the hypothalamus, the 11β-hydroxysteroid dehydrogenase type 2 (HSD2) neurons in the rat nucleus tractus solitarius (NTS) are activated in close association with sodium appetite (16). Here, we tested whether the HSD2 neurons are also activated in response to either of the two physiological stimuli for thirst: hyperosmolarity and hypovolemia. Hyperosmolarity, produced by intraperitoneal injection of hypertonic saline, stimulated a large increase in water intake and a substantial increase in immunoreactivity for the neuronal activity marker c-Fos within the medial NTS, but not in the HSD2 neurons. Hypovolemia, produced by subcutaneous injection of hyperoncotic polyethylene glycol (PEG), stimulated an increase in water intake within 1–4 h without elevating c-Fos expression in the HSD2 neurons. The HSD2 neurons were, however, activated by prolonged hypovolemia, which also stimulated sodium appetite. Twelve hours after PEG was injected in rats that had been sodium deprived for 4 days, the HSD2 neurons showed a consistent increase in c-Fos immunoreactivity. In summary, the HSD2 neurons are activated specifically in association with sodium appetite and appear not to function in thirst.

*Salt appetite; fluid ingestion; hypovolemia; mineralocorticoid; nucleus of the solitary tract*

FLUID INGESTION IS AN IMPORTANT component of the homeostatic response to disturbances in hydromineral balance. The behavioral drive to ingest fluid is stimulated in response to hyperosmolarity and hypovolemia (29, 31, 53). Experimentally, hyperosmolarity can be produced by administering a hypertonic solution of an active osmolyte like sodium, which draws water out of cells by osmosis. The resultant cell shrinking is sensed by central and peripheral osmoreceptors, which ultimately promote urinary water retention (antidiuresis) and thirst via neuroendocrine and behavioral output centers in the hypothalamus (29, 31, 54).

Experimental hypovolemia can be produced (with a minimal change in osmolar pressure) by subcutaneous injection of a colloidal solution of polyethylene glycol (PEG), which draws isotonic blood plasma out from the intravascular space via oncotic pressure (11, 48). The hypovolemia produced by this technique is free of confounds like anemia or acute hyponatremia, which occur after other methods, such as hemorrhage or peritoneal dialysis. Hypovolemia progressively stimulates antidiuresis and thirst via incompletely understood brain sensory pathways (44, 45).

Also, under certain conditions, prolonged hypovolemia stimulates sodium appetite many hours after the initial increase in thirst (52). The exact peripheral stimuli and brain mechanisms responsible for this delayed increase in sodium appetite remain poorly understood. In the absence of experimental evidence for critical sodium appetite-specific stimulatory mechanisms, it has been proposed that thirst and sodium appetite are driven by the same basic stimuli (particularly angiotensin II) and that their separate temporal dynamics result from specialized inhibitory mechanisms that block sodium appetite until water is ingested (52). Candidate inhibitory mechanisms have been proposed, including a central oxytocinergic influence from the hypothalamus (36).

Nonetheless, it remains possible that sodium appetite is stimulated by sensory mechanisms separate from those that increase thirst. Consistent with this possibility, various pharmacological and physiological manipulations can selectively increase the ingestion of salt. For example, a selective appetite for sodium arises after high-dose mineralocorticoid treatment (38) or chronic dietary sodium deprivation (7).

We have identified a group of brain stem neurons that are activated in close association with sodium appetite. These neurons, located in the nucleus tractus solitarius (NTS), express 11β-hydroxysteroid dehydrogenase type 2 (HSD2), which makes them uniquely sensitive to mineralocorticoids via intracellular inactivation of glucocorticoids (16, 17). The HSD2 neurons are activated in parallel with sodium appetite after high-dose mineralocorticoid administration and chronic dietary sodium deprivation and are then rapidly inactivated once salt is ingested (16, 20). Rather than innervating the expected autonomic reflex sites in the caudal brainstem, these neurons were found to project rostrally, to pontine and forebrain sites previously implicated in salt intake (18, 19).

Despite their unprecedented functional and anatomical links to sodium appetite, it remains possible that these neurons play a more general role in fluid ingestive behavior. Therefore, in the present study, we tested the possibility that the HSD2 neurons are activated not only in association with sodium appetite but also during hyperosmolar or hypovolemic thirst.
MATERIALS AND METHODS

All experimental protocols were approved by the Washington University School of Medicine Institutional Animal Care and Use Committee and conformed to the National Institutes of Health guidelines.

Fluid measurements and dietary manipulations. For every experiment, individually caged Sprague-Dawley rats (n = 113, 250–400 g, male; Harlan, Indianapolis, IN) were provided separate graduated drinking tubes containing distilled water (dH2O) and, in various PEG hypovolemia experiments, a 2 or 3% solution of NaCl. On injection of hypertonic saline or PEG (below), food was removed, and subsequent fluid intake volumes were recorded. All rats were killed by perfusion after each experiment (described below).

Before injection into individual cages for each experiment, group-housed rats (2–3/cage) were provided ad libitum access to tap water and normal rat chow (0.3% sodium, Picolab rodent diet no. 20; LabDiet, Richmond, IN). For some PEG hypovolemia experiments, rats were deprived of dietary sodium for 4 days before injection. Rats in these groups were caged individually and provided ad libitum access to dH2O and a low-sodium chow (~0.01% Na, no. 85292; Harlan-Teklad, Madison, WI) during the 4 days before PEG injection.

