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Water-deprived white-footed mice express c-*fos* on a day/night cycle graded according to the duration of deprivation

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Abstract

Mammals respond to electrolyte and water imbalance by a variety of neural and endocrine mechanisms that regulate water and salt intake and loss. We used the expression of c-*fos* and Fos-related antigens to indicate neuronal activation in hypothalamic neurons of members of an outbred laboratory population of white-footed mice (*Peromyscus leucopus*) deprived of water for biologically reasonable periods of time (6–18 h). We examined Fos-like immunoreactivity (Fos-LIR) in the supraoptic nucleus (SON) and paraventricular nucleus (PVN). During the dark period, when these animals are normally active, 6 h of water deprivation produced near-maximal increases in the number of cells positive for Fos-LIR in the SON and PVN. In contrast, during the light period, when these mice are normally inactive and do not have access to water, 6 h of water deprivation only slightly affected Fos-LIR. During the day, it required as much as 12 h of water deprivation to produce increases in Fos-LIR cells approaching those achieved at night. Plasma osmolarity was directly related to the number of Fos-LIR cells. In addition, mice lost weight more rapidly at night than during the day when water-deprived, and also recovered that lost weight more rapidly when access to water was returned. Our results show (1) that biologically reasonable levels of water restriction (and resulting changes in blood osmolarity) induce changes in Fos-LIR in this wild mouse species, and (2) that these mice have a daily cycle of sensitivity to water deprivation that is demonstrated by both behavioral, psychological and immunohistological assessment of reactions to water deprivation. © 1998 Elsevier Science B.V.

Keywords: Osmoregulation; Fos; Daily cycle; Water deprivation; Water balance; Thirst; *Peromyscus*

1. Introduction

Terrestrial mammals must maintain water balance in the context of a daily cycle of activity and inactivity. During part of their daily cycle, many mammals are forced to be inactive, due to such factors as changes in temperature, predation risk, or visibility. An animal that is gradually losing body water during its inactive period can either tolerate the loss of water or minimize water loss through physiological or behavioral mechanisms.

Water balance and electrolyte balance are maintained by osmoreceptors and baroreceptors that activate or suppress behavioral or physiological responses that counteract changes in the volume and osmolality of fluids by adjusting ingestion and excretion of water and salt (reviewed in Refs. [5,10,25]). In response to extracellular fluid (ECF) hyperosmolality or a reduction in fluid volume, magnocellular neurosecretory cells (MNC) in the supraoptic nuclei (SON) and paraventricular nuclei (PVN) secrete vasopressin (VP) and oxytocin (OT). VP reduces renal water excretion (reviewed in Ref. [5]). In rats, OT increases renal sodium excretion [3,8,17] and can also reduce renal water excretion [7]. Thus, these two hormones counter the effects of ECF hyperosmolality or fluid loss (reviewed in Refs. [5,10,25]). VP and OT (synthesis and release) are known to be affected by time of day, photoperiod, and melatonin [16,31]. This suggests that animals might respond differently, either physiologically or behaviorally, to changes in water balance according to time of day.

A commonly used marker for neural activity in response to changes in ECF osmolality or volume is a nuclear protein, Fos [15,27,28], the product of the c-*fos* proto-oncogene. This gene is one of a family of immediate-early genes which may act as third messengers regulating the expression of other genes [23]. Immunocytochemical identification of Fos and Fos-related proteins as markers for changes in water balance has been used extensively in laboratory rats [9,14,26,28,29]. Water deprivation in-

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duces Fos activation in the SON and PVN [9,28,29], while water intake inhibits Fos expression [13]. Other osmotic or cardiovascular stimuli that also trigger changes in neural activity in these neurons include hypertonic saline injections (both i.c.v. and i.p.) [11,26,28] and hemorrhage [9]. There is evidence that Fos expression increases in a graded fashion in relation to the neurosecretory activity of vasopressinergic and oxytocinergic magnocellular neurons (reviewed in Ref. [15]).

In this paper, we examine neuroendocrine and behavioral responses to biologically reasonable levels of water deprivation (0–18 h) in an outbred laboratory colony of white-footed mice (*Peromyscus leucopus*). We compare responses of mice during their active and inactive period in a light cycle of 16 h light and 8 h dark, chosen to approximate the light cycle of a long temperate-zone summer day (including twilight), when the effects of water deprivation might be the most extreme. This species is widely distributed in North America, living in a variety of woodland and field habitats that include areas that may lack easy access to water. White-footed mice cannot produce sufficient metabolic water to balance respiratory and urinary water loss [21], and they have relatively greater evaporative water loss than some other small rodents of equivalent size in similar habitats [12,22]. Therefore, this species is a good choice for assessing the potential involvement of Fos in a non-domesticated species given biologically reasonable challenges to water balance.

