DAY AND NIGHT DIFFERENCES
IN WATER REGULATION
OF *PEROMYSCUS LEUCOPUS*

A Thesis

Presented to

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by

Haejin Erin Chae

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APPROVAL SHEET

This thesis is submitted in partial fulfullment of

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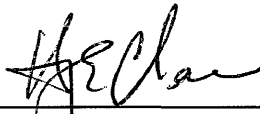

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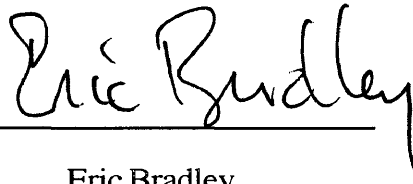

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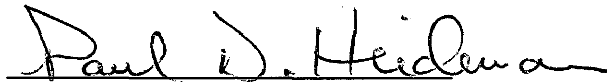

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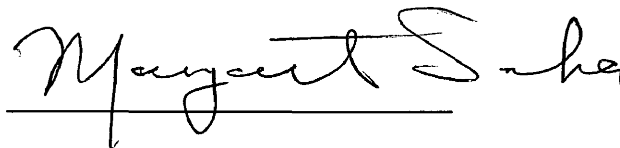

Margaret Saha

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ABSTRACT

This study was conducted to describe first some neuroendocrine responses to different levels of dehydration during physiologically relevant lengths of time without water and, second, to assess the time of day effects on responses to water deprivation in white-footed mice, *Peromyscus leucopus.* I examined fos-like immunoreactivity (FOS-LIR) in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) . Six hours of water deprivation did not significantly increase the FOS-LIR in the SON nor the PVN, while 12 and 18 h treatment group showed a dramatic increase. An examination of 6, 8, 10, and 12 h of water deprivation in the day and night show that there is a clear day and night difference in how the brain responds to water deprivation. The night water-deprived mice had more FOS-LIR cells in the SON and the PVN than day water-deprived mice . To observe how the whole animal responds to the absence of water during day and night, change in weight during the 6 and 12 h of water deprivation was measured. The results indicate that there exists a day and night difference in the amount of weight lost for the 6h but not for the 12h treatment groups. Returning water to these dehydrated animals and measuring the weight recovered caused the night 6 and 12 h water-deprived animals to gain more than the corresponding day treatment groups, even though none of them fully regained all the weight lost. These results suggest that *P. leucopus* maintains proper water balance by activating the SON and PVN more strongly and/or by making the hypothalmic areas more sensitive to water loss at night. It also suggests that the ability to recover from this type of dehydration depends on the time of day and, perhaps, on activity level.

DAY AND NIGHT DIFFERENCES IN WATER REGULATION OF *PEROMYSCUS LEUCOPUS*

INTRODUCTION

Mammals have evolved sophisticated behavioral and physiological responses to minimize or counteract changes in the volume and osmolality of their extracellular fluid (ECF). The maintenance of the ECF depends upon the coordination of activities related to fluid and electrolyte intake and loss. In situations of disequilibrium, first there is a physiological response and, if that is insufficient, behavioral actions arise in order to reestablish homeostasis. As in the case of many other physiological parameters, there are homeostatic responses serving to maintain a desired "set point". This regulation is achieved through osmotic activation or suppression of behavioral or humoral responses which collectively act to balance amounts of water and salt gained through ingestion and metabolism. Interactions of mechanisms controlling both water and sodium intake have not yet been fully clarified, and thus water intake/loss may or may not be associated with sodium intake/loss. Although it is difficult to isolate the events involved in the intake of water, in the following pages, I will limit my comments specifically to water balance with some discussion of salt balance where it is directly related to water balance.

In 1881, Nothnagel first suggested the existence of a "thirst center" located in the Central Nervous System (CNS) at the level of the medulla oblongata (Fitzsimons 1972). Many years later, the evidence indicates that the "thirst center" includes the hypothalamus (Franci 1994). Many later studies show that electrical stimulation or microinjections of hypertonic saline into the middle of the hypothalamus induces water intake (reviewed by Franci 1994). In fact, hypothalmic as well as extra-hypothamic structures have been found to be involved in the neuroendocrine control of water balance. Water deprivation studies in rats have shown that the following areas of the brain are involved in the regulation of water balance: supraoptic nucleus (SON), paraventricular nucleus (PVN)

(Sladek and Olschowka 1994, Sharp et al. 1991, Fenelon et al. 1993), organum vasculosum of the lamina terminalis (OVLT) (Hamamura et al. 1992), subfornical organ (SFO) (Giovannelli and Bloom 1992), median preoptic nucleus (MnPO) (Hamamura et al. 1992, Fenelon et al. 1993), and lateral preoptic nucleus (LPO) (Osaka et al. 1993). These findings indicate that there is no single, "thirst center", but rather an interacting complex of neural circuits involved in water control.

Much of what we know about the regulation of water balance comes from studies conducted on laboratory rats. Studies have shown that water deprivation (Sagar et al. 1988, Sladek and Olschowka 1994, Oldfield et al. 1991), hypertonic saline injections (Giovannelli and Bloom 1992, Sharp et al. 1991), hemorrhage (Hoffman et al. 1993), and certain dipsogenic injections or infusions (Blair-West et al. 1994) cause cellular dehydration which trigger neural activities. A commonly used marker for these activities is a nuclear protein, FOS (Sharp et al. 1991, Hoffman et al. 1993). FOS protein is a 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 (Morgan and Curran 1991). FOS, FOS-B, FOS-related antigens (FRA-1 and FRA-2), and possibly other FOS-like proteins form heterodimers with proteins of *c-jun* (Chiu et al., 1988) and the cAMP response element binding protein (CREB)/ AP-1 transforming factor (ATF) (Sassone-Corsi et al. 1988, Hoeffler et al. 1991). These heterodimers are recognized by and interact with target DNA sequences such as AP-1 and CRE consensus sites to induce transcription of mRNA of specific genes (Chiu et al. 1988). Activation of these genes is belived to be one of the earlier upstream events in regulation of water balance; thus, the presence of FOS appears to indicate neuronal activation at the cellular level in many areas of the mammalian nervous system that control water balance. Immunocytochemistry identifying FOS as a marker for changes in water balance has been used extensively in laboratory rats (Sladek and Olschowka 1994, Fenelon et al. 1993, Sharp et al. 1991, Herbert et al. 1992). In rats, water deprivation induces FOS activation in the SON, PVN (Sagar et al. 1988, Sladek and

Olschowka 1994) SFO, and OVLT (Oldfield et al. 1991). By applying this technique in my study, I was able to describe neurological changes after manipulating the hydration level of a non-domesticated animal model, the white-footed mouse, *Peromyscus leucopus*.

While most investigators have controlled for the time of day at which animals were perfused, no one has attempted to assess the effect of time of day on numbers of FOS-positive neurons of dehydrated animals. Although the limited information available on circadian changes within the hypothalamus indicates that rats exhibit some night and day changes in FOS expression, it is not at all related to water regulation. Kononen et al. (1990) showed that there is a circadian rhythm of c-FOS-like immunoreactivity in the rat brain; however, circadian variation only occurs in the hippocampus, the caudate putamen, and the suprachiasmatic nucleus (SCN), none of which are known to be involved in the regulation of water balance. In contrast, the SON, an area involved in regulating thirst, did not vary statistically in numbers of FOS positive cells throughout a 24h period. Similarly, the other major thirst-related hypothalamic area, magnocellular neurons of the PVN (mPVN), has not been found to exhibit any endogenous circadian rhythm. Although specific medial parvocellular oxytocinergic neurons in the PVN of female Sprague-Dawley rats have been suggested to be rhythmic (Arey and Freeman 1992), no magnocellular neurons, which specifically release peptides, oxytocin (OT) and vasopressin (VP), involved in water and salt balance, have been found to be involved in this rhythmic cycle.

P. leucopus is an ideal small mammalian model to investigate questions on the adaptive physiology of water balance. They live in a variety of habitats including dry bushy areas where there may be no easy access to water. MacMillen (1983) reports that even under conditions of water restriction, they lose water through evaporation. In particular, they have 35% greater evaporative water loss than other small rodents of equivalent size such as species of *Microtus* and *Dipodomys* (MacMillen and Garland 1989). One of the ways a mammal can replace lost water is to have a greater rate of metabolism and produce metabolic water. However, *P. leucopus* has a metabolic rate

similar to other small mammals; thus, it seems as though they are less efficient in water regulation (MacMillen 1983). This lack of well-developed physiological capacity is compensated by an omnivorous diet which includes succulent foods like plants, fruit, and insects. These succulent foods, however, are not always readily available and these mice spend some of their time in physiologically stressful environments.