Hypertonic saline injection. To produce intracellular dehydration, rats (n = 9) were injected intraperitoneally (ip) with 2 ml of 12% NaCl (~2 M), prepared in deionized H2O. Controls (n = 10) were injected with an identical volume of sterile isotonic NaCl (0.9%, ~0.15 M). On injection, food was removed from all cages, and a graduated drinking tube containing dH2O was provided to subsets of these rats (n = 4 hypertonic vs. n = 5 isotonic). Their fluid intake volumes were recorded at 15, 30, 60, and 120 min after injection. The remaining 10 rats (n = 5 hypertonic vs. n = 5 isotonic) were not provided any fluids and were perfused at 2 h postinjection for c-Fos immunohistochemical analysis (see below). A pilot experiment with a larger injected volume of hypertonic saline (5-ml ip injections of the same solution: 2 M NaCl, n = 5; 0.15 M NaCl, n = 5) produced results qualitatively identical to those reported here, although c-Fos activation in the medial NTS (exclusive of the HSD2 neurons) tended to be even greater after the larger sodium load.

PEG injections. Hypovolemia was produced by injecting a colloidal solution of 10, 15, 20, or 30% PEG (mol wt 15,000–20,000, no. P2263; Sigma, St. Louis, MO) prepared in sterile isotonic saline. Using an 18-gauge needle, a volume of 5.5 ml (warmed to body temperature) was injected into the interscapular subcutaneous space in rats anesthetized with halothane (3.5%). The entire length of the needle (1.5 cm) was inserted into the subcutaneous space before injection, and the PEG solution was then massaged rostrally as the needle was withdrawn to minimize any back leakage. The same protocol was followed for injection of vehicle solution (5.5 ml of sterile isotonic saline, warmed to body temperature).

Various PEG hypovolemia paradigms stimulate varying degrees of thirst or salt appetite (50), so we first ran a series of pilot experiments to determine the optimal experimental conditions for producing the intended changes in fluid ingestion and to determine which conditions, if any, might produce c-Fos activation of the HSD2 neurons. Only 30% PEG was used in these pilot experiments (later groups were given 10, 15, 20, or 30% PEG injections).

In these pilot experiments, four different conditions were tested: 1 and 2) 4-h postinjection survival, with or without 4 days of prior dietary sodium deprivation, and 3 and 4) 12-h postinjection survival, with or without 4 days of prior dietary sodium deprivation. Within each of these four conditions, three separate groups of rats (n = 4 each) were tested: 1) 30% PEG injection, no drinking fluids available; 2) 30% PEG, dH2O and 2% NaCl (~0.3 M) continually available; and 3) vehicle injection, dH2O and 2% NaCl continually available. For all 4-h groups, injections were made in the early morning (7–8 AM), and rats were perfused just before noon. For all 12-h groups, injections were made in the evening (7–9 PM), and rats were perfused the following morning (7–10 AM). Blood samples were not taken in any of these pilot tests, which were used merely to assess the general parameters of fluid ingestion and HSD2 neuron c-Fos activation under our laboratory protocols, which included a different strain of rats than those used in previous studies involving PEG hypovolemia as a stimulus for sodium appetite (Wistar rats and Zivic-Miller Sprague-Dawley rats; see Refs. 50, 52).

In these pilot experiments, consistent c-Fos activation in the HSD2 neurons was found only in groups deprived of dietary sodium during for 4 days before PEG injection, a manipulation reported to increase and accelerate sodium appetite in response to PEG hypovolemia (50). Four-day dietary sodium deprivation alone has been found to produce small increases in sodium appetite (26) and HSD2 neuronal activation (16).

Subsequent experimental groups were sodium deprived for 4 days and injected with either vehicle or PEG to test for an effect specific to the added hypovolemic stimulus. In these rats, which were perfused 12 h after injection, blood samples were drawn (described below) for determination of the plasma concentrations of total protein (as an index for relative differences in blood volume), sodium, and potassium. dH2O was provided during the 12-h postinjection period, because pilot rats that had drunk water tended to show greater HSD2 neuronal activation than rats without fluid access. No saline was provided, because the main parameter of interest in this experiment, HSD2 neuronal activation, is reduced by saline ingestion (16). In addition to two groups injected with 30% PEG (n = 13; two separate groups of 7 and 6) and one injected with vehicle (n = 6), these experiments were repeated in two additional groups receiving injections of 10% PEG (n = 7) and 20% PEG (n = 7) using the same experimental conditions.

Finally, additional groups (also 4-day sodium deprived) were given injections of 30% PEG (n = 12), 15% PEG (n = 4), or vehicle (n = 5) to test the effects of this PEG hypovolemia protocol on sodium appetite. Six of the rats injected with 30% PEG were given a saline intake test (2-h access to a tube of 3% NaCl and a tube of dH2O following 12-h postinjection access to only dH2O), while the other six (perfused without saline access) were used as positive controls for comparison with previous PEG-injected groups. Blood plasma and c-Fos and HSD2 results from these six positive control cases were included for analysis along with the previous cases injected with 30% PEG. All rats from the 15% PEG-injected and vehicle-injected groups were given a saline intake test.

Perfusions, histology, and blood samples. At the end of every experiment, each rat was anesthetized with pentobarbitial sodium (50 mg/kg ip) and perfused through the ascending aorta with 200 ml of isotonic saline, followed by 500 ml of paraformaldehyde (4% solution, prepared in 0.1 M sodium phosphate buffer, pH 7.4). Double-immunofluorescence staining was performed on a one-in-five series of frozen sections through the caudal NTS (9 transverse sections, 50-μm thick), using a sheep antibody raised against HSD2 (1:40,000, Chemicon, Temecula, CA; see Refs. 17 and 22), combined with a rabbit antibody that specifically labels c-Fos (1:10,000; c-Fos “Ab-5” from Oncogene, Cambridge, MA). In most cases, single-label immunohistochemistry for c-Fos was also performed in an adjacent one-in-five series of sections, using the ABC-DAB histochemical technique (for immunohistochemical protocols, see Ref. 17). In some experiments, another adjacent series of one-in-five sections was double stained for c-Fos along with the catecholamine synthetic enzyme tyrosine hydroxylase (TH), using an anti-TH serum raised in sheep (AB1542, Chemicon; diluted 1:1,000). TH immunofluorescence was found only within the well-established distributions of catecholaminergic neurons in the dorsomedial and ventrolateral medulla (A1 and A2 noradrenergic groups, area postrema, and C1, C2, and C2d adrenergic groups).