2. Materials and methods

2.1. Animals

Experiments were performed on young adult *P. leucopus* (approximately 15–25 g, 60–120 days old). The mice were produced in an outbred laboratory colony at the Population and Endocrinology Laboratory of The College of William and Mary. The founders of the population were captured in the vicinity of Williamsburg, VA. Mice were raised with same-sex siblings under an L16:D8 cycle (lights on at 0500 and lights off at 2100 h). Food (Agway Prolab Rat/Mouse/Hamster 3000, Syracuse, NY) and water were provided ad libitum except as described for the experiments below. During periods over which experiments were performed, the relative humidity of the animal rooms averaged $49 \pm 6\%$ (mean $\pm$ S.E.) and the room temperature was 22 ± 2°C. At the beginning of all experimental treatments, all mice were separated into individual cages, and controls were disturbed by cage movements at the same time as experimental animals. On the morning of perfusions, mice were transported to an animal room in a second building (relative humidity $45 \pm 4\%$; temperature 22 ± 2°C) and held until euthanasia. All animals were anesthetized with an overdose of sodium pentobarbital (200 mg/kg body weight i.p.) prior to perfusion.

2.2. Immunocytochemistry

Expression of Fos-like immunoreactivity (Fos-LIR) in the SON and the PVN was detected using a single-labeled avidin–biotin-peroxidase-complex method. Mice were perfused through the left ventricle at approximately 4 ml/min using a Harvard Apparatus perfusion pump and bled via the right atrium. Perfusion of 5 ml of 0.1 M phosphate-buffered saline (PBS) at a pH of 7.4 with 15 IU heparin/ml was followed by perfusion of 50 ml of fresh cold (5°C) 4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) in PBS. Brains were removed and post-fixed at 4°C for 2 h and then placed overnight at 4°C in 0.1 M PBS with 30% sucrose for cryoprotection.

Frozen coronal sections (40 μm) were cut on a cryostat and rinsed three times for 10 min each in cold PBS. All subsequent treatments were conducted with gentle agitation at room temperature unless otherwise noted. Tissue was incubated with 1.4% normal goat serum (NGS; Vector Laboratories, Burlingame, CA) and 0.2% Triton X-100 (Fisher Scientific) in PBS for 20 min followed by 42 h incubation at 4°C with a rabbit anti-Fos polyclonal antibody (K-25, catalog #sc-253, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:10,000 in PBS with NGS and Triton X-100. This antibody is broadly reactive with c-Fos, Fos B, FRA-1, and FRA-2. Sections were given three 10-min rinses in PBS and incubated in biotinylated goat anti-rabbit immunoglobulin G (1:200 in PBS with NGS and Triton X-100, Vector Laboratories) for 1 h at room temperature. After three more rinses in 0.1 M PBS, sections were incubated in avidin–biotin-peroxidase (Vector Laboratories Elite ABC-Peroxidase kit) in PBS for 1 h. Sections were given three rinses in Tris buffered saline gel (TBS gel) (0.1 M, pH 7.6, FisherBiotech, Pittsburgh, PA and Sigma Chemical, St. Louis, MO) and placed in 1.5 ml of a solution of diaminobenzidine (DAB; 0.2 mg/ml), $NiSO_4$ (24 mg/ml), and H_2O_2 (0.83 μl/ml of freshly prepared 3% H_2O_2; Sigma Chemical) in Tris buffer (0.1 M, pH 7.6). The color reaction was allowed to proceed for approximately 10 min. After three rinses in Tris buffer, sections were mounted on gelatin-coated slides and air dried, dehydrated in xylene, and coverslipped with Permount (Fisher Scientific). Following mounting, any brain in which the hypothalamic regions of interest were damaged (by an assessment blind with respect to treatment) was removed from the study.

In order to test whether VP and OT were present in the SON and PVN and whether they colocalize with Fos in white-footed mice, Fos-LIR-stained tissue from four mice was further treated, before slide mounting, by incubation in rabbit anti-arginine–VP, rabbit anti-OT polyclonal primary antibodies (both 1:10,000 dilution in 0.1 M PBS with 1.4% NGS and 0.2% Triton X-100, catalog #AB1565 and #AB911, respectively; Chemicon International, Temecula, CA), or mouse anti-arginine–VP (1:500 dilution, catalog #MAB018-45/1; Chemicon) at room temperature for 1 h

and then at 4°C overnight. On the following day, the DAB staining protocol (above) was repeated without $NiSO_4$ in order to produce a red–brown color localized to the cytoplasm and distinguishable from the blue/black nuclear staining for Fos. Analysis of these sections was limited to confirmation of the presence of all three kinds of staining in the SON and PVN.

Tests for specificity of the primary antibodies used were conducted using a control Fos peptide, arginine–VP peptide, and OT peptide (catalog #sc-253 P, for Fos protein, Santa Cruz Biotechnology; catalog #O4375 and V9879 for VP and OT, respectively, Sigma Chemical). In each case, antibody was preincubated for 1 h with specific antigen (1 mg/ml) in PBS with normal serum and Triton-X prior to incubation with tissue. Additional tests for specificity were conducted by omitting the primary antibody, the secondary antibody, or both. None of these tests produced specific staining.

