

c-fos-Like Immunoreactivity in the Subfornical Organ and Nucleus of the Solitary Tract following Salt Intake by Sodium-Depleted Rats

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Received 2 June 1997; Accepted 25 September 1997

HOUP^T, T. A., G. P. SMITH, T. H. JOH AND S. P. FRANKMANN. *c-fos-like immunoreactivity in the subfornical organ and nucleus of the solitary tract following salt intake by sodium-depleted rats.* *PHYSIOL BEHAV* **63**(4) 505–510, 1998.—Acute sodium depletion by furosemide induces a robust salt appetite in the rat which is satiated rapidly by ingestion of sodium chloride (salt) solutions. To identify neuronal populations activated by sodium depletion and by salt intake, we quantified c-fos-like immunoreactivity (c-FLI) in the subfornical organ (SFO) and nucleus of the solitary tract (NTS) after sodium depletion and at time intervals from 30 min to 12 h after 1 h of access to 0.3 M NaCl. Rats drank 10 ± 1.6 mL over 1 h, with most of the intake occurring by 30 min. Increased numbers of c-FLI-positive cells were observed in the SFO 24 h after sodium depletion; c-FLI remained elevated for 90 min after 0.3 M NaCl intake and then declined until the number of c-FLI-positive cells at 12 h was not significantly different from mock-depleted levels. Sodium depletion alone did not significantly elevate c-FLI in the NTS, but the number of c-FLI-positive nuclei in the NTS was significantly increased after 0.3 M NaCl intake. The cellular location and temporal pattern of c-FLI expression are consistent with activation of neural circuitry sensitive to humoral, gustatory, and postingestive stimuli accompanying sodium depletion and 0.3 M NaCl ingestion. c-FLI in the SFO and NTS may serve as quantifiable markers in the central nervous system of the state of sodium depletion and of ingestive (orosensory and gastrointestinal) sensory stimulation, respectively. © 1998 Elsevier Science Inc.

Immediate-early genes Time course Furosemide Satiety Sodium appetite

HYPERTONIC NaCl (salt) solutions are normally rejected by the rat, but under conditions of sodium depletion they are avidly ingested. The depletion-induced acceptance of salt is rapidly reversed when the salt appetite is satiated. The neural mechanisms which underlie the initiation, maintenance, and termination of this salt appetite are poorly understood. Numerous candidate mechanisms for the arousal of salt appetite have been proposed, including increased circulating angiotensin II (AII) and aldosterone (22), decreased brain sodium concentration (30), and decreased central oxytocin release (26). There is less evidence for specific satiation mechanisms, although roles for orosensory feedback, mediated by the chorda tympani nerve (24), and postingestive feedback, mediated by the hepatic branch of the vagus nerve [(28), but see (7)] and bombesin-like peptides (5,6), have been suggested. The central integration of this information in the natural sequence of salt appetite and satiation has yet to be elucidated.

The arousal of salt appetite occurs 5–8 h after initiation of sodium depletion by injection of the diuretic–natriuretic drug furosemide. After initiation of salt intake, however, complete long-term satiation occurs within 30–45 min (21,25). The humoral

correlates of depletion (plasma renin, angiotensin, and aldosterone) return to replete levels only 60–120 min after salt intake (28). The different time courses of appetite arousal and satiation suggest a dissociation of appetitive and satiating signals, such that signals of salt appetite are slow to arise and slow to decay, but the signals of postingestive satiation are rapid. The brain regions that detect the appetitive and satiating signals arising in the periphery are also thought to be distinct based on lesion, pharmacological, and electrophysiological studies. Thus in the forebrain, the circumventricular organum vasculosum of the lamina terminalis (OVLT) and subfornical organ (SFO) respond to humoral factors in the periphery correlated with sodium depletion; in the hindbrain, central gustatory and visceral sensory relays such as the nucleus of the solitary tract (NTS) respond to the immediate orosensory and postingestive consequences of salt intake.