In experiments involving PEG injection after 4-day sodium deprivation, blood samples were drawn immediately before perfusion. To prevent sample clotting, heparin was first infused (50 U in 0.5 ml; Abbott, Chicago, IL) via a femoral catheter inserted after the rat was...
anesthetized with pentobarbital sodium. Then, a 3- to 4-ml blood sample was drawn from the left ventricle of the heart, followed immediately by perfusion as described above. Blood plasma was immediately separated by centrifugation (5,000 rpm, 5 min) and sent for determination of the plasma concentrations of total protein, sodium, and potassium (all performed in the clinical laboratories at Washington University/Barnes-Jewish Hospital, St. Louis, MO). Because of the time required to draw blood and perfuse each rat, the postinjection survival times of rats in “12-h” groups were scattered between 12 and as much as 14 h postinjection, but cases with longer survival times did not exhibit any consistent difference in any of the parameters measured in these studies.

Cardiorespiratory failure under anesthesia occurred in one rat in the 10% PEG-injected group during catheterization for heparin infusion, just before blood sampling and perfusion, and this case was discarded. Blood samples from two other rats (one each from the 20 and 30% PEG-injected groups) showed evidence of severe hemolysis (red plasma) and were excluded from analysis because of the confounding influence of lysed red blood cell contents on plasma measurements.

**Data analysis.** In each case, the number of HSD2 neurons containing nuclear c-Fos immunoreactivity was counted and divided by the total number of HSD2 neurons counted. This quotient provided a percent estimate of neuronal activation within this NTS subpopulation. Between 141 and 222 HSD2 neurons were counted in each case (mean 173.3 ± 2.5, n = 113), always found in an identical distribution within the NTS (as described in Ref. 17), spanning 6–7 transverse sections (in a series of 9 rostrocaudal sections from each case). Although circumstances involving chronic changes in mineralocorticoid levels (such as chronic sodium deprivation, adrenalectomy, or daily mineralocorticoid injections) can influence the intensity of HSD2 immunoreactivity, changing the number of HSD2 neurons visible for counting (see Refs. 20, 21), no consistent group differences were found in the present experiments.

Data are presented as group means ± SE. Student’s two-tailed t-test (for comparison between 2 means), ANOVA (for determining the significance of a treatment across multiple groups), and Pearson’s linear correlation coefficient were used for statistical assessments, with a type I error probability of P < 0.05 considered significant.

**RESULTS**

**Hypertonic saline.** Dehydration with hypertonic saline (2 M, 2 ml ip) rapidly stimulated a large increase in water intake (see Fig. 1C). Hypertonic saline-injected rats drank an average of 8.3 ± 1.3 ml of water in 2 h, primarily within the first 30 min (5.5 ± 1.8 ml), while isotonic saline-injected control rats drank very little during the entire 2-h period (2.9 ± 1.1 ml, P = 0.02 vs. hypertonic saline).

As shown in Fig. 1A, hypertonic saline injection substantially increased c-Fos immunoreactivity in the NTS and area postrema (in a separate group of rats perfused 2 h postinjection with no access to water, n = 5) relative to isotonic saline-injected controls (Fig. 1B). Despite the robust c-Fos activation found throughout a large extent of the medial NTS in hypertonic saline-injected rats, only a few of their HSD2 neurons contained weak c-Fos-immunoreactivity (0–3 of 151–176 neurons per case). Indeed, hypertonic saline-injected rats contained even fewer activated HSD2 neurons on average than isotonic saline-injected controls (0.5 ± 0.1 vs. 1.8 ± 1.4%, see Fig. 1D), although this difference was not statistically significant (P = 0.33). Figure 2 shows the rostral group of HSD2 neurons (adjacent to the caudal fourth ventricle) from a hypertonic saline-injected rat, surrounded by dense and intense c-Fos-immunoreactive nuclei in the medial NTS.

This lack of HSD2 neuronal activation was not a false-negative result of our double-immunofluorescence labeling technique. As reported in a previous study of medullary c-Fos activation during hypernatremia (25), many TH-immunoreactive neurons within the nearby A1 and A2 noradrenergic groups contained c-Fos-immunoreactivity (Fig. 3).

**PEG hypovolemia.** Thirst was increased in the hours immediately following PEG injection. As shown in Fig. 4A, rats made hypovolemic by injection of 30% PEG drank significantly more water than controls (PEG-injected saline).
significantly more water than vehicle-injected controls (3.6 ± 0.4 vs. 0.4 ± 0.2 ml, n = 4 each, P = 0.0006). Most water intake occurred between 1 and 3 h postinjection. In this experiment, a 2% NaCl solution was available in addition to water, and the small volumes ingested by rats in both groups (1.5 ± 0.8 ml, 30% PEG, vs. 1.9 ± 0.4 ml, vehicle; see Fig. 4B) were not significantly different (P = 0.7), consistent with an absence of sodium appetite in the early hours following PEG injection.