2.3. Quantification of Fos

Nuclear staining for Fos-LIR in the SON and PVN was analyzed using an image analyzer (Bioquant IV) under 400 × magnification. Analyses were conducted in random order by a single individual blind with respect to treatment. Four mid-nuclear SON and four mid-nuclear PVN sections were chosen from each animal. The total number of Fos-LIR cells in the four SON and PVN sections of each animal was used in the statistical analyses. To assess repeatability and accuracy of the data collection, two additional observers collected data on a subset of all sections. In these, all counts were within 15% of the original (and most were much more similar) over a range of 1 to 105 positive neurons/section ($N = 54$ sections recounted).

2.4. Experiment 1: levels of water deprivation

The first experiment tested for effects of different periods of water deprivation without controlling for time of day of water deprivation. Water bottles were removed from the cages of male mice for 6, 12, or 18 h ($\pm 1/2$ h). A fourth treatment group of euhydrated animals, defined here and below as those with ad libitum access to water, served as negative controls. Finally, a fifth group of animals injected with hypertonic saline solution (0.37 ml of 1.5 M NaCl i.p.) 1.5 h before perfusion served as positive controls. In this and all other experiments, lights came on at 0500 h on a light cycle of L16:D8. All mice were perfused during the light period between 0900 and 1600. Mice in the 6-h treatment were water-deprived during the light period (water removed between 0700 and 1200 h), and mice in the 12 and 18-h groups were water-deprived during both their light and dark period (water removed between 1600 and 2100 h). Not all brains could be processed for immunocytochemistry simultaneously, so the experiment was conducted over a series of experimental runs balanced across treatments. The final sample size was six animals per treatment group. Fos-LIR was analyzed in the SON and PVN.

2.5. Experiment 2: time of day differences

This experiment investigated Fos-LIR in response to water deprivation during subjective day ('day', hereafter) and subjective night ('night', hereafter) for various lengths of time between 6 and 12 h. Male mice were water deprived for 6, 8, 10 or 12 h ($\pm 1/2$ h), with euhydrated animals serving as negative controls. Thus, this experiment had a 2×5 design (two times of day by five treatments) with final sample sizes of five or six in each treatment. Our longest periods of water deprivation were longer than the 8-h dark period. In order to provide a graded series of dark-period water-deprivation treatments, 2/3 of each night water-deprivation treatment occurred during the dark period, and 1/3 during the light. Thus, only the 12-h night treatment included the entire 8-h dark period (water removed at 2030 h); the 10-, 8-, and 6-h night treatments included 7, 5.5, and 4 h of the dark-period, respectively (water removed at 2200, 2330, and 0100, respectively). Water was removed from the cages of the 12-, 10-, 8-, and 6-h day treatments at 0630, 0730, 0830, and 1000 h, respectively. For both night and day water-deprived animals, euhydrated controls were perfused at similar times (0600–0900 for the night control, and 1600–1900 for the day control).

Not all brains could be processed for immunocytochemistry simultaneously, so the experiment was conducted over a series of experimental runs balanced across treatments.

2.6. Experiment 3: plasma osmolarity during water deprivation

In order to determine the relationship between Fos-LIR and electrolyte balance during subjective day and night, we measured the plasma osmolarity during water deprivation (0, 6 and 12 ± 0.5 h). Again, because the subjective short night of a summer day was only 8 h in duration, the night water-deprivation groups included both light and dark periods. As in the second experiment, 2/3 of each 'night' water-deprivation treatment occurred during the dark period, and 1/3 during the light. Removal of water occurred at 0100 (6-h night treatment), 2030 h (12-h night treatment), 0830 h (6-h day treatment), and 0630 h (12-h day treatment) on a L16:D8 cycle with lights on at 0500. In this experiment, female mice were included to increase sample size ($N = 4$ males and 4 females per treatment group), and the statistical analysis included sex as a variable (see below).

Mice were rendered unconscious in Halothane, following which trunk blood was collected in heparinized collection tubes. Osmolarity of plasma samples was measured in

duplicate using two Wescor 5500 vapor pressure osmometers (Wescor, Logan, UT).

2.7. Experiment 4: weight loss during water deprivation

In order to investigate how mice responded to the absence of water during subjective day and night, we measured the weight lost during water deprivation (0, 6, and 12 ± 0.25 h) and weight gained after the return of water for 2 h. The timing of water removal followed that for experiment 3. The rehydration periods for all groups occurred during the light period. During the 2 h for rehydration, food was removed from all cages, including the euhydrated controls, in order to prevent increases in body weight due to food consumption alone. Controls were disturbed and weighed at times matching disturbances to water-deprived mice.

Water loss was estimated by the difference in body weight, relative to controls, before and after the deprivation period [21]. As in experiment 3, female mice were included to increase sample size, and the statistical analysis included sex as a variable. Sample sizes were four females and four males in all except one treatment group with four females and three males. This experiment was conducted using two runs with 23 or 24 animals per run. Each run was balanced across treatments.