This study was undertaken to characterize markers of neuronal activation correlated with sodium depletion and the satiation of salt appetite in rats. We quantified c-fos-like immunoreactivity (c-FLI) in two brain regions, the SFO and NTS, known to be involved in body fluid balance and the processing of ingestive stimuli under

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conditions of sodium depletion by furosemide injection and repletion by intake of 0.3 M NaCl solution. Because c-FLI can be employed as a marker of neuronal activation (15), visualizing c-FLI in the central nervous system may identify discrete neuronal populations involved in the processing of peripheral signals leading to initiation and satiation of salt appetite. The SFO was chosen as a marker of appetite initiation because it is sensitive to peripheral humoral signals of depletion and because it is necessary for the acute expression of a sodium appetite after diuretic depletion (27,31). The NTS was chosen a marker of satiation because it is the first central relay of taste and visceral sensations that mediate the termination of salt intake. We also characterized the time course of c-FLI expression in the SFO and NTS up to 12 h after repletion.

METHODS

Sodium Depletion and Salt Appetite Test

Thirty adult male Sprague–Dawley rats (300–400 g) were housed individually under a 12-h light, 12-h dark cycle with ad lib. access to tap water and Purina rodent chow. All the rats had been sodium-depleted and allowed access to 0.3 M NaCl 3–4 times prior to this study, the last depletion being at least 1 week earlier. Sodium depletion was induced in 25 rats by two subcutaneous injections of furosemide (Lasix, 5 mg in 0.5 mL of isotonic NaCl) 2 h apart early in the lights-on period. The remaining 5 rats were mock-depleted with two subcutaneous injections of isotonic NaCl. At the time of the first injection, rats were food- but not water-deprived. Overnight body-weight changes and water intakes were recorded.

Twenty-four hours after the first furosemide injection, 20 rats were given access to 0.3 M NaCl solution for a maximum of 1 h. Salt intakes were recorded to the nearest 0.1 mL at 5, 10, 15, 30, and 60 min. Five rats were sacrificed at each of four time points: 30, 90, 270, and 720 min after the start of 0.3 M NaCl access. Thus, the 90-, 270-, and 720-min conditions had 1-h access whereas the 30-min condition had only 30-min access. The mock-depleted rats and five of the sodium-depleted rats were also sacrificed 24 h after the first injection, but without access to 0.3 M NaCl.

Tissue Collection and Immunohistochemistry

Rats were overdosed with sodium pentobarbital, and, when completely unresponsive, transcardially perfused first with 100 mL of heparinized isotonic saline containing 0.5% NaNO₂ and then with 400 mL of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB). The brains were dissected, blocked, postfixed for 2 h, and transferred into 30% sucrose for cryoprotection. Forty-micron coronal sections were cut on a freezing, sliding microtome through the rostral–caudal extent of the NTS, the pons at the level of the PBN, and the forebrain at the level of the SFO and anterior hypothalamus. Thirty-six sections were cut in the NTS from the caudal subpostremal NTS (bregma – 14 mm) to caudal extent of the rostral NTS (bregma – 12.8 mm). Ten sections were cut through the extent of the SFO. Every other section collected from the NTS and SFO were processed for c-FLI. All coordinates are based on (18).

Free-floating tissue sections were washed twice for 15 min in 0.1 M sodium phosphate buffered saline (PBS) and then permeabilized in 0.2% Triton, 1% bovine serum albumin (BSA) in PBS for 30 min. Then the sections were washed in PBS–BSA twice and incubated overnight with a sheep anti-c-fos peptide antibody (Cambridge Research Biochemicals) at a dilution of 1:3000. Sections were washed in PBS–BSA twice and incubated for 1 h with