Very few c-Fos-activated HSD2 neurons were counted from rats in this experiment, indicating that the HSD2 neurons were not activated by hypovolemia at this 4-h time point. PEG-injected rats with access to water and 2% NaCl contained only 0–3 HSD2 neurons with nuclear c-Fos immunoreactivity (0.9 ± 0.4% from a sample of 156–183 per animal). If anything, the data shown in Fig. 4C suggest a tendency toward greater HSD2 neuronal activation in the control group: 2–10 HSD2 neurons were activated in vehicle-injected rats (2.8 ± 0.8% from a sample of 169–203), but this difference was not significant (P = 0.08). An additional group of 30% PEG-injected rats, which were denied access to drinking fluids postinjection (n = 4), once again contained very few activated HSD2 neurons (1.9 ± 0.4%; 2–5 c-Fos positive from a sample of 171–204 HSD2 neurons per animal). Among these three groups of rats perfused 4 h after PEG injection, no significant differences in HSD2 neuronal activation were found by ANOVA [F(2,9) = 2.73, P = 0.12].

On the basis of a previous report (50) and a large series of PEG pilot experiments involving a variety of pre- and postinjection conditions and times (see MATERIALS AND METHODS), we next examined HSD2 neuronal activation in rats that had been sodium deprived for 4 days before PEG injection to boost sodium appetite. Sodium-deprived rats were injected with PEG at 30% (n = 18), 20% (n = 6), or 10% (n = 6) and provided only water until they were perfused 12–14 h later. PEG injection produced a wide range of hypovolemia, as evidenced by the elevated plasma protein concentrations in these rats (range 5.8–8.4 g/dl) relative to vehicle-injected controls (5.3–5.6 g/dl).

As shown in Fig. 5C, the HSD2 neurons were activated similarly in all PEG hypovolemic groups in this experiment. PEG-injected rats contained an elevated percentage of HSD2 neurons with c-Fos-immunoreactive nuclei (18.8–63.0%, see example in Fig. 5A) relative to the mild activation found in 4-day sodium-deprived vehicle-injected control rats (12.7 ± 2.9%, see example in Fig. 5B). HSD2 neuronal activation was similarly elevated in all three PEG hypovolemic groups (all P ≤ 0.001 vs. vehicle-injected controls), but the differences among these three groups themselves were not significant [F(2,27) = 1.57, P = 0.23].
statistically significant differences were found relative to normovolemic vehicle concentration ([K⁺] activation during extended hypovolemia. The plasma potassium concentration (with or without postinjection access to drinking fluids, until many hours after the onset of hypovolemia (52)). Among the PEG hypovolemic cases themselves, immunoreactive HSD2 neurons ([Na⁺] vs. %c-Fos-activated HSD2, \( \rho = -0.25, P > 0.05 \)). Only rats from the 30% PEG-injected group showed a significant difference in [Na⁺] (138.1 ± 0.7 mM) relative to the vehicle-injected group (142.5 ± 0.4 mM, \( P = 0.002 \)) and the 10% PEG group (143.0 ± 0.37, \( P = 0.006 \); Fig. 5F). Rats injected with 20% PEG (140.7 ± 1.2 mM) or 10% PEG (143.0 ± 0.4 mM) showed no significant group differences vs. vehicle-injected controls (\( P = 0.17 \) and \( P = 0.39 \), respectively). Thus, despite the fact that a net loss of sodium (extracellular fluid volume) results in delayed activation of the HSD2 neurons, their activation does not require a decrease in the extracellular sodium concentration.

None of the three PEG-injected groups showed a statistically significant difference in postinjection (12 h) water intake relative to one another or to vehicle-injected controls \( (F(3,32)=0.0679, P = 0.99) \); see Fig. 5G). Overall, water intake during the 12-h period after PEG injection tended to be reduced in cases with more severe hypovolemia and hyperkalemia, showing weak negative correlations with both the plasma protein concentration and plasma [K⁺] (\( \rho = -0.38 \) and \( \rho = -0.33 \), respectively; both \( P < 0.05 \)). Water intake was not significantly correlated with plasma [Na⁺] (\( \rho = 0.17, P > 0.05 \)) or with the percentage of c-Fos-activated HSD2 neurons (\( \rho = 0.004, P > 0.05 \)). Nonetheless, the availability of water after the onset of hypovolemia seemed to play some role in boosting HSD2 neuronal activation, because more c-Fos-activated HSD2 neurons were found in 4-day sodium-deprived, 30% PEG-injected rats that were provided drinking water in these experiments (44.1 ± 2.4%, \( n = 18 \)) than in pilot rats that had been treated identically but were provided no drinking fluids after PEG injection (24.4 ± 3.4%, \( n = 4 \), \( P = 0.002 \)).

Sodium appetite stimulated by PEG hypovolemia after 4-day sodium deprivation. Increased ingestion of concentrated saline (3% NaCl) is indicative of an elevated sodium appetite and has been observed previously after PEG hypovolemia (50). Because of our slightly different experimental paradigm (12-h postinjection access to water before testing), additional experiments were tested to learn whether sodium appetite is increased under the present conditions. An additional group was sodium deprived for 4 days, injected with 30% PEG, and then, after 12-h access to dH₂O, provided an additional graduated drinking tube containing 3% NaCl for 2 h (\( n = 6 \)). Run simultaneously with this group was a positive control group (\( n = 6 \), treated identically, including 30% PEG injection, but not given access to saline) and a negative control group (\( n = 5 \), injected with vehicle, but otherwise treated identically, including the 3% NaCl access test).