In the present study, I have examined water deprivation levels for different durations of time and for different times of the day using a small mammal, the white-footed mouse (*Peromyscus leucopus*), from a laboratory colony at The College of William and Mary. I have used immunocytochemical analysis of FOS and FOS-like proteins in the SON and PVN to visualize water stress in these white-footed mice. No one has attempted to describe different levels of dehydration or to assess the time of day effects in water regulation by depriving mice of water. My emphasis here is to apply the FOS immunocytochemical techniques developed in laboratory rats: 1) to detect neurological changes and assess timed water stress during a 24 h period in a novel nocturnal animal model, *P. leucopus*; 2) to investigate the effects of water deprivation for physiologically relevant lengths of time, and; 3) and to apply this to the investigation of water balance variations in the daily life of these mice.

LITERATURE REVIEW

There are two ways in which the brain perceives changes in water balance. Cellular dehydration is provoked by hyperosmolarity of ECF, and extracellular dehydration is provoked by hypovolemia of ECF without a change in osmolarity. Osmoreceptors may be responsible for the detection of the signal produced by the variation in osmolarity; specifically, receptors sensitive to variation in sodium ions have been suggested (Kadekaro et al. 1995). Furthermore, vascular baroreceptors detecting variations in blood volume and/or central angiotensin II release due to hypovolemia are the likely causes for extracellular dehydration (Epstein 1976). The two different ways of detecting dehydration, osmotic and volumetric, seem to have two different neurophysiological controls as well. To illustrate this point, Andersson and Larson (1956), made lesions of the LPO and abolished the water intake response induced by hypertonic saline (change in osmolarity). However, this lesion did not affect responses to dehyration induced by water deprivation (change in osmolarity and ECF volume). Some studies suggest that medial regions of the hypothalamus regulate water intake induced by volumetric or extracellular stimuli (i.e. dehydration of extracellular compartments), and lateral hypothalamic regions regulate water intake induced by osmotic or cellular stimuli (i.e. an increase in osmolality of ECF with consequent dehydration of cellular compartments) (Kucharczyk and Morgenson 1975) suggesting that different stimuli activate different circuits for control of water intake.

In response to cellular dehydration, osmoreceptors outside the blood-brain barrier (BBB) and others within the brain react to the sodium concentration or the overall osmolality of the cerebrospinal fluid (CSF) or the brain ECF (McKinley et al. 1978). A region of the hypothalamus just anterior and ventral to the third ventricle (AV3V) is known to play a role in the osmotic control of the neuronal activity of the hypothalamic magnocellular neurosecretory neurons, in particular neurons within the SON and the PVN (Honda et al. 1989, Leng et al. 1989). These magnocellular neurosecretory neurons

synthesize principally vasopressin (VP) and oxytocin (OT) (Bourque et al 1994). These neurons project into the neurohypophysis where secretion of hormone into the blood occurs in proportion to the frequency of the action potentials generated at the cell body (Poulain and Wakerley 1982). The release of the primary mammalian antidiuretic hormone, VP, is increased by ECF hyperosmolality and hypovolemia. VP acts on the kidney to change the rate of renal water excretion; it is an important contributor to fluid homeostasis. The other hormone product of the magnocellular neurosecretory neurons is OT. OT release is caused most commonly by parturition and suckling. However in the rat, the release of OT increases as a function of plasma osmolality (Blackburn 1993, Kjaer et al. 1995, Chou et al. 1995).

Another prominent structure that plays a role in osmotic control is the OVLT. The OVLT is thought to lack a BBB and is thus a likely osmoreceptor site (Thrasher and Keil 1987). Electrolytic damage to this structure impairs the osmotically induced release of VP (Sladek and Johnson 1983, Thrasher and Keil 1987)) and OT (Blackburn et al 1987). Similar lesions reduced the osmosensitivity of the PVN (Honda et al. 1989) and SON (Chaudhry et al. 1989). Using electrical stimulus pulses applied to the AV3V and MnPO, Honda et al. (1989) have found that the AV3V region provides excitatory influence to the SON. They also conclude that since an injection of local anaesthetic into the MnPO blocked that effect, the connection between the AV3V and SON is mediated by MnPO neurons. In addition, electrical stimulation of the SON excited the neurons of the ventral AV3V which were also excited by raised plasma osmotic pressure (Chaudhry et al. 1989). Neuroanatomical studies with retrograde tracers suggest a direct projection of fibers from the OVLT to the PVN. These and other investigations indicate that the neuronal connections of the circuits involved in the response of cellular dehydration is possibly as sketched in Figure 1.

As noted above, it is difficult, if not impossible, to separate the events involved in cellular and extracellular dehydration (i.e. osmotically and volumetrically stimulated

dehydration). Many connections and areas involved in the neural response to cellular dehydration are also important for extracellular dehydration. One area not mentioned in relation to cellular dehydration is the SFO. The SFO lies outside of the BBB and, as with the OVLT, is likely to be a site involved in receiving information from the ECF. Using retrograde and anterograde techniques it has been suggested that the MnPO sends fibers to the SFO (Lind et al. 1982) (not shown in Fig. 1). Tritiated amino acids injected into the SFO produce terminal labeling of the MnPO, OVLT, SON, and PVN (Miselis et al. 1979, Miselis 1981). These findings are supplemented by results of HRP injections into the SON in which labels were found in the OVLT, MnPO, and SFO (Lind et al. 1982).

The SFO has been found to mediate drinking behavior induced by angiotensin II (AII) (Simpson 1981). When AII is infused i.c.v. into mammals, birds, reptiles and even fish, this powerful dipsogen acts as a neurotransmitter or a modulator in circuits subserving water and salt appetite (Fitzsimon 1986). Some researchers suggest that water intake caused by increasing the concentration of sodium ions is mediated by brain AII, because by infusing losartan, an AII antagonist, i.c.v, it has been shown that water intake caused by i.c.v. infusion of 0.5M NaCl in a variety of mammalian species is decreased (reviewed by Blair-West et al. 1994). However, while AII levels seems to increase when osmolality increases, AII is best known for its role in volume homeostasis (Epstein et al. 1970). It has been shown that only in extreme situations of dehydration does peripheral AII reach plasma concentrations capable of inducing water intake (Philips 1987). In contrast, Epstein et al. (1970) have found that hypovolemic dehydration significantly increased the central AII level. It seems that while AII increases in response to decreases in volume those changes trigger central AII rather than peripheral AII. The site at which central AII causes the release of VP and OT (Ferguson and Kasting 1988) is unknown (Keil et al. 1975). There are two possibilities for AII site of action suggested by Phillips et al. (1979). One possibility is that there is a localized release of the AII in the SON, which contains the magnocellular neurons that secrete VP and OT. Although the SON seems to contain some

AII receptors, autoradiographs show relatively weak binding of AII in SON (Mendelsohn et al. 1984). The other possibility is that the OVLT or SFO are the site of action. They both contain receptors for AII (Simpson 1981), and they both have been demonstrated by HRP tracer studies to be connected to the SON (Miselis 1981). However, studies of OVLT or SFO lesioned animals have found that AII still causes the release of VP (Sladek and Johnson 1983) suggesting that there is more than one pathway regulating water balance.

It has been found that dehydration-induced drinking, in which osmolality and volume have both been disturbed, is mediated by cholinergic pathways as well as AII (Bonjour and Malvin 1970). The cholinergic pathways represent an alternative parallel pathway for water regulation. This has been suggested because there are fibers containing acetylcholinesterase (AchE) that project to the SFO and the OVLT (reviewed by Lind et al. 1982) and there is substantial AchE activity in VP neurons of the hypothalamus (Morris and Pow 1993). Other studies have shown that while AII induces water and salt intake, carbachol (a cholinergic agonist) induces only water intake via the release of VP and OT from the neurohypophysis (Phillips 1987). Hoffmann et al. (1978) have found that dehydration-induced drinking can be abolished by simultaneously blocking the cholinergic pathway and AII using atrophine and saralasin (a cholinergic antagonist and an AII inhibitor, respectively).