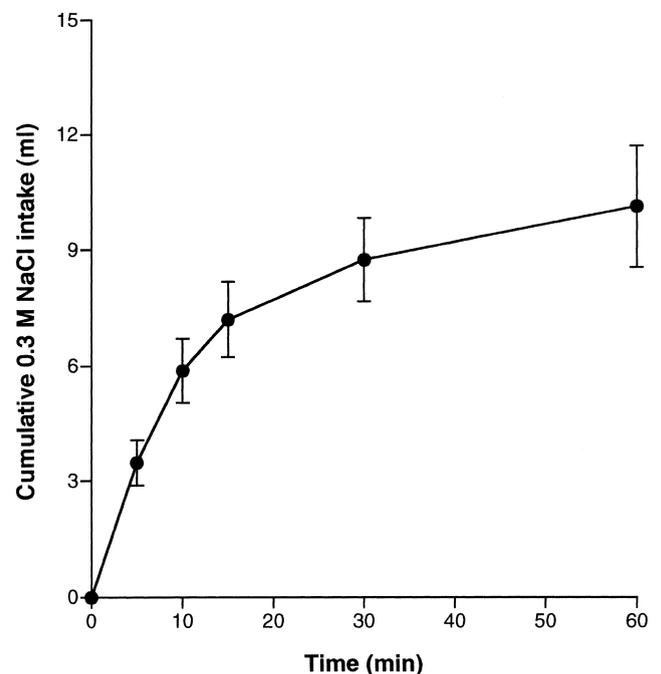


FIG. 1. Cumulative intake of 0.3 M NaCl (mean \pm SEM) by sodium-depleted rats ($n = 30$ at time 0–30 min, $n = 25$ at 60 min).

a biotinylated anti-sheep rabbit antibody (Vector Laboratories); bound secondary antibody was then amplified with the Vector Elite ABC kit. Antibody complexes were visualized by a 5-min 0.05% diaminobenzidine reaction.

Cells expressing positive, nuclear c-FLI were counted by hand in the SFO and NTS. In both the NTS and the SFO, only dark, punctate nuclear staining was counted; diffusely stained cell bodies were not counted. The NTS was divided into three subregions: caudal (ventral and caudal to the area postrema, AP), intermediate (abutting the IVth ventricle), and rostral (where the NTS separates from the IVth ventricle). Each of these three NTS subregions was represented by approximately 6 sections of the 18 NTS sections collected from each rat. Cell counts for all sections within each SFO and each NTS subregion of each rat were averaged, and the individual mean counts for each region averaged across rats by subregion within experimental groups. All data were analyzed by ANOVA with Statview software; subsequent post hoc analyses were performed using the Fisher PLSD test.

RESULTS

Depletion and Appetite

Twenty-four hours after furosemide treatment, depleted rats had consumed 26 ± 1.6 mL of water and had lost 41 ± 1.2 g of body weight, compared to mock-depleted animals, who consumed 15 ± 4.9 mL of water and lost 32 ± 5.1 g of body weight. Given 1 h of access to 0.3 M NaCl, depleted rats drank on average 10 ± 1.6 mL, typically with latencies of less than 1 min. Most drinking occurred within the first 15 min (Fig. 1).

Pattern and Time Course of c-FLI

Subfornical organ. c-FLI was limited to large cells in the highly vascularized medial central region of the SFO (Fig. 2). c-FLI was also observed extending ventrally from the level of the