The percentage of c-Fos-immunoreactive HSD2 neurons increased with the severity of hypovolemia up to plasma protein values of roughly 7 g/dl (see Fig. 5H). In this range, HSD2 neuronal activation showed a positive linear correlation with the plasma protein concentration (\( \rho = 0.86, P < 0.001 \)), when cases up through 7.0 g/dl are considered. No further correlation with the percentage of c-Fos-activated HSD2 neurons ([Na⁺] vs. %c-Fos-activated HSD2, \( \rho = -0.76, P < 0.001 \); Fig. 5K). However, no statistically significant relationship was found between [Na⁺] and the percentage of c-Fos-activated HSD2 neurons ([Na⁺] vs. %c-Fos-activated HSD2, \( \rho = -0.25, P > 0.05 \)).

Plasma electrolyte changes associated with HSD2 neuronal activation during extended hypovolemia. The plasma potassium concentration ([K⁺]) was elevated above the control range in every PEG-injected rat (4.6–7.4 vs. 3.7–4.1 mM in the vehicle group) and was positively correlated with the severity of hypovolemia (PP vs. [K⁺], \( \rho = 0.81, P < 0.001 \); Fig. 5J). As shown in Fig. 5E, [K⁺] was elevated similarly in all three PEG hypovolemic groups (all \( P < 10^{-4} \) vs. vehicle-injected controls), while no significant differences were found among these three groups themselves by ANOVA \( (F(2,27) = 1.8959, P = 0.17) \). Across all cases, [K⁺] showed a positive correlation with the percentage of c-Fos-activated HSD2 neurons (\( \rho = 0.63, P < 0.001 \)), but this simply reflected the separate clustering of values from vehicle-injected controls with low [K⁺] and PEG-injected rats with high [K⁺], as shown in Fig. 5K. Among the PEG hypovolemic cases themselves (\( n = 30 \)), these two variables were not significantly correlated (\( \rho = 0.008, P > 0.05 \)).

In moderately hypovolemic animals and control cases, variation in the plasma sodium concentration ([Na⁺]) was small, but a number of rats from the 30% PEG group and two cases from the 20% PEG group exhibited larger decreases in [Na⁺] (see Fig. 5J), similar to the decline in [Na⁺] previously reported for rats given access only to water after 30% PEG injection (see Ref. 52). Overall, [Na⁺] was negatively correlated with the severity of hypovolemia (PP vs. [Na⁺], \( \rho = -0.76, P < 0.001 \); Fig. 5K). However, no statistically significant relationship was found between [Na⁺] and the percentage of c-Fos-activated HSD2 neurons ([Na⁺] vs. %c-Fos-activated HSD2, \( \rho = -0.25, P > 0.05 \)). Only rats from the 30% PEG-injected group showed a significant difference in [Na⁺] (138.1 ± 0.7 mM) relative to the vehicle-injected group (142.5 ± 0.4 mM, \( P = 0.002 \)) and the 10% PEG group (143.0 ± 0.37, \( P = 0.006 \); Fig. 5F). Rats injected with 20% PEG (140.7 ± 1.2 mM) or 10% PEG (143.0 ± 0.4 mM) showed no significant group differences vs. vehicle-injected controls (\( P = 0.17 \) and \( P = 0.39 \), respectively). Thus, despite the fact that a net loss of sodium (extracellular fluid volume) results in delayed activation of the HSD2 neurons, their activation does not require a decrease in the extracellular sodium concentration.

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Fig. 4. A: polyethylene glycol (PEG) hypovolemia increased thirst within 2 h postinjection, as shown by the significantly elevated water intakes of 30% PEG (PEG30)-injected rats (\( n = 4 \)) relative to vehicle (Veh)-injected rats (\( n = 4 \)). *\( P \leq 0.004. B: \) ingested volumes of saline (2% NaCl) were small and similar in both groups, consistent with the previously reported lack of sodium appetite until many hours after the onset of hypovolemia (52). C: very few c-Fos-immunoreactive HSD2 neurons were found in hypovolemic rats killed at 4 h after PEG injection (with or without postinjection access to drinking fluids, \( n = 4 \) each). No statistically significant differences were found relative to normovolemic vehicle-injected control rats (\( n = 4 \)).
Results from the positive control group (n = 6) were included with the 30% PEG data presented above (Fig. 5). HSD2 neuronal activation and plasma values measured in these six rats were generally similar to those from two previous groups injected with 30% PEG, although they tended to have larger volume deficits with higher plasma protein concentrations (7.8 ± 0.2 g/dl, range 6.8–8.4), as well as larger increases in plasma [K⁺] (6.5 ± 0.3 mM, range 5.8–7.4). Their [Na⁺] (135.5 ± 0.8 mM, range 132–138) and percentage of c-Fos-activated HSD2 neurons (38.9 ± 4.7%, range 26.5–56.1) tended to be slightly lower as well.

In any case, this positive control data established that the hypovolemia produced by this final series of PEG injections was at least as severe as in previous 30% PEG-injected cases. For this reason, it came as a surprise that most of the rats given access to saline drank relatively minimal amounts. Four rats only consumed 0.5–1.5 ml of 3% NaCl, while one drank 7.5 ml and another 12 ml. Because of this wide variance in the 30% PEG-injected group, their saline ingestion was not significantly different from that of vehicle-injected controls (Fig. 6A). The saline ingestion of the four cases with smaller intake volumes fell well within the control range (with the exception of one high value of 5.5 ml, vehicle-injected controls drank 0.5–1.5 ml).