2.8. Identification of VP- and OT-ergic neurons

Because both VP and OT have been shown to play roles in the regulation of water balance [2,5,9,30], we examined the distribution of VP and OT and their colocalization with Fos-like proteins within the PVN and SON of white-footed mice. Four male mice were given 12 h of water restriction including the subjective night (water bottle removed at 2030 h). Serial sections from each brain were divided among four staining treatments using the following antibodies or combinations of antibodies: Fos-polyclonal followed by OT-polyclonal, VP-polyclonal, or VP-monoclonal. Some sections were pretreated with OT, VP, and/or Fos control peptide as negative controls; no specific staining was found in these sections.

2.9. Statistical analyses

Analyses were conducted using Statview (1.04 A) or Super Anova (1.11; both from Abacus Concepts, Berkeley, CA) on a Macintosh computer. Data sets were tested for homogeneity of variances; where variances were not homogeneous (experiments 1 and 2), data analyses were carried out on log-transformed data. In all analyses, data were considered to be significant at $P < 0.05$. In experiment 1, one-way analysis of variance (ANOVA) was used to compare numbers of Fos-LIR cells in the different treatment groups. Preplanned pair-wise comparisons between treatment groups and the control were made using Fisher's Paired Least Significant Difference. Post hoc comparisons were conducted to compare the 6-h vs. 12-h and 6-h vs. 18-h treatments.

In experiment 2, two-way ANOVA was used to compare numbers of Fos-LIR cells found in water-deprived mice for various time lengths during day or night. Preplanned pair-wise comparisons were made using Fisher's Paired Least Significant Difference. In addition, comparisons between control and water-deprivation treatments were carried out to identify the minimum period of water deprivation necessary to cause a significant increase in Fos-LIR during either the day or night period.

In experiments 3 and 4, three-way ANOVA was used to analyze the effects of water deprivation (or return) for various time lengths during day or night cycles across gender. The analyses were carried out on plasma osmolarity and weight loss following water deprivation and weight gain upon return of water. The following post-hoc pair-wise comparisons were conducted: all day treatment groups were compared to their corresponding night treatment groups, and the 6-h night group was compared with the 12-h day group and with the 12-h night group.

3. Results

3.1. Experiment 1: water deprivation over time

In the SON, water deprivation resulted in large increases in Fos-LIR cells over time ($F = 66.1$; $P < 0.0001$; Fig. 1A). Both 12 and 18 h of water deprivation resulted in increased levels of Fos-LIR ($F = 140.6$, $P < 0.0001$; $F = 114.6$, $P < 0.0001$, respectively), while 6 h of water deprivation had no effect ($F = 1.9$, $P > 0.10$). A post-hoc comparison showed that there was a significant difference between 6 and 12 h of water deprivation ($F = 108.6$, $P < 0.0001$) and between 6 and 18 h of water deprivation ($F = 85.9$, $P < 0.0001$).

Similarly, in the PVN, water deprivation caused increases in Fos-LIR cells over time ($F = 8.2$; $P < 0.001$; Fig. 1B). Both 12 and 18 h of water deprivation resulted in increased levels of Fos-LIR cells ($F = 14.6$, $P < 0.001$; $F = 10.0$, $P < 0.005$, respectively), while 6 h of water deprivation had no effect ($F = 0.1$, $P > 0.10$). A post-hoc comparison showed a significant difference between the Fos-LIR in the PVN of 6-h and 12-h water-deprived mice ($F = 17.5$, $P < 0.001$) and between 6-h and 18-h water-deprived mice ($F = 12.5$, $P < 0.005$).

3.2. Experiment 2: water deprivation during the day and night

There was a large day and night difference in the number of Fos-LIR cells in the SON ($F = 27.3$; $P < 0.0001$; Fig. 2A and Fig. 3). Water deprivation that included the dark period produced more Fos-LIR than water

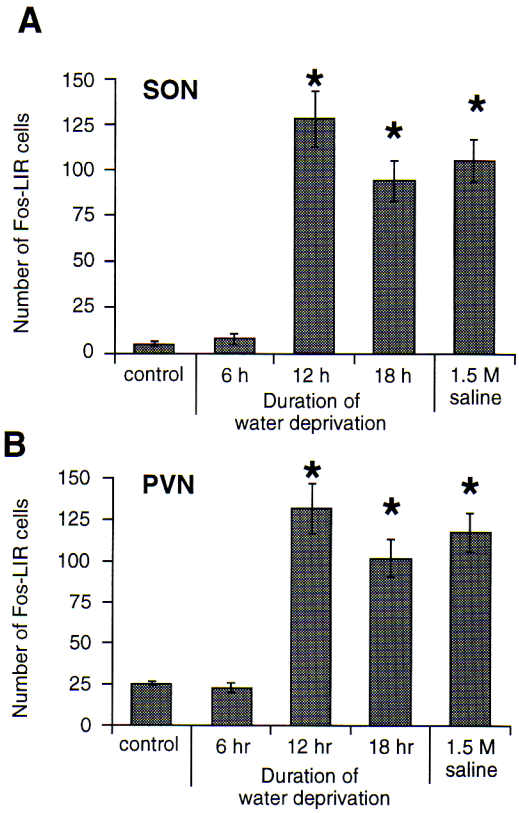


Fig. 1. Number (mean ± S.E.) of Fos-LIR cells/section in the SON (A) and in the PVN (B) after water deprivation for 0, 6, 12, and 18 h and for a 1.5 M NaCl-injected positive control (* indicates a significant difference from 0-h negative control).