In summary, evidence suggests that there is a complex system for water regulation. Dehydration can be stimulated osmotically or volumetrically. Extracellular dehydration can be mediated by two types of pathways: AII or cholinergic pathways. Behavior modifications of salt and/or water intake can be made to counteract changes in fluid homeostasis. Separately but cooperatively, all areas and circuits involved are coordinated to maintain an appropriate state of fluid balance. In this study, I use our understanding of how the brain perceives and responds to water imbalance and the immunocytochemical

labelling techniques developed to study water balance to investigate how different levels of dehydration can effect the brain and body in a nondomestic species.

MATERIALS AND METHODS

Animals

Experiments were performed on both male and female *P. leucopus* (approximately 15-25 g). The animals were raised with same-sex siblings and were kept under a L16:D8 cycle (lights on at 0500 and lights off at 2100) in the Population and Endocrinology Laboratory of The College of William and Mary. At the time of use, all animals were about 60 - 90 days old. They were given food (Agway Prolab Rat/Mouse/Hamster 3000, Syracuse, NY) and water *ad libitum* except during experimental procedures described below. Over the period of this study, the relative humidity of the animal rooms averaged 47 ± 4% (mean ± standard error) and the room temperature was 69 ± 1° F (mean ± standard error). At the begining of experimental treatments, all mice were separated into individual cages. On the morning of perfusions, they were transported to an animal room in a different building under similar conditions, in which relative humidity averaged 41 ± 3% and the room temperature averaged 71 ± 1° F until their time of sacrifice. All animals were anesthetized with an overdose of sodium pentobarbital (200 mg/kg body weight, i.p.) prior to perfusion.

Experiment 1: Levels of Water Deprivation

To visualize and quantify the effects of water deprivation within the hypothalamus in *P. leucopus* over time, water bottles were removed from the cages of male mice for 6, 12, or 18 h and FOS-like immunoreactivity (FOS-LIR) in the SON and PVN analyzed. A fourth group of euhydrated animals, defined as those with *ad libitum* access to water, served as

negative controls. 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 experiment, all mice were perfused between 0900 and 1600. Not all brains could be processed for immunocytochemistry simultaneously without sacrificing quality, so this experiment was conducted using 7 experimental runs with an average of 8 animals per run. Prior to analysis, each brain was evaluated for staining quality by an evaluator blind to the treatment. Brain sections showing signs of poor perfusion or damage in the SON or the PVN were removed from the study. All runs were balanced across treatment groups.

Experiment 2: Time of Day Differences

The second experiment investigated FOS-LIR in response to water deprivation during day and night cycles and for various lengths of time between 6 and 12 h. "Day cycles or periods" refer to treatments which were entirely in the light period. Our longest periods of water deprivation were longer than the 8 h dark period; thus, "night cyles or periods" refer to treatments in which 2/3 of the water deprivation fell in the dark period and 1/3 in the light period. Male mice were water deprived for 6, 8, 10, or 12 hrs during their day or night cycle. Euhydrated animals were sacrified as negative controls. Night controls were perfused between 0600 and 0900 and day controls between 1600 and 1900. This experiment was performed not only to study the FOS-LIR occuring between 6 and 12 h without water, but also to investigate differences in the way the brain responds to water deprivation during different times of the day (i.e. night vs. day period). All treatment groups had sample sizes of 5 or 6. This experiment was conducted using 7 runs with an average of 11 animals per run. Each brain was evaluated using the same criteria as in the previous experiment, and those with poor perfusions or damage in the area of interest were rejected prior to analysis, with the evaluator blind as to treatment. Each experimental run was balanced to control for order effects, and all runs were again balanced across treatment groups. The day treatment animals were perfused at the end of their light cycle between

1600 and 2000 and the night treatment animals were perfused following their dark cycle between 0630 and 0900.

The last major disturbance before perfusion occurred during handling of the mice during separation from siblings into individual cages and movement of the cages while transporting the mice from the one building to another. All treatment group animals for the day were transported at the same time in the morning; thus times of perfusion relative to the time of transport differed for the day and night treatment groups. The average time between disturbance and perfusion was 9 - 13 h for the day treatment animals and only 0.5 - 2 h for the night treatment animals. In order to test for handling/transport bias, night controls were disturbed 1 - 3 h before perfusion and day control groups disturbed 9 - 11 h before perfusion. These control groups showed nearly identical levels of FOS-LIR in the SON and the PVN. Therefore, there is no evidence to support the idea that differential stress due to time of transportation affected basal FOS expression.

Experiment 3: Weight Loss during Water deprivation

In order to investigate how mice responded to the absence of water during day and night periods, I measured the weight lost during water deprivation (0, 6, 12 h) and weight gained after the return of water for 2 h. One of the ways to observe water regulation in small mammals subjected to water deprivation is measuring weight loss and weight gain (MacMillen 1983); therefore, water intake was estimated by the change in body weight relative to controls after the return of water. Removal of water occurred at 0100 (6 h night), 1930 (12 h night), 0830 (6 h day), and 0630 (12 h day). As in previous experiments, the mice were given a L16:D8 cycle (lights on at 0500). This was followed by returning water bottles for 2 h. During the 2 h drinking period, food was removed from all cages in order to prevent weight gain due to food consumption. In addition, the weight of the water bottle was recorded before and after it was returned to each animal in order to provide an alternative set of data for water consumption.

In this experiment, females were included to increase sample size (N = four males and four females in each treatment group). Statistical analysis included gender as an additional variable. One male within the control night group died of unknown causes the day after data collection and its data were excluded from the analyses. As in the previous experiment, night and day negative control groups consisted of euhydrated animals which were weighed during similar time intervals as their respective treatment groups. This investigation was conducted using 2 runs with 24 animals per run. Each run was balanced across treatments.

Isolation of VP- and OT-ergic neurons

It is known that VP and OT play an integral part in the control of water balance (Verbalis et al. 1986, Bennelli et al. 1991, Fenelon et al. 1993, Bourque et al. 1994). This part of the study was conducted to test for colocalization of VP and OT with FOS-like proteins within the hypothalamic magnocellular neurons of SON and PVN. Four subjects were given 12 h of water restriction at night (water bottle removed at 2030). Serial sections from each brain were divided among 4 staining treatments using the following combination of antibodies: FOS ployclonal followed by OT-polyclonal, VP-polyclonal, or VP-monoclonal. Pretreatment of OT, VP, and/or Fos peptide served as negative controls, no specific staining was found in the control sections.

Immunocytochemistry

Expression of FOS-LIR in the SON and the PVN were detected using a single-labeled Avidin-Biotin-Peroxidase-Complex method. Mice were perfused through the left ventricle with 5 ml of heparinized saline buffer (15,000 IU heparin per liter of 0.1 M phosphate-buffered saline (pH 7.2) (PBS)) at approximately 4.4 ml/min using a Harvard Apparatus perfusion pump and bled via the right atrium (Côté et al. 1993). They were then immediately transferred to a tray of ice, and perfusion continued with 50 ml of ice-cold,

freshly prepared 4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) in 0.1 M PBS approximately at 4.4 ml/min. The brains were taken out of the skull and post-fixed in the same fixative with gentle agitation at 4° C. After 2 h of post-fixation, they were transferred to 30% sucrose solution made with 0.1 M PBS for cryoprotection and were incubated overnight at 4° C on an orbital shaker.

Frozen coronal sections (40 μm) were obtained using a cryostat set at -15° C. After all sections of one brain were cut, they were immediately rinsed 3 times for 10 min each in cold 0.1 M PBS (pH 7.4) on an orbital shaker at room temperature (225 rpm). At each subsequent rinse or reagent treatment procedure, sections were agitated on the orbital shaker at room temperature unless otherwise noted. The tissue was then treated with 1.4% normal goat serum (NGS)(Vector Laboratories, Burlingame, CA) and 0.2% Triton X-100 (Fisher Scientific, Fair Lawn, NJ) in 0.1 M PBS for 20 min. Sections were incubated with a rabbit anti-fos polyclonal antibody which is broadly reactive with c-FOS, FOS B FRA-1, and FRA-2 (1:10,000 in PBS/NGS/Triton X-100 solution; K-25, catalog # sc-253, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour followed by approximately 42 hours on the shaker at 4° C (170 rpm).