The two 30% PEG-injected rats that drank 7.5 and 12 ml of concentrated saline (followed by 4 and 8 ml of water, respectively) exhibited substantial reductions in their plasma protein concentrations (5.8 and 5.3 g/dl) and plasma [K⁺] (5.5 and 6.1 mM), as well elevations in plasma [Na⁺] (144 and 148 mM). The other four 30% PEG-injected rats, which drank only 0.5–1.5 ml of 3% NaCl (along with 0–2.5 ml of water), remained hypovolemic, hyperkalemic, and somewhat hypotonic: PP (6.8–8.4 g/dl), [K⁺] (6.0–7.4 mM), [Na⁺] (129–139 mM). These four rats did not exhibit any apparent behavioral deficits that would have prevented them from ingesting larger volumes; they all showed signs of alertness, and all but one of them sampled the 3% NaCl solution shortly after it was mounted in their cages. Nonetheless, severe hypovolemia, hyperkalemia, and/or hypotonicity may have negatively influenced salt ingestion in these cases.

Consistent with this possibility, a more uniform increase in 3% NaCl ingestion (and subsequent water intake) was observed when 15% PEG was injected instead of 30% PEG to produce less severe volume deficits. These rats drank 6.3 ± 1.0 ml of 3% NaCl (range 3.5–8 ml, n = 4, P = 0.02 vs. vehicle-injected controls; see Fig. 6A). As shown in Fig. 6B, they also drank larger amounts of water after saline was made available (9.4 ± 1.1 ml, Fig. 6B) relative to groups injected with vehicle (2.0 ± 0.9 ml, P = 0.001) or 30% PEG (2.7 ± 1.2, P = 0.005).

**DISCUSSION**

The HSD2 neurons are activated under various conditions that stimulate sodium appetite, but not thirst. They are currently the only neurons for which such a specific functional association has been demonstrated and also the only cells in the brain with directly confirmed sensitivity to aldosterone (16), which selectively enhances sodium appetite (38, 60).

_Sodium appetite vs. thirst: separate sensory mechanisms in the brain?_ The brain tissue surrounding the rostral third ventricle is necessary for the stimulation of thirst by hypovolemia (1, 27). Some of these sites also play a role in the stimulation of thirst by hypovolemia, at least in part by responding to circulating levels of angiotensin II (5, 12, 47). The possibility that neurons within this region also regulate sodium appetite has been explored in a number of previous investigations. In support of this possibility, angiotensin II increases saline ingestion under certain conditions (reviewed in Ref. 13). Also, various lesions involving thirst-associated regions in and around the anterior hypothalamus can either decrease or augment salt ingestion in response to a variety of stimuli (1, 8, 10, 15, 27, 57). Cells within at least one of these sites, the subfornical organ, appear to play an important role in inhibiting salt intake in response to elevated extracellular [Na⁺] (24).

The key sensory neurons for stimulating sodium appetite, however, are probably located outside the anterior hypothalamus. During hypovolemia, neurons in this brain region are activated long before sodium appetite, with a time course similar to thirst (41). Also, we and others have found no evidence for the expression of HSD2 (the glucocorticoid-inactivating enzyme required for mineralocorticoid sensitivity) in or around the anterior hypothalamus (17, 40), suggesting that, at physiological concentrations, mineralocorticoids do not exert a direct stimulatory influence within this region. Consistent with this prediction, spontaneous salt intake and mineralocorticoid-stimulated saline ingestion are both enhanced, not reduced, by various lesions involving the anteroventral third ventricular region and subfornical organ (1, 10, 15, 36). Most importantly, in both rats and sheep, the mechanisms that stimulate sodium appetite persist despite large lesions within this region that successfully eliminate the homeostatic stimulation of thirst (2, 8, 58).

**HSD2 neuronal activation is sodium appetite specific.** The HSD2 neurons in the NTS were identified on the basis of their unique mineralocorticoid sensitivity (16, 17). Mineralocorticoids subserve sodium balance in two ways: they 1) increase sodium retention by the kidney and 2) enhance the behavioral drive to ingest sodium. Mineralocorticoids are not required for sodium appetite (39), nor are they required for the activation of HSD2 neurons in response to sodium deficiency (16), but they represent the only class of hormones capable (in pharmacological doses) of recreating the selective appetite for sodium that occurs naturally after an actual physiological deficiency (14, 38, 51, 59, 60).

Activation of these mineralocorticoid-sensitive HSD2 neurons occurs in parallel with sodium appetite under a variety of experimental conditions, including high-dose deoxycorticosterone administration, chronic dietary sodium deprivation, and diuresis or adrenalectomy followed by short-term sodium deprivation (16). The present results add prolonged PEG hypovolemia (after 4-day sodium deprivation) as another experimental paradigm in which the HSD2 neurons are activated in association with sodium appetite.

One well-supported theory asserts that hypovolemic thirst and sodium appetite are driven by similar input signals, yet the delayed onset of salt ingestion results from a central inhibitory mechanism that prevents sodium appetite until body fluids are sufficiently diluted by the ingestion and retention of water (52, 56). With respect to this possibility, an important finding of the
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present study is that the HSD2 neurons are not activated under hypovolemic conditions that stimulate thirst, indicating that these cells do not convey a simple nonspecific signal for hypovolemia. Instead, by transmitting a signal specifically associated with the delayed appearance of sodium appetite, they represent a unique central sensory group.

The HSD2 neurons are positioned to integrate multiple neural and humoral input signals that arise during prolonged hypovolemia. They are located within the NTS, which is the primary recipient of neural viscerosensory information. Also, the distribution of the HSD2 neurons overlaps a subregion of the NTS with a diminished blood-brain barrier (4, 23), which may allow them increased sensitivity to various humoral changes associated with prolonged sodium deficiency.