deprivation during the light period. The control animals, in contrast, showed no difference in Fos-LIR ($F = 3.9$; $P > 0.05$). As in the first experiment, water deprivation over time also caused a significant increase in the number of Fos-LIR cells ($F = 16.4$; $P < 0.0001$) with a non-significant interaction between time of day and hours of deprivation ($F = 0.4$; $P > 0.10$). Water deprivation for as little as 6 h in the night-treatment group produced a large and highly significant increase in the number of Fos-LIR cells ($F = 18.1$; $P < 0.0001$; Fig. 2A and Fig. 3). During the day, 6 h of water deprivation produced a smaller but significant effect ($F = 9.3$; $P < 0.01$). Fos-LIR was always lower during the day ($F = 11.9$, $P < .005$; $F = 5.9$, $P < 0.05$; $F = 9.5$, $P < 0.005$; $F = 4.6$, $P < 0.05$ for 6-, 8-, 10-, and 12-h water deprivation, respectively), and it required 12 h of water deprivation during the day to approach the number of Fos-LIR cells observed in the 6-h night group. In contrast, 6 h of water deprivation during the night induced near-maximal numbers of Fos-LIR cells (Fig. 2A and Fig. 3).

In the PVN, there were also day and night differences in the number of Fos-LIR cells ($F = 31.3$, $P < 0.0001$; Fig. 2B), and there was a significant effect of duration of water deprivation ($F = 6.3$, $P < 0.0005$). No interaction was found between time of day and hours of deprivation ($F = 1.4$, $P > 0.10$). For every duration of water deprivation, there were more Fos-LIR cells in the PVN during the night ($F = 13.7$, $P < 0.001$; $F = 8.2$, $P < 0.01$; $F = 9.3$, $P < 0.005$; and $F = 6.0$, $P < 0.05$ for 6-, 8-, 10- and 12-h water deprivation, respectively). As in the SON, water for as little as 6 h in the night treatment group lead to a large increase in the Fos-LIR cells in the PVN ($F = 6.6$, $P < 0.05$). During the day, in contrast, water deprivation for even 12 h did not produce a significant increase over the euhydrated control ($F = 4.06$, $P > 0.05$).

3.3. Experiment 3: plasma osmolarity during water deprivation

There were no significant differences in plasma osmolarity between the sexes ($F = 1.26$, $P > 0.10$), nor were any trends apparent. The data from males and females were combined for further analyses.

Water deprivation over time caused a significant increase in plasma osmolarity ($F = 7.1$, $P < 0.05$; Fig. 4), and this difference depended on the time of day at which the water deprivation occurred ($F = 10.6$, $P < 0.001$). The control and 6-h water-deprivation groups did not differ in plasma osmolarity ($F = 0.3$, $P > 0.10$). The 12 h of water