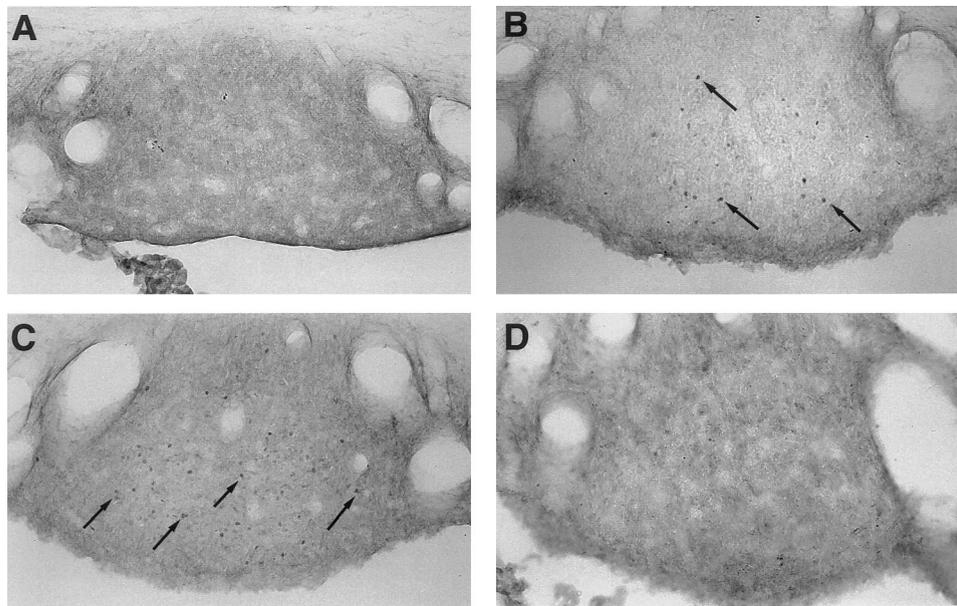


FIG. 2. Examples of c-FLI expression in coronal sections of SFO before and after sodium depletion. (A) Mock-depleted rat; no c-FLI can be seen. (B) Sodium-depleted rat; sodium depletion induces moderate numbers of scattered c-FLI-positive nuclei only in the well-vascularized medial central SFO. (C) Ninety minutes after access to 0.3 M NaCl; no significant changes in the number of c-FLI-positive cells were seen (compared to panel B). (D) Twelve hours after access to 0.3 M NaCl; c-FLI is reduced. Arrows indicate examples of c-FLI-positive nuclei.

SFO into the OVLT, paraventricular thalamus, and medial preoptic area. There was an overall significant effect of condition on the number of c-FLI cells in the SFO ($F(5, 23) = 3.65, p < 0.05$; Fig. 3). The number of c-FLI-positive cells was significantly increased compared to the mock condition 0 and 90 min after 0.3 M NaCl access ($p < 0.05$). The number of c-FLI-positive cells at 30 and 270 min approached but did not achieve a significant difference

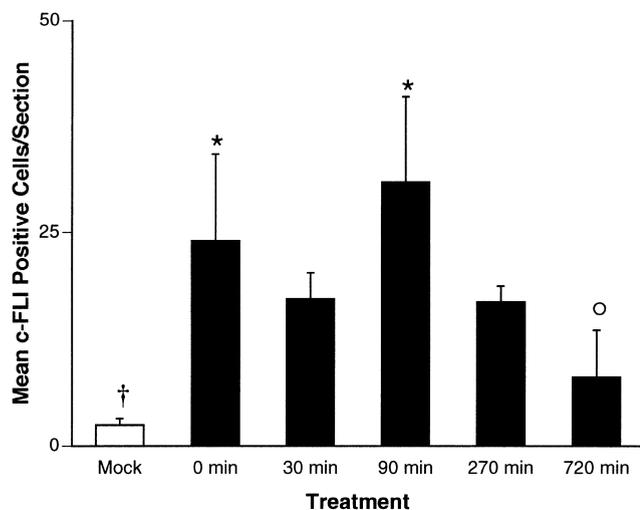


FIG. 3. Mean \pm SEM c-FLI-positive cells per section in the SFO for mock-depleted rats, sodium-depleted rats (0 min), and four time points (30, 90, 270, and 720 min) after the start of 1 h of access to 0.3 M NaCl. (* $p < 0.05$ vs. mock-depleted; † $p < 0.05$ vs. 0 min; ° $p < 0.05$ vs. 90 min.)

compared to the number in the SFO of mock-depleted rats. The number of stained cells in the SFO at 720 min after 0.3 M NaCl access was significantly lower than at 90 min after 0.3 M NaCl and was not significantly different from mock-depleted levels.

Nucleus of the solitary tract. For those groups given access to 0.3 M NaCl, positive cells were present in the medial part of the NTS, although scattered cells were present in the lateral part, most commonly just lateral to the solitary tract (Fig. 4). In the caudal NTS, c-FLI-positive nuclei in the medial region were densest ventrally, forming a thin strip of cells immediately dorsal to the dorsal motor nucleus of the vagus. Very few c-FLI-positive nuclei were seen in the AP or on the NTS-AP border. The intermediate medial NTS displayed the most pronounced induction of c-FLI, in a dense circular cluster of nuclei centered within the medial NTS, and a small number of c-FLI cells extended along the ventral lateral edge of the NTS adjacent to the solitary tract fibers but not along the medial and dorsal edges. In the rostral NTS, a significant number of cells were seen, but these were scattered throughout the medial-lateral and ventral-dorsal extent ventral to the solitary tract, with no obvious pattern of clustering. Very few c-FLI-positive cells (<10 per section) were observed in the NTS of mock-depleted rats or sodium-depleted rats not allowed access to 0.3 M NaCl.