After incubation in primary antibody, the sections were removed from the refrigerator and left on a shaker at room temperature for an hour. Then after rinsing three times, 10 min each with 0.1 M PBS, the sections were incubated in biotinylated goat anti-rabbit immunoglobulin G (1:200 in PBS/NGS/Triton X-100 solution, Vector Laboratories) for 1 hr at room temperature. Following 3 more rinses in 0.1 M PBS, avidin-biotin-peroxidase solution (Vector Laboratories Elite ABC-Peroxidase kit) was added for 1 hr. The sections were washed in TRIS Buffered Saline Gel (TBS gel)(0.1 M, pH 7.6, FisherBiotech, Pittsburg, PA and Sigma, St. Louis, MO) and stained with 1.5 ml of a solution of diaminobenzidine (DAB)(0.2 mg/ml) $NiSO_4$ (24 mg/ml) and freshly prepared 3% H_2O_2 (0.83 μl/ml) (all from Sigma Chemical Co. St. Louis, MO), in TRIS buffer (0.1 M, pH 7.6). The color reaction was allowed to proceed for approximately 7 to 12

minutes to a uniform level of non-specific background staining in the cortex. All sections within one run received the same incubation time. The sections were then washed 3 times for 10 minutes each in cold TRIS buffer to stop the color reaction.

For the co-localization of VP and OT neurons, the FOS-LIR stained tissue was treated once again with 1.4% NGS solution (PBS/NGS/Triton X-100) for 20 min. The sections were then incubated in rabbit anti-arginine-vasopressin or rabbit anti-oxytocin polyclonal primary antibodies (1:10,000 in 0.1 M PBS/1.4% NGS/0.2% Triton X-100, catalog #AB1565 and #AB911, Chemicon International Inc. Temecula, CA), first at room temperature for an hour then at 4° overnight on the shaker. On the following day the DAB staining protocol used above was repeated without $NiSO_4$ in order to give a red-brown color localized to the cytoplasm of cell bodies and axons that was distinguishable from the blue/black nuclear staining of FOS.

After staining, brain sections were mounted on gelatin coated slides and air dried. The sections on slides were further dehydrated with xylene and coverslipped with permount. Each slide was scanned by an observer blind to treatment using an Olympus CH-2 brightfield microscope; any sections showing tissue damage or poor quality of staining was discarded.

Tests for the specificity of the primary antibodies used were conducted using a control FOS peptide, arginine-vasopressin peptide, and oxytocin peptide (catalog # sc-253 P, for FOS protein, Santa Cruz Biotechnology; catalog # O4375 and V9879, for VP and OT peptide, Sigma Chemical Company). In each case, peptide was added (1 μg/ml) to normal serum/Triton-X/PBS solution before the antibody was added to the correct titer. The sections incubated in this antigen-antibody solution showed no specific staining in any area of the brain. Further tests for specificity of staining were conducted by omitting either the primary antibody or the secondary antibody, or both. The results for these tests also showed no specific staining.

Quantification of FOS

Nuclear staining for FOS-LIR in SON and PVN was identified and counted using an image analyzer (Bioquant IV) under 400x magnification. All quantitative analyses were conducted in random order by a single individual blind to the treatment conditions. Four mid-nuclear SON and PVN sections (either the right or the left side) were chosen for each animal. The number of FOS-LIR were counted for the four sections within the individual treatment group, and the average was calculated. To assess repeatability and accuracy of counts of FOS-and FOS-like positive neurons, two additional observers recounted sections for FOS-LIR neurons on a subset of all treatment groups. On average, these counts were 9% different, and all were within 15% of the original counts over a range of 1 to 105 neurons/section (N = 54 sections).

Statistical analyses

Experiment 1. One-way analysis of variance (ANOVA) was used to compare mean cell counts of FOS-LIR in different treatment groups. Pre-planned (a priori) 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 mean value for 6 vs. 12 and 6 vs. 18 h of water deprivation. For this and subsequent statistical analyses in this study, data were considered to be significant at $P < 0.05$.

Experiment 2. A two-way ANOVA was used to evaluate the differences in the mean number of FOS-LIR cells found in water-deprived mice for various lengths during day or night. This analysis was followed by the same set of post hoc pair-wise comparisons for both SON and PVN data. For each treatment time, night and day comparisons were made. Comparisons between control and treatment groups were conducted in order to identify the minimum amount of water deprivation period needed to cause a significant increase in the

number of FOS-LIR cells within either the day or the night period. In addition, 6 h of deprivation at night was compared with the 12 h of deprivation at night and day.

Experiment 3. A three-way ANOVA was used to analyze water deprivation for various time lengths during day or night cycles across gender. The statistical analysis was conducted for weight loss during water deprivation, the weight gain upon return of water, and differences in the bottle weight before and after the 2 h drinking period. A series of 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 the 12 h night group.

RESULTS

Water deprivation over time

In the SON, water deprivation resulted in large increases in FOS-LIR cells over time ($F = 32.358$; $P = 0.0001$; Fig 2A). Twelve and 18 h of water deprivation resulted in increased levels of FOS-LIR ($F = 63.95$, $P = 0.0001$; $F = 60.85$, $P = 0.0001$, respectively), while 6 h of water deprivation had no effect ($F = 0.7$, $P = 0.4237$). A post-hoc comparison showed that there was a significant difference between 6 and 12 h of water deprivation ($F = 63.95$, $P = 0.0001$) and between 6 and 18 h of water deprivation ($F = 54.6$, $P = 0.0001$).

Similarly, in the PVN, water deprivation caused significant increases in FOS-LIR cells over time ($F = 5.918$; $P = 0.0017$; Fig 2B). Twelve and 18 h without water resulted in increased levels of FOS-LIR cells ($F = 14.94$, $P = 0.0031$; $F = 7.55$, $P = 0.0206$, respectively), while 6 h of water deprivation had no effect. ($F = 0.05$, $P = 0.8233$). A post-hoc comparison showed a significant difference between the FOS-LIR in the PVN of

6 and 12 h of water deprived mice ($F = 14.94$, $P = 0.0031$) and between 6 and 18 h of water deprived mice ($F = 8.05$, $P = 0.0176$).

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 ($F = 27.262$; $P = 0.0001$; Fig. 3A). Night water deprivation produced more FOS-LIR than day deprivation. In contrast, control animals showed no day and night difference ($F = 2.94$; $P = 0.125$). As in the first experiment, water deprivation over time also caused a significant difference in the number of FOS-LIR cells ($F = 7.968$; $P = 0.0001$) with a nonsignificant day/night interaction ($F = 1.262$; $P = 0.2994$). Water deprivation of as little as 6 h in the night produced a significant difference in the number of FOS-LIR cells ($F = 26.73$; $P = 0.0009$). However, during the day, animals needed to be deprived of water for 8 h before a significant difference was found ($F = 12.05$; $P = 0.0084$) and that difference was smaller than that produced at night. Furthermore, it took 12 h of water deprivation during the day to reach a similar number of FOS-LIR cells to that of the 6 h night group ($F = 0.75$, $P = 0.4113$). This 6 h night group induced nearly the same number of FOS-LIR cells as the night 12 h treatment ($F = 1.8$, $P = 0.2122$).

In the PVN there were day and night differences of the number of FOS-LIR cells ($F = 23.598$, $P = 0.0001$; Fig 3B), and there was a significant effect of duration of water deprivation ($F = 5.514$, $P = 0.0012$). No interaction between time of day and hours of deprivation was found ($F = 1.189$, $P = 0.3302$). For moderate lengths of water restriction, 6 and 8 h, there was a day and night difference in the FOS-LIR cells ($F = 14.29$, $P = 0.0043$; $F = 7.18$, $P = 0.0279$). However, for the most extreme periods of water restriction (10 and 12 h) the FOS-LIR did not show a significant day and night difference, although a trend was present ($F = 4.35$, $P = 0.0704$; $F = 3.44$, $P = 0.1007$, respectively). As in the SON, water deprivation for as little as 6 h in the night treatrment group lead to a marked increase in the FOS-LIR cells in the PVN ($F = 6.61$, $P = 0.0331$). However,

during the day, water deprivation for even 12 h did not produce a significant increase over the control ($F = 2.57$, $P = 0.153$). Again, as in the SON, 6 h water deprivation during the night produced an increase in FOS-LIR cells similar to 12 h of deprivation during the day ($F = 3.44$, $P = 0.1007$).