Aldosterone is clearly one of these inputs, although this hormone is neither necessary nor sufficient (in physiological amounts) for stimulating the HSD2 neurons or sodium appetite, both of which continue to respond to dietary sodium deprivation when aldosterone production is eliminated by removal of the adrenal glands (16). A decrease in plasma \([\text{Na}^+]\) does not appear to be necessary for their stimulation either, despite the intuitive appeal of such a mechanism. Many conditions that activate the HSD2 neurons and elevate sodium appetite, including dietary sodium deprivation, do not reduce plasma \([\text{Na}^+]\) (7). If anything, the blunted HSD2 neuronal activation and reduced saline intake found here in the more severely hyponatremic rats (30\% PEG injected) would be more consistent with an inhibitory role for hyponatremia, similar to its inhibition of thirst (49).

In contrast to the lack of any consistent relationship with plasma \([\text{Na}^+]\), prolonged sodium deprivation does produce a consistent elevation in plasma \([\text{K}^+]\). The present data confirm that \([\text{K}^+]\) rises during PEG hypovolemia after short-term dietary sodium deprivation (see also Table 1 of Ref. 55). In fact, elevated \([\text{K}^+]\) is one of the few physiological changes observed consistently across the various physiological conditions that increase sodium appetite. Chronic dietary sodium deprivation elevates both \([\text{K}^+]\) and sodium appetite in rats (7). Adrenalectomy causes a baseline elevation in \([\text{K}^+]\) that progressively worsens on sodium deprivation (in parallel with an increasing sodium appetite) and then falls back to baseline after salt ingestion (see Table 2 of Ref. 26). Peritoneal dialysis initially produces hyponatremia, but whereas plasma \([\text{Na}^+]\) quickly returns to normal, \([\text{K}^+]\) slowly increases up to the time that an increased sodium appetite becomes apparent (6). It has even been suggested that baseline \([\text{K}^+]\) may be related to a preference for more concentrated saline solutions in sheep (32, 33).

This elevation in \([\text{K}^+]\) is the primary stimulus for the massive increase in aldosterone production caused by dietary sodium deprivation (35), suggesting that potassium could exert an indirect influence on the HSD2 neurons (and sodium appetite) by boosting aldosterone production. The fact that \([\text{K}^+]\) is persistently elevated indicates that even maximal aldosterone production is not sufficient to combat hyperkalemia under such conditions, and that sodium intake is not only important for

![Fig. 6. Three groups of rats were given access to 3% NaCl for 2 h, following 4-day sodium deprivation, injection of PEG (30 or 15%) or vehicle, and 12-h postinjection access to only distilled water. A: moderate volume deficits, produced by injection of 15% PEG, uniformly increased 3% NaCl relative to vehicle-injected controls \((P = 0.02)\). More severe volume deficits, produced by injection of 30% PEG, produced a widely divergent pattern of saline intake: 7.5 and 12 ml were ingested by two rats in this group, while the other four only drank 0.5–1.5 ml (see RESULTS). B: 15% PEG-injected rats also drank a significantly elevated amount of water after 3% NaCl became available \((P = 0.001)\) vs. vehicle, \(P = 0.005\) vs. PEG30), largely following their initial bouts of saline intake.](http://ajpregu.physiology.org/)

Fig. 5. Many HSD2 neurons became c-Fos-immunoreactive 12 h after PEG injection in 4-day sodium-deprived rats. A: an example of c-Fos-immunoreactivity (red) in HSD2 neurons (green), adjacent to the caudal 4th ventricle (4V) in a case that had been injected with 30\% PEG. B: in vehicle-injected control cases, a small percentage of HSD2 neurons contained weak c-Fos immunoreactivity. Scale bar = 100 \(\mu\)m for both A and B. C: HSD2 neuronal activation was similarly elevated in all 3 PEG-injected groups. PEG30, \(n = 18\); PEG20, \(n = 6\); PEG10, \(n = 6\); vehicle, \(n = 6\). \(P < 0.001\) vs. vehicle; no significant differences were found among the PEG-injected groups themselves. D: PEG-injected rats were hypovolemic, relative to vehicle-injected controls, as reflected by their significantly elevated plasma protein values. \*\(*P < 10^{-4}\) vs. vehicle and \(P < 0.03\) vs. 10\% PEG. \*\(*P = 0.0066\) vs. vehicle. E: plasma potassium concentrations were similarly elevated in all 3 PEG-injected groups. \*\(*P < 10^{-4}\) vs. vehicle; no significant differences were found among the 3 PEG groups themselves. F: only the group injected with 30\% PEG showed a statistically significant decrease in the plasma concentration of sodium. \*\(*P = 0.002\) vs. vehicle and \(P = 0.006\) vs. 10\% PEG. G: there were no significant differences in total 12-h water intake during the postinjection period. H: in cases with increasing plasma protein \((PP)\) values up through 7 g/dl, c-Fos activation in the HSD2 neurons showed a positive linear correlation with the severity of hypovolemia (regression equation: \(y = 0.262x - 1.264, \rho = 0.86, P < 0.001\)), while cases with more severe volume deficits \((PP >7 g/dl)\) showed a nonsignificant trend toward decreased HSD2 neuronal activation. I and J: plasma sodium concentration was negatively correlated with the severity of hypovolemia \((PP vs. [\text{Na}^+])\); \(y = -2.787x + 158.73, \rho = 0.76, P < 0.001\), whereas a positive correlation was found for the plasma potassium concentration \((PP vs. [\text{K}^+])\) regression equation: \(y = 0.781x - 0.074, \rho = 0.81, P < 0.001\). K: despite the concurrent increases in both plasma \([\text{K}^+]\) and the percentage of c-Fos-activated HSD2 neurons in all PEG-injected cases, these 2 variables showed no apparent correlation among the PEG-injected cases themselves \((\rho = 0.008, n = 30)\).
restoring blood volume but also for preventing dangerous elevations in plasma [K\(^+\)] by normalizing its excretion by the kidney (34, 37).