There was an overall significant effect of condition on the number of c-FLI cells in each subregion of the NTS (Fig. 5): rostral ($F(5, 21) = 12.56, p < 0.0001$), intermediate ($F(5, 24) = 9.62, p < 0.0001$), and caudal ($F(5, 24) = 13.15, p < 0.0001$). The cell counts at 0 min were not different from mock-depleted rats in any NTS region, and the cell counts increased above mock levels at 30 min only in the intermediate NTS; the number of c-FLI-positive cells at 30 min was not different from 0 min in any NTS region. In all three subregions the number of c-FLI-positive cells at 90 min was significantly higher than at all other time points. The

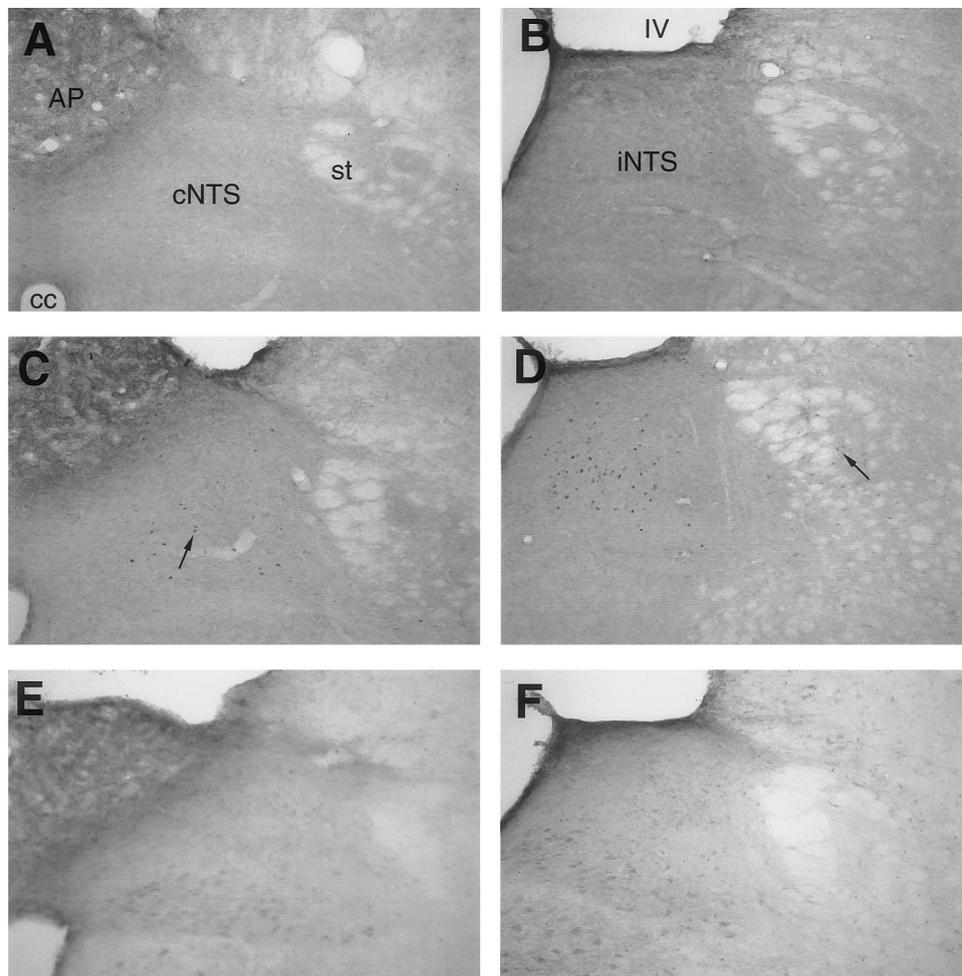


FIG. 4. Examples of c-FLI in the NTS of 24-h sodium-depleted rats sacrificed (A, B) without 0.3 M NaCl access, (C, D) 90 min after the start of 1 h of access to 0.3 M NaCl, and (E, F) 720 min after the start of 0.3 M NaCl access. Left panels show the caudal NTS; right panels show the intermediate NTS. Arrows indicate c-FLI-positive nuclei. (AP = area postrema, st = solitary tract, cNTS = caudal NTS, iNTS = intermediate NTS, IV = IVth ventricle, cc = central canal.)

number of cells at 270 min remained above mock-depleted levels in the rostral NTS and above mock-depleted and 0-min levels in the intermediate and caudal NTS. By 720 min, the number of stained cells was not significantly different from 0-min levels, although it remained above mock-depleted levels in the rostral and caudal NTS. Thus c-FLI was not increased by sodium depletion, but after access to 0.3 M NaCl peaked at 90 min and returned to 0-min or mock-depleted levels after 270-720 min.