Effects of Water Deprivation on Weight Loss and Recovery

There were no significant differences in weight loss or in weight gain between the sexes ($F = 0.282$, $P = 0.5986$; $F = 0.654$, $P = 0.424$, respectively; data not shown) 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 ($F = 39.833$; $P = 0.0001$; Fig 4A), and this difference depended on the time of day at which the water deprivation occurred ($F = 10.021$; $P = 0.0003$). The control animals showed no day and night effect in their weight change ($F = 1.87$; $P = 0.1947$). Six hours without water during the night produced significantly more weight loss than during the day ($F = 20.16$; $P = 0.0005$). Yet, for the 12 h of water deprived mice, there was no weight loss difference when comparing the day and night groups ($F = 1.01$, $P = 0.3326$). Further post-hoc comparisons showed that 6 h without water during the night produced as much weight loss as the 12 h of water deprivation during either the day or night ($F = 0.75$, $P =$ 4007).

Measurements of weight gained by return of water show that again there was a time of day effect ($F = 12.069$; $P = 0.0012$; Fig. 4B) and a duration of water deprivation effect ($F = 22.52$; $P = 0.0001$). Mice drank more following the night water deprivation period than those who drank following the day water deprived period for both 6 h and 12 h of deprivation ($F = 8.68$; $P = 0.0106$; $F = 9.74$; $P = 0.0075$). In fact 6 h night treated mice gained more weight than the 12 h day treated subjects ($F = 19.25$; $P = 0.0006$) and as much as the 12 h night treatment ($F = 0.56$; $P = 0.4675$).

Water bottle weight measurements were made along with body weight measurements to serve as an alternate method for assessing the amount of water consumed during the drinking period. However, bottle weight data showed nonsignificant differences for time length treatments and for day/night treatments($F = 1.137$, $P = 0.3307$; $F = 0.581$, $P = 0.4503$; respectively, not shown). Observations indicate that water bottles leaked or dripped varing amounts when placed on and taken off of the cages. This was deemed an inaccurate method of measuring water gain.

Co-localization of VP and OT peptides with FOS-like protein

Additional mice were water deprived at night for 12 h and the serial sections of these brains were stained for VP and OT in order to examine their distribution and to assess whether the type of hypothalamic neurons that was being marked by the FOS-LIR cells includes these cell types. Double-labeled ABC immunocytochemistry of VP and FOS or OT and FOS showed the FOS and FOS-like proteins were found consistently contained within the nucleus of the cell whereas the VP or the OT antigens were labeled only in the surrounding cytoplasmic areas of the cell body as well as the ascending and decending fibers of those neurons. Both VP and OT positive cells were abundant in the SON and PVN, areas in which FOS-LIR cells were also abundant, and co-localization of FOS-LIR with VP and OT was common. These observations are consistent with a study using laboratory rats showing that FOS-LIR cells activated by water restriction are predominantly VP and OT positive cells (Fenelon et al. 1993). Figure 5 shows the FOS-positive nucleus of SON and PVN (red/brown) surrounded by cytoplasmic VP or OT in the cell bodies and in the axons (blue/black).

DISCUSSION

These results show that during biologically reasonable periods of dehydration there is a physiological and behavioral difference in the response to day and night water stress. During the night, as little as 6 h without water caused a near maximum increase in the FOS-LIR cells of SON and PVN. In contrast, during the day, 8 h of water deprivation was needed to significantly increase the FOS-LIR neurons of the SON and PVN, and even 12 h water deprivation did not produce a response as large as that produced at night. These results suggest that the mice are more sensitive to the changes in their water balance at night, during their active period in comparison to day. In fact,water deprivation at night, induced more FOS-LIR in the SON and PVN water-deprived for every comparable period during the day (Fig. 3). Finally, 12 h and 18 h of water deprivation produced similar levels of FOS-LIR, indicating that 12 h may have caused a maximal level of activation for neural regulation of water balance.

Our results suggest that the FOS-LIR in the SON may be more sensitive to changes in water balance than the PVN. In the SON of euhydrated mice, the number of FOS-LIR cells were very low where as in the PVN, the numbers were higher (i.e., the increase in FOS-LIR cells were less dramatic in the PVN). The SON consists essentially of magnocellular OT and VP neurons (Palkovits and Brownstein 1988, Fenelon et al. 1993) while the PVN consists of parvocellular and magnocellular neurons known to be involved in many other neural circuits. For example, the parvocellular PVN (pPVN) has been found to be involved in the rhythmic patters of prolactin secretion (Arey and Freeman 1992). It has also been shown that the parvocellular PVN has corticotropin-releasing factor-containing neurons that are activated during periods of stress in addition to the neurons in which vasopressin is induced following osmotic simulation (Bondy et al. 1989, Ceccatelli et al. 1989). This suggests that the results from the SON may reflect more accurately the effects of varying water deprivation levels.

The third experiment tested day/night differences in the rates of water loss and in the amount of recovery of water thereafter. Body weight was measured to estimate the relative amount of fluid lost and subsequent replenishment during different water deprivation states. Water loss can reflect changes in food intake and fecal loss as well as changes in body fluid. However, any increase in body weight after the return of the water bottle should be due to water intake only since food was not available during the 2 h rehydration period. The night water-deprived mice lost more weight and lost that weight more rapidly than day water deprived mice, suggesting that FOS-LIR reflects, in part, the rate of water loss. This is supported by the fact that mice also gained more weight after the night deprivation period than the day deprivation period. All treated animals regained their lost weight, but none recovered completely. It may be that stored energy (e.g. fat) was lost and could not be recovered by drinking alone, or that feeding might be required to make up additional weight loss from defecation

Although the negative controls of the study control for any effects of handling or general disturbance, it could be that the activation of SON and PVN in response to water deprivation is a stress response to the loss of water. However, studies on stress response using FOS as a marker indicate that patterns of FOS expression resulting from stress alone are different from those seen here (Sharp et al. 1991). Stress caused by water-avoidance tasks increased FOS positive cells (Bonaz and Tache 1994) and stress caused by handling and injections induced *c-fos* mRNA (Sharp et al. 1991) in the pPVN (but not the SON). Thus, an ACTH stress-response may have contributed to the day/night differences found in the PVN. Other studies on stress and thirst indicate that release of dopamine during water deprivation represents an arousal response to stress in rats (Horvitz et al. 1993, Zabik et al. 1993). It has been shown that administration of a dopaminergic agonist, pergolide, initiates drinking (Zabik et al. 1993), and dopamine receptor blockade decreases the latency to initiate drinking after water deprivation (Horvitz et al. 1993). Results of these studies suggest that what is seen in the present study might be due to stress of water

removal and not strictly due to dehydration. Nevertheless, it is clear that water deprivation affected FOS-LIR in SON and PVN. Regardless of why FOS-LIR is present following water deprivation, there is a day and night difference in the way the brain is activated in reponse to removal of water .

Manipulations of water availability at night caused a greater response at the level of the hypothalamus when compared with water deprivation during the day. This day/night thirst difference could be due to a circadian rhythm. Yasin et al. (1993) has shown that melatonin induces modulation of VP and OT peptide release, *in vitro*. Although secretion of VP and OT has been shown to exhibit a circadian rhythm there has been no research that has investigated the effects of circadian rhythm on Fos activity in response to water imbalance. The fact that the SON shows no rhythm of FOS-LIR suggests that some other factor induces this rhythm of secretion. It could also be that the activity level rather than the amount of light has an effect on the neural activity of water deprived *P. leucopus*. This day/night difference could be due either to greater rates of water loss at night or lower tolerance of water loss at night. Theoretically, increased activity during the night, their active period, could cause mice to lose more body fluid, creating a need for more water to replenish the loss; therefore, water regulation and intake may become increasingly important during the night. This is supported by observations of increases in metabolic VO_2 of caged *Peromyscus* during their night, relative to their day time resting period (E. L. Bradley, personal communication).

This study provides evidence for involvement of FOS-LIR in the response of animals to modest but physiologically relevent day/night periods of water deprivation. Although there is no endogenous FOS-LIR rhythm within the magnocellular nuclei of the SON and PVN, as little as 6 hours of water deprivation produces a time of day effect in the *P. leucopus*. Over a range of levels of dehydration, night time water restriction produces a greater effect than day time manipulations. This suggests that in *P. leucopus* the brain has adapted to respond appropriately to the water needs of the activity period rather than how

much water the body has actually lost. Finally, our results suggest that FOS-LIR could be used to compare physiologically relevent changes in water and salt changes and that controlling for time-of-day effects in planning and interpreting studies on water balance is important.