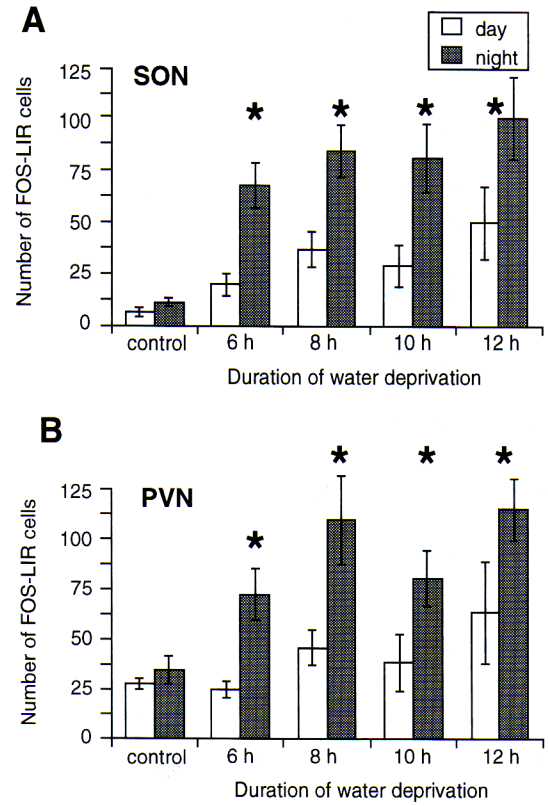
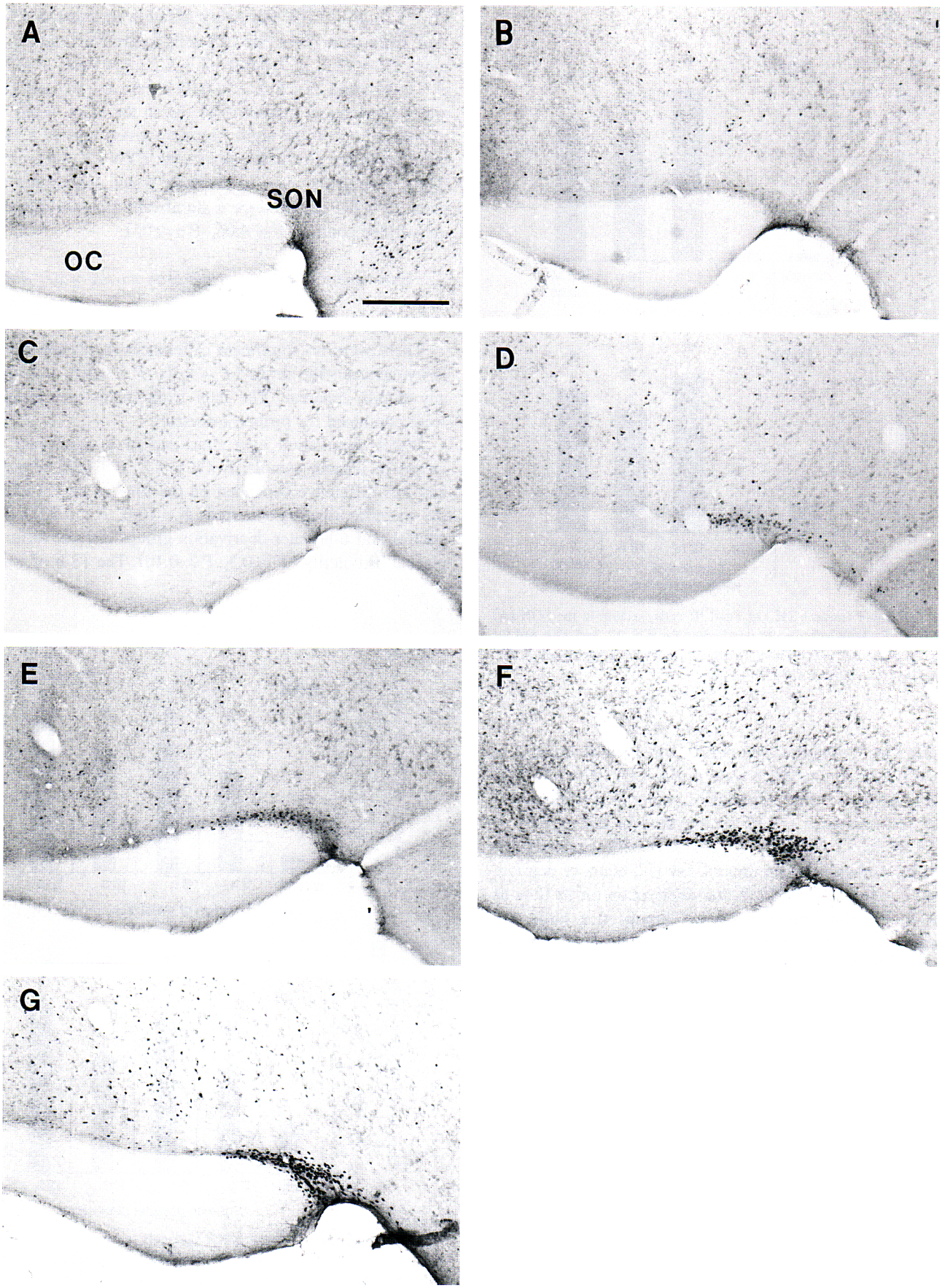


Fig. 2. Number (mean ± S.E.) of Fos-LIR cells/section in the SON (A) and in the PVN (B) after water deprivation for 0, 6, 8, 10, and 12 h including either subjective day or subjective night (see Section 2). Gray bars represent dark-period water restriction; open bars indicate light-period water restriction (* indicates a significant day/night difference).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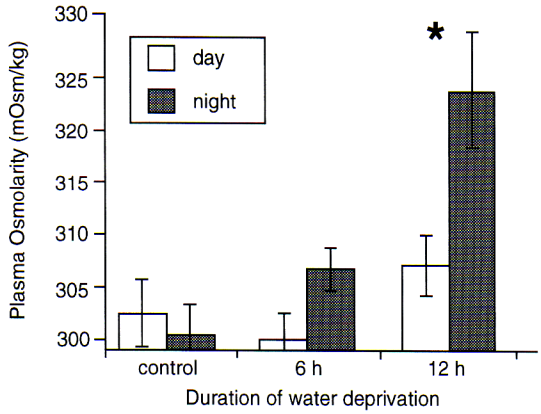

Fig. 4. Plasma osmolarity during the light period and dark period after 0, 6 or 12 h of water deprivation. Gray bars represent dark-period water restriction; open bars indicate light-period water restriction (* indicates a significant day/night difference).

deprivation at night produced much higher plasma osmolarity than other group ($P < 0.05$ for all comparisons; Fig. 4).

3.4. Experiment 4: effects of water deprivation on weight loss and recovery

There were no significant differences in weight loss or in weight gain between the sexes ($F = 0.28$, $P > 0.10$; $F = 0.65$, $P = 0.10$, respectively), nor were any trends apparent. The data from males and females were combined for further analyses.