DISCUSSION

The present study characterized the time course and regional expression of c-FLI in the two brain areas known to be involved in arousal and satiation of sodium appetite: the SFO, which is sensitive to circulating levels of angiotensin and aldosterone that are elevated by sodium depletion, and the NTS, which can integrate peripheral taste and visceral sensory signals (NTS) during intake of 0.3 M NaCl. Sodium depletion increased the number of c-FLI-positive cells in the SFO, but not in the NTS. Thirty minutes after intake of 0.3 M NaCl, c-FLI increased in the intermediate NTS, with maximal levels observed 90 min after access to 0.3 M NaCl

and declining after 270 min. In the rostral and caudal NTS significant increases of c-FLI were observed at 90 min. Thus sodium depletion alone increased c-FLI in the SFO, whereas sodium repletion by drinking 0.3 M NaCl increased c-FLI in the NTS.

We interpret these results to suggest that the c-FLI in the SFO reflects a state of hydromineral imbalance, whereas c-FLI in the NTS reflects the consequences of 0.3 M NaCl intake (orosensory or postingestive stimulation) for the sodium-depleted rat. In general, it can be observed that the pattern of neuronal activity as assessed by c-FLI shows a depletion-induced elevation of activity in the SFO which begins to decline 270 min after 0.3 M NaCl intake and increasing activation of neurons in the NTS following 0.3 M NaCl intake which peaks at 90 min and declines thereafter. Thus, the state of sodium depletion seems to activate the SFO whereas sodium repletion appears to activate the NTS.

Others have demonstrated increased c-FLI in the SFO in response to sodium depletion and osmotic challenges. Acute sodium depletion by peritoneal dialysis (29) and 24-h sodium depletion after furosemide treatment (10,19) induces c-FLI in the SFO; acute depletion by polyethylene glycol, however, does not induce c-FLI

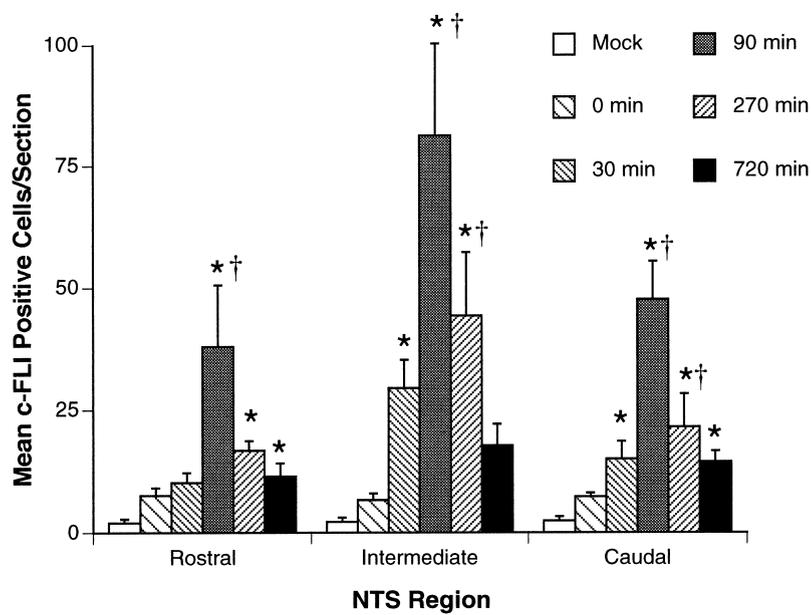


FIG. 5. Mean \pm SEM c-FLI-positive cells per section in the caudal, intermediate, and rostral NTS for mock-depleted rats, sodium-depleted rats (0 min), and four time points (30, 90, 270, and 720 min) after the start of 1 h of access to 0.3 M NaCl. (* $p < 0.05$ vs. mock-depleted, † $p < 0.05$ vs. 0 min; all time points different from 90 min, $p < 0.05$.)

in the SFO (10). The combination of furosemide and Captopril (an angiotensin-converting enzyme inhibitor), which induces a salt appetite more rapidly than furosemide alone (4), has also been reported to induce c-fos rapidly in the SFO (33). The SFO also expresses c-fos in response to osmotic stimulation such as hypertonic NaCl injection (9,17), or electrical stimulation of baroreceptors (14).