FUTURE DIRECTIONS

To provide additional test of my conclusions and to expand on day/night influence on water regulation, I have briefly described below some additional experiments.

MacMillen (1983) discussed two methods of measuring the efficiency of water regulation in water deprived small mammals,weight loss and concentration of urine. The present study used one of these methods, weight loss and gain measurements, to assess changes in body fluid. The other method, collecting urine samples after periods of water deprivation, would give more detailed information on the condition of the animals to support or dispute my interpretation of the weight change results. In addition, osmotic concentration as well as the sodium ion concentration could be measured. This would serve as a non-intrusive method of estimating the fluid lost due to water deprivation. The greater weight loss found in the night water-deprived group suggests that there would be more urine volume collected for the night treatment group than for the day treatment group. In direct contrast, FOS-LIR suggests that less urine would be collected after night water deprivation because greater neural activation at night indicates greater release of antidiuretic hormone such as aldosterone and VP acting on the kidney to increase water resorption and sodium release. It may be (a) that the increasing FOS-LIR does generate greater hormonal release to increase water resorption and decrease urine volume but that the mice are so active at night that even the increased amount of FOS-LIR seen is not enough to compensate, or (b) it may be that FOS-LIR does not lead to an increase in kidney

efficiency. In either case, I expect to see greater urine volume collected. On the other hand, if the increasing FOS-LIR does create enough VP and OT to partially compensate for the loss of water, then I expect less urine would be collected after night water deprivation.

The FOS-LIR labelling method used in this study identified the amount of neural activity and its location but not the type of activation (i.e. the end product, the peptide produced). The co-localization of VP and OT to FOS-LIR neurons of the hypothalamus identifies the active neurons as VP- or OT-ergic in this study; however, it did not determine if FOS-LIR actually does lead to an increase in VP and OT. Therefore, measurements of circulating VP and OT could be taken to reveal whether FOS-LIR affects kidney activity by leading to an increase in VP and OT. If the circulating VP and OT increases after a night without water in accordance with the increase in FOS-LIR, it suggests that the amount of FOS-LIR cannot fully compensate for the water lost suggesting that the activity level is so high that the kidney cannot conserve the vast amount of water it produces. However, if the circulating VP and OT does not increase, then it would suggest that the increase in FOS-LIR after night deprivation does not lead to an increase in VP or OT release, but that FOS-LIR produces some other neural signal in order to tolerate the water loss.

This study was conducted using a small nocturnal animal, *P. leucopus*. It was concluded that the night and day difference detected in water regulation might be due to the activity level rather than to circadian rhythms. I hypothesize that repeating this study using a small diurnal mammal would produce the opposite result. A diurnal animal, being active during the day, might be more sensitive to the change in water balance during the day rather than to the changes imposed during the night, when their metabolic rate is lower. Therefore, they might be found to tolerate water loss better (i.e. less FOS-LIR cells) during the night. Another interesting experiment that could be conducted is the investigation of water deprivation induced FOS-LIR on a free-running circadian cycle. Daily rhythms could be altered by exposure to pinealectomy or a SCN lesion, and these free-running mice could be water deprived for various lengths of time so that neuronal activation can be

analyzed. The results of the present study suggest that water deprivation of free-running mice would show time duration and activity period effects but might lose day/night effects of FOS-LIR in the SON or the PVN.

The present study was conducted using an outbred population of *P. leucopus*. Although the length of water deprivation was estimated to be physiologically relevent, it is not certain that 6 or 12 hours without access to water actually occurs in the wild. Thus, capturing wild *P. leucopus* at different times of the day and assessing FOS-LIR in the SON and PVN would shed light on what a wild *P. leucopus* in nature really experiences in a given day. This particular study was actually attempted during summer of 1995. Subterranean nestboxes were placed in many different wooded locations. Samples of mice were to have been taken at dawn, dusk, midnight, and mid-afternoon. Unfortunately, the population of these mice during the summer are extremely low (C. R. Terman 1993), and those mice present in the summer of 1995 did not use the nest boxes. Nevertheless, this experiment is worth another attempt in the fall or early spring with better-drained nest boxes. These mice would be taken immediately into the lab, perfused, and fixed for later sectioning and staining of FOS-LIR. The results might indicate when these wild mice normally undergo water deprivation , and if they also experience high water stress at night as was seen for the laboratory colony.

Another experiment attempted was the use of food consumption to create dehydration by increasing osmotic concentration of the blood and ECF. Hamamura et al. (1991) have shown that i.p. injection of cholecystokinin (CCK) produces expression of c-fos mRNA in the SON and PVN magnocellular OT neurons. CCK is an octapeptide which is released during food consumption and activates the gastric vagal nerve endings (Hamamura et al. 1991). It has also been shown that food intake produces significant numbers of FOS-LIR cells in the magnocellular region of the PVN and the SON in laboratory rats (Li et al. 1994). It is believed that sudden consumption of dry rat chow will increase the blood osmolality, thus producing cellular dehydration and causing enough

osmotic disequilibrium to initiate neuronal activation similar to those found in the water deprivation experiments. This and the other follow-up experiments suggested above would extend to the findings of the present study on day/night differences in water regulation of *Peromyscus leucopus.*