Water deprivation over time caused a significant decrease in the weight of the mice ($F = 39.8$, $P < 0.0001$; Fig. 5A), and this difference depended on the time of day at which the water deprivation occurred ($F = 10.0$, $P < 0.0005$). The control groups did not differ in their weight change ($F = 1.9$, $P > 0.10$), although they lost small amounts of weight. 6 h without water during the night produced more weight loss, relative to controls, than the same deprivation during the day ($F = 20.2$, $P < 0.001$; Fig. 5A). In fact, 6 h without water during the night produced as much weight loss as 12 h of water-deprivation during either the night or day (differences not significant; $F = 0.8$, $P > 0.10$ for both comparisons). The weight loss induced by 12-h of water deprivation was similar in the night- and day-deprived groups ($F = 1.0$, $P > 0.10$; Fig. 5A).

Measurements of weight gained following the return of water also showed a time of day effect ($F = 12.1$, $P < 0.005$; Fig. 5B) and an effect of duration of water deprivation ($F = 22.5$, $P < 0.0001$). Mice rehydrated more following dark period water deprivation than following light-period water deprivation after either 6 h or 12 h of deprivation ($F = 8.7$, $P < 0.05$, and $F = 9.7$, $P < 0.01$, respectively). Even after 12 h of water deprivation during the day, rehydrating mice did not recover as much weight as those subjected to only 6 h of water deprivation at night ($F = 19.3$, $P < 0.001$).

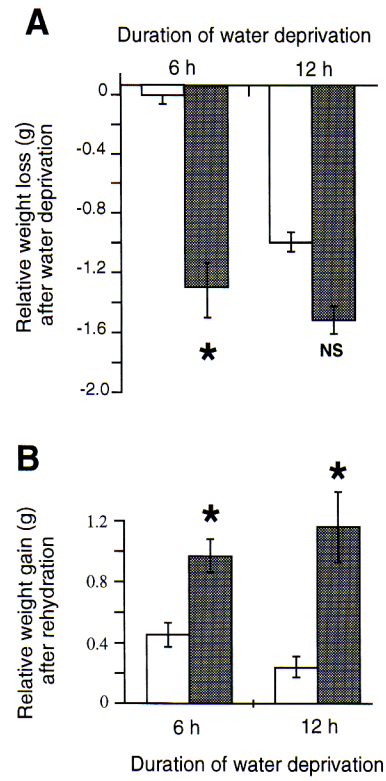

Fig. 5. Changes in weight, relative to controls, during the light period and dark period after 6 or 12 h of water deprivation (A), and following 2 h of rehydration (B). Gray bars represent dark-period water restriction; open bars indicate light-period water restriction. Changes in weight are shown after subtracting the weight changes of euhydrated controls (* indicates a significant day/night difference).

3.5. Distribution of VP- and OT-ergic neurons

Both VP- and OT-positive cells were abundant in the SON and PVN, areas in which Fos-LIR cells were also abundant. In addition, Fos-LIR commonly colocalized with VP or OT immunoreactivity. These observations on white-footed mice are consistent with previous work on rats showing that the Fos-LIR cells activated by water restriction are predominantly VP- and OT-ergic neurons [9].

Fig. 3. Light micrograph showing Fos-LIR in SON sections from representative animals. In each case, the section shown has Fos-LIR cell numbers near the mean for that treatment group. (A) SON of a euhydrated, day control mouse. (B) SON of a euhydrated, night control mouse. (C) SON of a 6-h day water-deprived mouse. (D) SON of a 6-h night water-deprived mouse. (E) SON of a 12-h day water-deprived mouse. (F) SON of a 12-h night water-deprived mouse. (G) SON of a hypertonic-saline injected mouse. Abbreviations: supraoptic nucleus (SON), optic chiasm (OC). Scale bar = 250 μm.

4. Discussion

Our results show that white-footed mice respond differently during the day and the night to biologically reasonable periods of dehydration, and these differences were apparent by both behavioral and physiological measures. In a previous study carried out on rats, water deprivation for 8 h or more (which included 6 h or more of a 12-h night) induced modest increases in c-*fos* mRNA, while shorter periods had no effect [6]. We found that slightly shorter periods had significant effects on Fos expression in white-footed mice, but that those effects were very slight unless they included part of the dark period (Figs. 2 and 3). During the night, however, 6 h without water caused a near-maximal increase in Fos-LIR cells in the SON and PVN (Figs. 2 and 3). Similarly, water deprivation at night produced greater effects on plasma osmolarity than did deprivation during the day (Fig. 4).