Despite these intriguing associations, however, it appears unlikely that elevated [K\(^+\)] is an important stimulus for the HSD2 neurons or sodium appetite. For example, ingestion of concentrated saline is also increased by dietary potassium deprivation (3, 61). Our preliminary results indicate that HSD2 neuronal activation and sodium appetite are just as robust when plasma [K\(^+\)] is decreased, rather than increased, by combined dietary deprivation of potassium and sodium (unpublished observations, Geerling JC and Loewy AD). Although these data do not rule out the possibility that increases in [K\(^+\)] modulate the activation of HSD2 neurons and/or sodium appetite by other stimuli, they indicate that elevated [K\(^+\)] is not critical for their activation.

It is possible that the HSD2 neurons are activated directly by some other unidentified peripheral stimulus, but this possibility would not preclude a simultaneous role for central inhibitory mechanisms in shaping their sodium appetite-specific activity. For example, the HSD2 neurons receive a substantial descending projection from inhibitory neurons in the central nucleus of the amygdala (18), which exerts an important modulatory influence over sodium appetite (28). Also, neurons in the medial and subpostremal NTS receive input from neurons in the paraventricular hypothalamic nucleus, including many that express oxytocin as a cotransmitter (30, 42), suggesting that the central inhibition of sodium appetite by oxytocin (56) could involve projections that target the HSD2 neurons (or their associated microcircuitry within the NTS; see Ref. 46). Conversely, the activation of the HSD2 neurons after prolonged hypovolemia may help to relieve sodium appetite from oxytocinergic inhibition via their direct and relayed output to the ventrolateral bed nucleus of the stria terminalis (BSTvl; Ref. 19). BSTvl contains GABAergic (inhibitory) neurons that heavily innervate, among other sites, the same regions of the paraventricular hypothalamic nucleus that contain brain stem-projecting oxytocinergic neurons (9).

**Technical considerations: saline ingestion and c-Fos activation after PEG hypovolemia.** In 2-h access tests, the rats in our PEG experiments did not drink as much concentrated saline (3% NaCl) as might be expected based on previous reports (50, 52). Insufficient hypovolemia is clearly not the reason for this difference, because our PEG injections produced substantial volume deficits. On the basis of their plasma protein concentrations, all rats injected with 30% PEG had blood volume deficits ranging between 8 and 53% (relative to vehicle-injected controls), and the rats with the largest volume deficits seemed to exhibit the weakest sodium appetites. Possible reasons for attenuated saline ingestion are differences in the strain of rats used (Harlan Sprague Dawley rats here vs. Wistar rats and Zivic-Miller Sprague Dawley rats, respectively, in Refs. 50 and 52) and testing protocols (12-h water access before sodium appetite testing here vs. continuous postinjection access to water and saline in prior studies).

The more uniform increase in sodium ingestion stimulated after 15% (vs. 30%) PEG injections suggests that, past a certain point, greater volume deficits do not increase sodium appetite. Consistent with this possibility, chronic (8 day) sodium deprivation produces an appetite for sodium that is at least as strong as that measured here, in rats that were considerably less hyponatremic (Ref. 16, see also Fig. 8 of Ref. 43).

It is possible that the extreme physiological changes that follow 30% PEG injections produced competing signals, which inhibited sodium appetite. In this regard, it should be noted that severe hyperkalemia and hyponatremia were observed in a number of these rats, including the 30% PEG-injected rats that failed to drink more than control amounts of 3% NaCl during a 2-h drinking test. Importantly, c-Fos activation in the HSD2 neurons did not show any greater increase in cases with extreme hypovolemia, hyponatremia, and hyperkalemia. On the contrary, their activation appeared to level off or possibly decrease in cases with more severe volume deficits (see Fig. 5H).

It is impossible to know the key differences, before saline access, between the two 30% PEG-injected rats that drank large volumes of 3% NaCl (7 and 12 ml) and the four that drank no more than control rats, other than to say that the latter remained severely hypovolemic, hyperkalemic, and somewhat hyponatremic (see RESULTS). The hypothesis that sodium appetite was inhibited by such sequelae of their extreme hypovolemia is supported by the more consistent increase in 3% NaCl ingestion after 15% PEG was used to produce less severe hypovolemia.

Finally, the HSD2 neuronal activation measured here was greater than what we previously measured after 8 days of dietary sodium deprivation (16). This difference suggests that the degree of HSD2 neuronal activation is not necessarily proportional to the strength of sodium appetite, particularly in the setting of severe, prolonged hypovolemia. One important factor that should be considered when comparing the present data with our previous report, however, is the time of day at which rats were killed for c-Fos analysis. Rats in the present experiments were perfused at 7 AM, just after lights-on (6:30 AM, the end of the 12-h dark period, during which rats spend most of their time awake), whereas the experiments in our previous study were conducted later in the day, when the animals had spent most of their time asleep during the hours before perfusion. We have since observed that the intensity of c-Fos immunoreactivity is consistently greater when rats are perfused earlier in the morning after 8-day sodium deprivation (40–80%, unpublished observations, Geerling JC and Loewy AD).

In conclusion, the HSD2 neurons in the NTS are unlike most other viscerosensory neurons in the brain stem or thirst stimulatory neurons in the hypothalamus because they are activated specifically in association with sodium appetite, not thirst. This property, in combination with their unique mineralocorticoid sensitivity and their axonal projections to other sodium appetite-associated brain sites, indicates that the HSD2 neurons are activated by peripheral and/or central inputs specifically associated with prolonged sodium deficiency and that they play a key role in generating sodium appetite.

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