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FIGURE 1

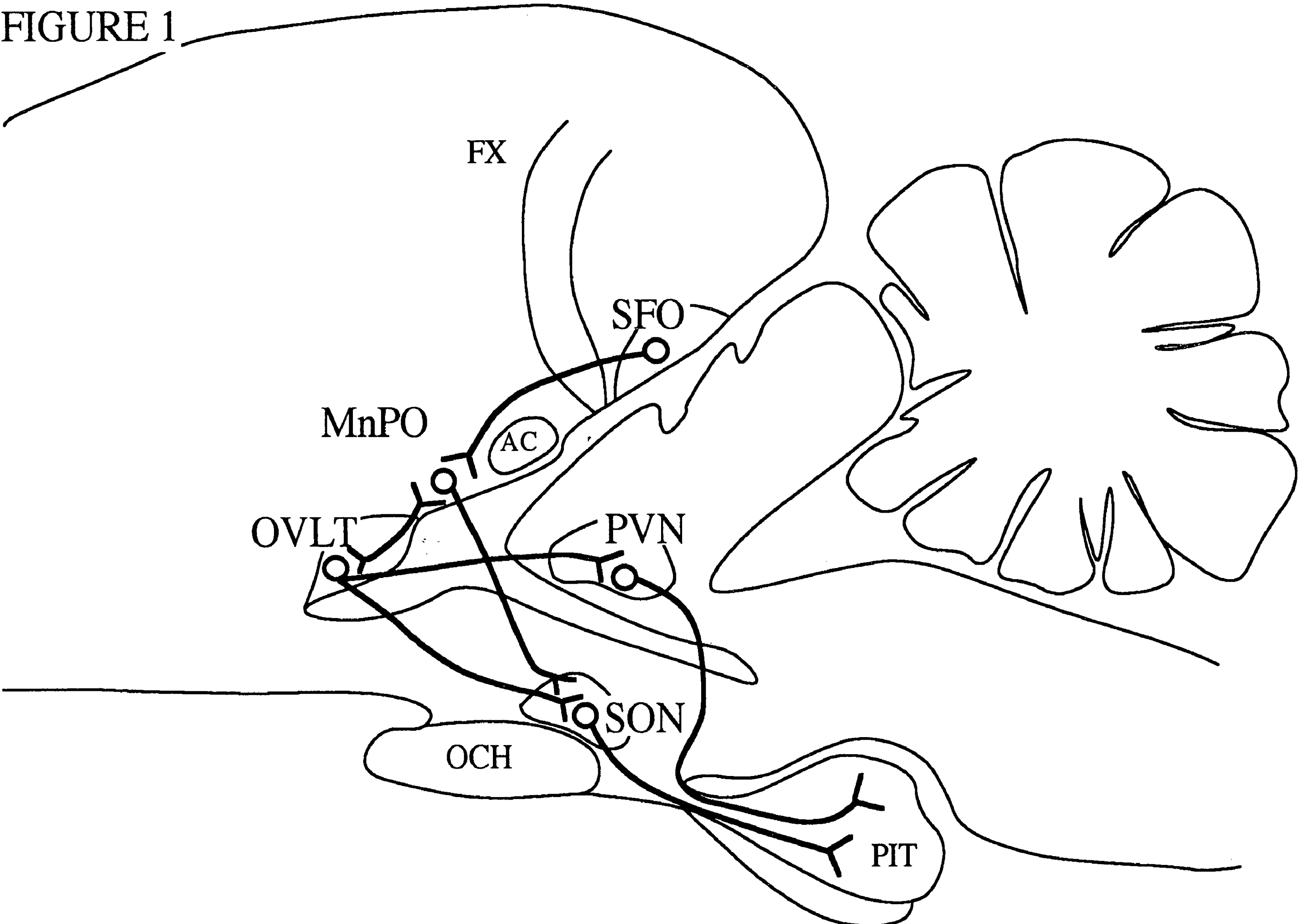


FIGURE 2

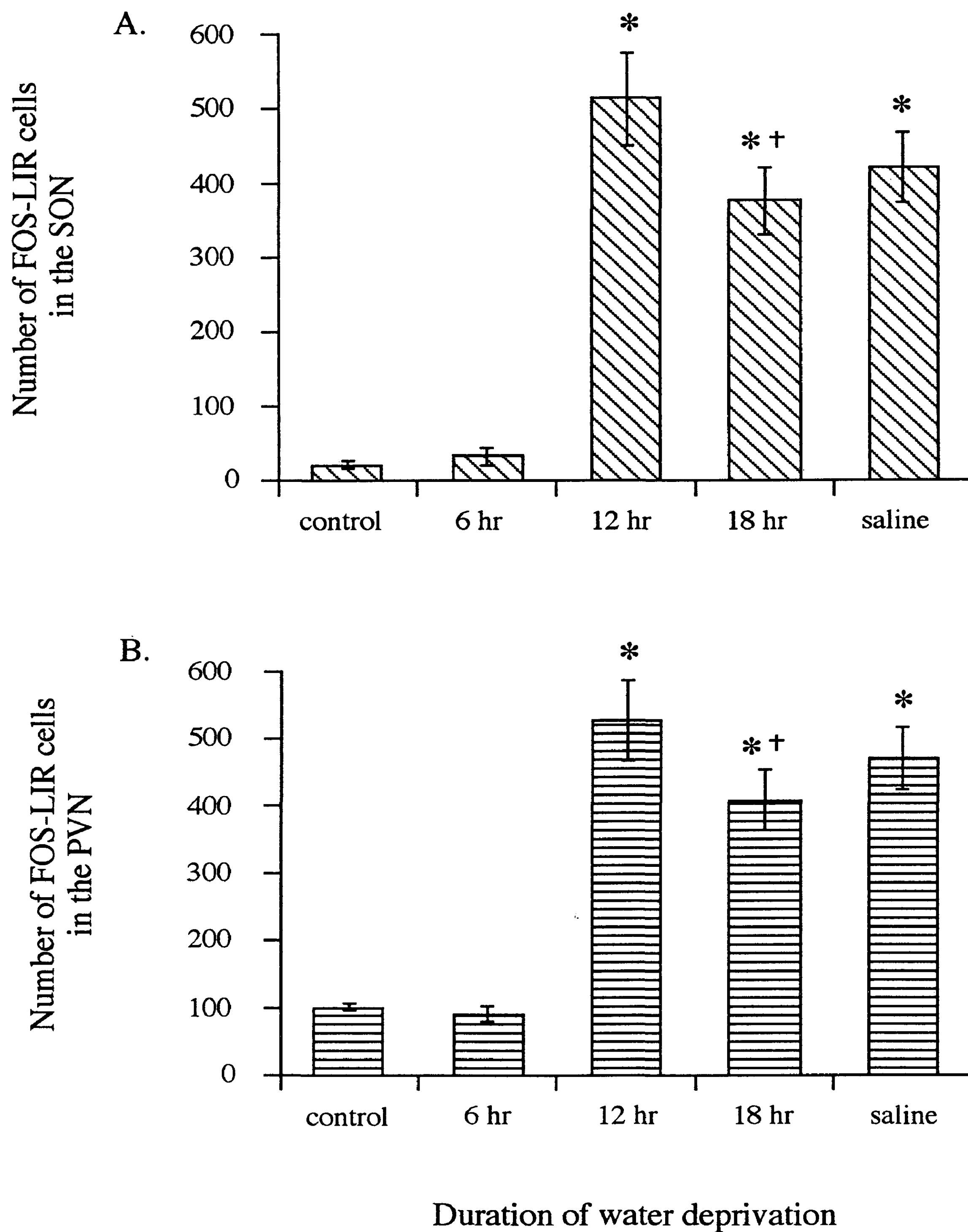
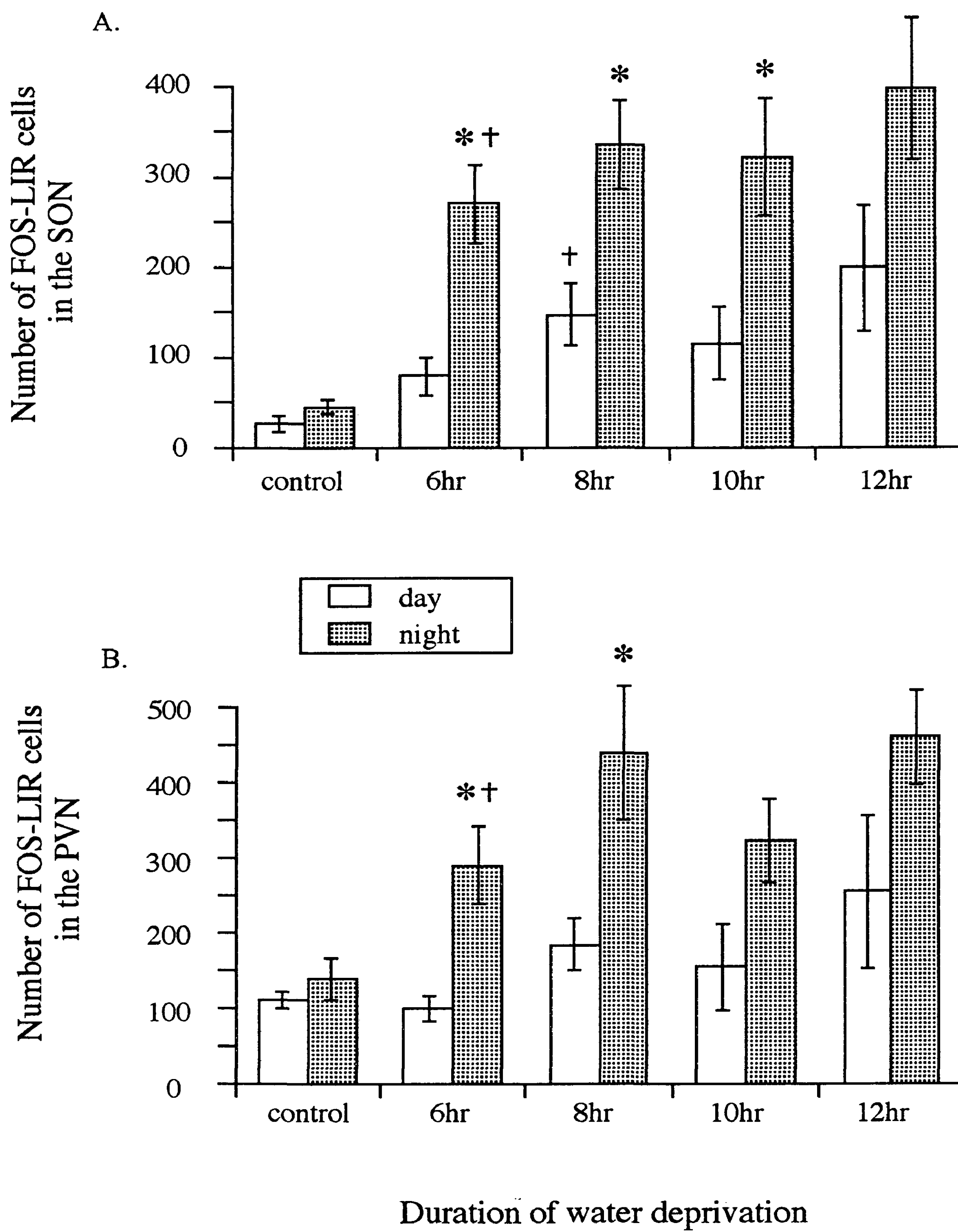