Our results suggest that Fos-LIR in the SON may be a more sensitive indicator of changes in water balance than is Fos-LIR in the PVN. In the SON of euhydrated mice, the numbers of Fos-LIR cells were extremely low (Figs. 1 and 2), and increased approximately 20-fold in mice water deprived for 12 h. In the PVN of euhydrated controls, in contrast, the numbers of Fos-LIR cells were higher, but increased only about five-fold in mice water-deprivation for 12 h. The PVN could be a less sensitive indicator of water deprivation than the SON because these two areas of the hypothalamus are associated with different aspects of osmotic and water balance, or there may be subpopulation of neurons in the PVN that are not involved in water or osmotic balance. The SON of rats consists largely of magnocellular OT and VP neurons [9,24], while the PVN includes parvocellular and magnocellular neurons known to be involved in many other neural circuits.

Both the SON and PVN had a greater response to water restriction during the night, the active period for these mice. This day/night difference in response to water restriction was due to greater rates of water loss at night, resulting in higher plasma osmolarity (Fig. 4), and not to lower tolerance of water loss during the night. The greater loss of body fluid at night could be due to increased activity at night. This is supported by observations of increases in metabolic VO_2 of caged *Peromyscus* during night relative to their daytime resting period (E.L. Bradley, personal communication). Mice lost more water during the night (Fig. 5), but were able to maintain near-normal plasma osmolarity (2% increase over controls) even after substantial weight losses during 6 h at night or 12 h during the day. The 12 h of water deprivation at night produced only a modest additional loss of weight (Fig. 5), but mice deprived of water for 12 h were unable to maintain osmotic homeostasis and had undergone an 8% increase in plasma osmolarity (Fig. 4). In a previous study on laboratory rats, an 8% increase in plasma osmolarity was also accompanied by a large increase in Fos-LIR [20].

Our test for day/night differences in rates of water loss and water recovery using estimates from weight measurements (following Ref. [21]), may have been affected by changes in food intake and fecal losses as well as changes in hydration. However, increases in body weight following return of the water bottle in this study should be due entirely to water intake, because food was not available during the 2-h rehydration period. Relative to the controls, water-deprived mice lost weight more rapidly at night than during the day (Fig. 5), suggesting that the day/night difference in Fos-LIR reflects differences in the rate of water loss. This interpretation is reinforced by the fact that mice also gained more weight after dark-period water deprivation, recovering much of their lost weight, than after light-period deprivation (Fig. 5).

Daily rhythms of Fos expression occur in rodent brains (e.g., Refs. [18,19]), but these are not typically found in the SON or PVN (but see Ref. [1]), nor did we see any evidence of day/night differences in the SON or PVN of our euhydrated control animals (Fig. 2). Stress can induce Fos-LIR in the PVN [4], in some cases by handling and injection alone [28]. Thus, stress responses, either to handling alone or specifically to water deprivation, could contribute to Fos-LIR, especially in the PVN. However, handling stress could not be the cause of the responses to water deprivation we observed, because handling stress should have been induced in our euhydrated controls as well as in our experimental animals. We cannot rule out the possibility that stress responses specific to water deprivation (e.g., loss of homeostasis) contribute to the increases in Fos-LIR we observed in the PVN, however.

In the context of water and electrolyte balance, we interpret our results as follows. Water deprivation produces changes in intra-cellular fluid volume and osmolality that stimulate peripheral osmoreceptors and baroreceptors, acting by way of brain areas such as the subfornical organ, organum vasculosum of the lamina terminalis, medial preoptic area, and other brain regions [5]. These areas then stimulate neurohypophyseal VP and OT release from magnocellular neurosecretory neurons in the SON and PVN, thereby increasing water retention and sodium excretion. Our results show that the Fos family of transcription factors is involved in the physiological response to water deprivation following relatively short periods of water deprivation during the active period (night), but not during the inactive period (day) of these mice. This may also be true for other mammals. While laboratory rats have been reported to undergo only modest increases in c-*fos* expression following 8–16 of water deprivation [6], it may be that stronger responses would obtained following water restriction at night, the active period for rats.

A number of studies on rats and Syrian hamsters have demonstrated that light or the pineal gland and its hormone, melatonin, can modulate the synthesis or secretion of VP and OT (e.g., Refs. [16,31]). We hypothesize that these effects of light, acting via the suprachiasmatic nu-

cleus and/or melatonin and the pineal, act to produce appropriate responses to changes in water or electrolyte balance according to time of day and the freedom of the animal to seek a source of water. Because an animal's options for defending or regaining water and electrolyte balance change over the day, the animal's responses, both physiologically and at the level of the brain, should also change on a day/night cycle. If our hypothesis is correct, then we predict that diurnal rodents would show responses opposite to the nocturnal rodents studied to date—rats [31], Syrian hamsters [16], and white-footed mice (this study).

This study is the first to provide evidence for the involvement of Fos in the normal, physiological response of animals to modest day vs. night periods without water. Most studies on rats or other mammals use paradigms in which access to water is removed for 24 to 72 h or more, a condition which probably would be rare for most of these species in the wild. Our results suggest that Fos immunocytochemistry could be used to provide comparisons of responses to biologically reasonable changes in water and salt balance within and between species. Finally, the presence of day/night differences in response to short periods of water deprivation (this study) and saline injection (e.g., Ref. [31]) emphasizes the importance of controlling for time-of-day effects in planning and interpreting studies on water balance.

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