The present study extended these reports by characterizing the pattern of c-FLI which occurs during recovery from sodium depletion. The depleted rats avidly consumed 0.3 M NaCl, and satiation occurred within 15–30 min (Fig. 1). Despite the rapid cessation of the depletion-induced salt intake, the number of c-FLI-positive cells was not significantly decreased in the SFO until 720 min after salt access. Thus continuing SFO activation is not sufficient to maintain the salt appetite after 0.3 M NaCl intake. Some other neural consequence of 0.3 M NaCl intake (taste or postingestive) acts to inhibit the salt appetite despite continuing SFO activation. (An important caveat to this interpretation is that the persistence of c-FLI in the SFO may reflect a long half-life of the c-fos protein rather than persistent neuronal activation. Note, however, that the number of c-FLI-positive neurons fell faster in the NTS than the SFO, suggesting either persistent SFO activation or a longer half-life for c-FLI in the SFO than the NTS.)

The expression of c-FLI in the SFO following sodium depletion may be a response to the increased levels of AII that occur during sodium depletion both in the circulation and centrally (20). The SFO possesses AII receptors and is responsive to circulating AII (23): intraventricular AII induces c-fos expression in the SFO (11) and intraventricular injection (1) or local application of AII to the SFO (16) elicits robust water and salt intake. c-FLI expression in the SFO also parallels the aldosterone response to sodium deple-

tion and repletion (28). The effects of aldosterone on c-fos expression have not yet been characterized, however. Taken together, these observations support the hypothesis that the c-FLI activity in the SFO reflects a neural response to peripheral humoral and neural changes during sodium depletion. The extended expression of c-FLI in the SFO after repletion may be a consequence of the gradual decline in circulating renin, angiotensin, and aldosterone, which only return to replete levels 60–120 min after salt intake (28).

Whereas the expression of c-FLI in the SFO is persistent and correlated with depletion state, c-FLI in the NTS is transient and follows the behavior of salt intake. Ingestion (32) or gastric loads (12) of hypertonic NaCl have been shown to induce c-fos in the medial NTS. Since visceral afferents (the vagus nerve and, indirectly, spinal afferents) project to the medial NTS, c-FLI in the NTS may reflect activation of primary and secondary sensory relays by NaCl in the gut. Interestingly, the pattern of c-FLI in the caudal NTS is similar to that induced by peripheral bombesin administration in sodium-replete rats (2,13). Bombesin and its mammalian analogs can acutely attenuate depletion-induced salt appetite (5,6). It may be that the pattern of c-FLI observed here reflects, in part, the role of endogenous bombesin analogs in the natural sequence of satiation of salt appetite.

Some c-FLI-positive cells were also present in the rostral NTS and the lateral caudal NTS adjacent to the solitary tract after salt ingestion. The rostral and lateral NTS receive afferent input from taste nerves innervating the tongue and oral cavity. Expression of c-FLI in the rostral and lateral NTS may therefore reflect central activation by the orosensory component of salt ingestion.

The NTS also has dense reciprocal connections with forebrain structures such as the central nucleus of the amygdala (3) which play a role in sodium appetite (8). Thus, some of the c-FLI-positive

cells observed in the NTS after satiation of the sodium appetite may be secondarily activated by descending forebrain inputs to the brainstem.

The present study is a preliminary survey of the time course of c-FLI induction in central sites following sodium depletion and repletion. More studies will be needed to extend these results from correlation to mechanism. These results are consistent, however, with proposed mechanisms by which a sodium deficit is detected by central neurons (possibly in the SFO) and the satiation of salt appetite by the activation of taste and

postingestive relays in the brainstem during and following salt intake. Thus c-FLI in the SFO and in the NTS may serve as useful and quantifiable markers of depletion state and ingestive sensory (taste or gastrointestinal) stimulation, respectively, in the experimental analysis of sodium appetite and satiation.

ACKNOWLEDGEMENTS

This study was supported by NIMH 528382 (T.A.H.), NIH DK-39180 (S.P.F.), MH 24285 (T.H.J.), and MH 00149 (G.P.S.).

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