FIGURE 3



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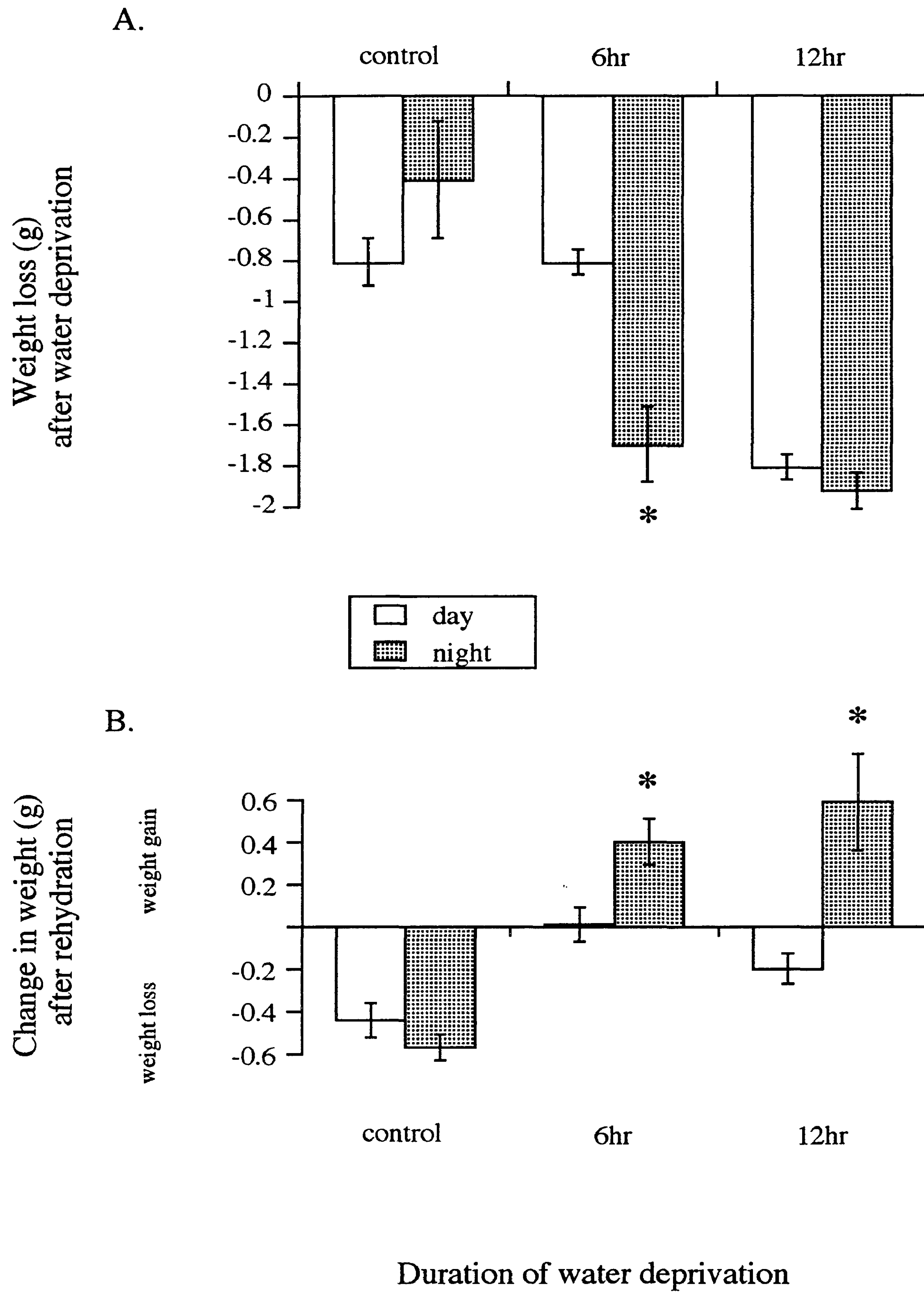

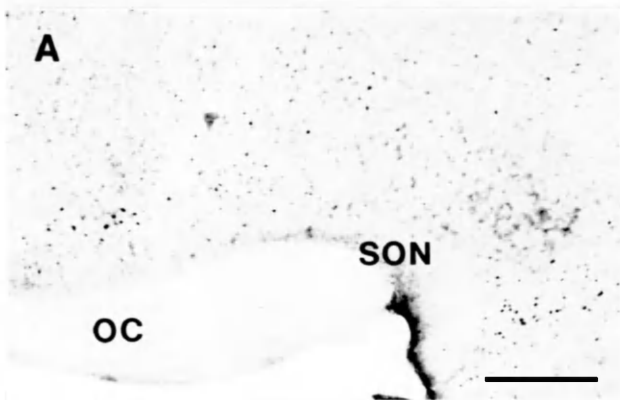
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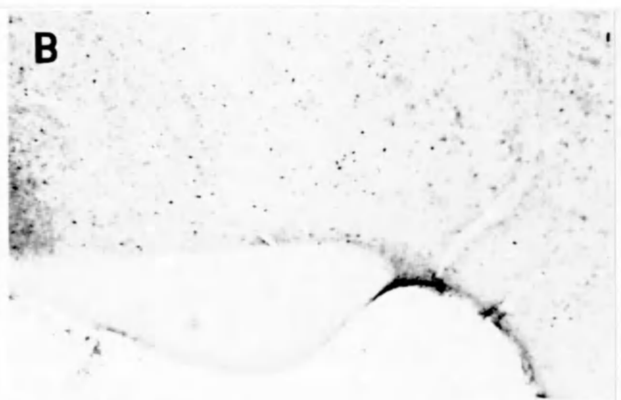
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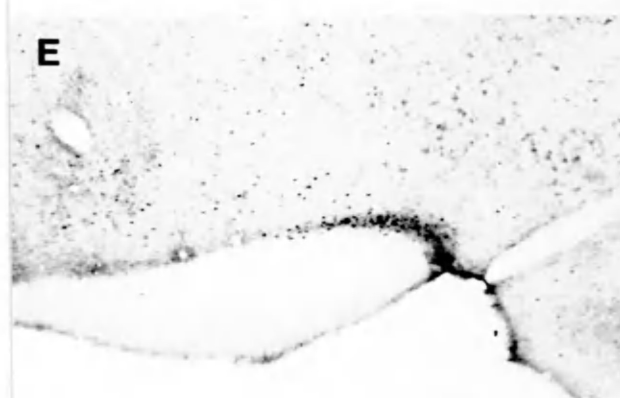
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FIGURE CAPTIONS

<u>Figure 1.</u> Schematic representation of selected neural pathways subserving water regulation. The major sensory area involved in initially detecting dehydration are the SFO and OVLT. These two area seem to be mediated by MnPO. All of these areas, either by direct means or by way of an intermediate, project down to the PVN and SON. These two area contain neurons which release VP and OT to control water balance. Abbreviations: AC, anterior commissure; MnPO, median preoptic nucleus; OVLT, organum vasculosum lamina terminalis; SON, supraoptic nucleus; PVN, paraventricular nucleus; SFO, subfornical organ; FX, fornix; OCH, optic chiasma; PIT, pituitary.

<u>Figure 2.</u> Amount of FOS-LIR A) in the SON and B) in the PVN after water deprivation for 0, 6, 12, 18 hours, and for 1.5 M NaCl injected positive control. * $P < 0.05$ compared with the negative control. † $P < 0.05$ compared with the 6 hour water deprived treatment.

<u>Figure 3.</u> Amount of FOS-LIR A) in the SON and B) in the PVN after water deprivation for 0, 6, 8, 10, and 12 hours. The gray bars represent water restriction during their dark cycle (night) and the open bars represent water restriction during their light cycle (day). *$P < 0.01$ indicates significant day/night differences. †$P < 0.01$ compared with the negative control.

<u>Figure 4.</u> A) Change in weight, relative to controls, during the light period and dark period after 6 or 12 h or water deprivation. B) and after 2 hours of rehydration. The gray bars represent water restriction during their dark period and the open bars represent water restriction during their light period. *$P < 0.01$ indicates significant day/night differences.

Figure 5. Light micrograph showing FOS-LIR in SON sections from representative animals. A) Day negative control SON. B) Night negative control SON. C) Day 6hr water deprivation in the SON. D) Night 6hr water deprivation in the SON. E) Day 12 hr water deprivation in the SON. F) Night 12 hr water deprivation in the SON. G) SON of a hypertonic-saline injected mouse. Abbreviations: Supraoptic nucleus (SON), optic chiasm (OC). Scale bar = 250 μm.

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