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Molecular- and Immuno-histochemical Study on Expressions of Vasopressin and Oxytocin Genes Following Water Deprivation

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ABSTRACT—We investigated the effects of 1 to 7 day water deprivation on expressions of vasopressin (AVP) and oxytocin (OXT) genes in neurosecretory neurons of the supraoptic (SON) and the paraventricular (PVN) nuclei in rats with the oligonucleotide-mRNA *in situ* hybridization (ISH) and the immunohistochemical avidin-biotin-peroxidase complex methods. Autoradiographic hybridization signals that indicate the localization of AVP mRNA were significantly increased in both nuclei after 4 day water deprivation. The water deprivation induced a rapid and marked increase in signals for OXT mRNA especially in the SON. Meanwhile, immunoreactivity of AVP neurons in the mirror image sections of those used for ISH was promptly decreased in both the SON and the PVN after day 1 of the treatment. Stainability of immunoreactive OXT neurons in the PVN was also reduced but after day 4, while the number of stained OXT neurons in the SON was decreased after day 4. Significant hypertrophy was first found in both AVP and OXT neurons in the PVN after day 2 and then in the SON after day 4. The present results thus indicate that both supraoptic and paraventricular AVP neurons are responsible for adapting to water deprivation, and that OXT neurons, especially supraoptic ones, may have some physiological role in the early phase of response to water deprivation. Paraventricular AVP neurons may be more sensitive to water deprivation than to sodium loading by which the elevation of AVP mRNA level in the PVN was not so conspicuous as that in the SON in our previous study.

INTRODUCTION

Arginine vasopressin (AVP) and oxytocin (OXT) are mammalian neurohypophysial hormones synthesized in the hypothalamic magnocellular neurosecretory neurons. AVP has important roles in regulation of plasma osmolality and blood pressure, although any roles of OXT in homeostatic regulation are not clear. In the previous study, we examined effects of sodium loading on AVP and OXT gene expressions with cellular and immunocytochemical changes of neurosecretory neurons in the supraoptic (SON) and the paraventricular (PVN) nuclei by applying the oligonucleotide-mRNA *in situ* hybridization (ISH) and the immunohistochemical methods [1]. After sodium loading, the AVP mRNA level was markedly increased in supraoptic AVP neurons, while the increase in the AVP mRNA level in the PVN was much less than that in the SON. Hypertrophy of AVP neurons was also marked in the SON [1]. On the other hand, it is well known that neurosecretory neurons are responsive not only to sodium loading but also to water deprivation. Although there is no detailed analysis of the effects of water deprivation on expressions of AVP and OXT genes, the AVP mRNA levels in both the SON and the PVN were actually increased after 4 day water deprivation [2, 3]. These results suggest that responses of supraoptic and paraventricular neurosecretory neurons to water deprivation differ from those to sodium loading mentioned above.

The OXT mRNA level was increased rapidly and conspicuously in both the SON and the PVN after sodium loading [1]. OXT release was also stimulated after sodium loading [4, 5]. In addition, water deprivation elevated plasma OXT concentrations [6]. The OXT content in the neurohy-

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pophysis was decreased not only by sodium loading but also by water deprivation [7]. These results indicate that OXT neurons are also responsive to water deprivation other than sodium loading. However, changes in the OXT mRNA level after water deprivation have not been investigated yet.

In the present study, effects of water deprivation on the levels of AVP and OXT mRNAs in magnocellular neurons of the rat hypothalamus were examined by the ISH method using synthetic oligonucleotide probes following the time course for 1 to 7 days. Mirror image sections were immunohistochemically stained, and stainability and sizes of single AVP and OXT immunoreactive (ir) neurons were determined for better understanding of synthetic activity. Present results were compared with those of the previous sodium loading study [1]. Preliminary results appeared elsewhere [8].

MATERIALS AND METHODS

Animals

Male Wistar-Imamichi rats (Imamichi Institute for Animal Reproduction, 140–160 g) were housed in individual cages with 14 L:10 D light schedule. Animals were allowed free access to tap water and standard laboratory chow (Charles River) for at least 5 days prior to the start of the treatment. They were divided into 5 experimental groups, each of them included 7 rats, and were deprived of water for 0, 1, 2, 4 and 7 days prior to sacrifice. Urine samples were collected daily, their volumes were measured, and they were kept at −20℃ until measurements of osmolalities and Na^+ concentrations. The animals were killed by decapitation between 10:00 to 12:00 to avoid possible circadian fluctuations in AVP and OXT gene expressions [9]. Their hypothalami and pituitaries were immediately removed and were immersed in a fixative solution containing 2% paraformaldehyde, 1% glutaraldehyde and 1% picric acid in 0.05 M phosphate buffer (pH 7.3). At the same time, blood was collected and centrifuged. Plasma samples were stored at −20°C. Plasma and urine Na^+ concentrations were measured later with an atomic absorption spectrometer (Hitachi 180–50). Their osmolality were measured with a vapor pressure osmometer (Wescor 5500).

In situ hybridization and quantitation of autoradiographic signals

The individual tissues were paraffin-sectioned, divided into several groups, and were processed for *in situ* hybridization and immunohistochemistry. The precise procedures for tissue preparation and ISH were described previously [10]. Three types of 22mer synthetic deoxyoligonucleotide probes, complementary to the loci of rat mRNAs encoding AVP (2–9), AVP-neurophysin (NP) (1–8) and OXT-NP (1–8), were used in this study. A mixture of the AVP and AVP-NP probes was used as a mixed probe for ISH study of AVP mRNA, since the mixed probe could markedly improve hybridization sensitivity [10]. Thus, we tried to detect changes in AVP mRNA, if occur, in parvocellular AVP neurons in the suprachiasmatic nucleus (SCN) and the parvocellular part of the PVN. The precise procedure for semiquantitative expression of hybridization signals was described previously [1]. In brief, the numbers of autoradiographic silver grains in 100 μm × 100 μm squares settled in each of the SON and the PVN were counted. Then the numbers of grains in the areas adjacent to the SON and the PVN, that is, the background levels, were counted, and were subtracted from the corresponding values in the SON and the PVN. Thereafter, the single-cellular numbers of grains were calculated by dividing the specific numbers of silver grains by the numbers of immunoreactive neurons within the 100 μm × 100 μm squares.

Immunohistochemistry

The mirror image sections to those used for grain counting were immunohistochemically stained by the avidin-biotin-peroxidase complex (ABC) method, the detailed procedure of which was described previously [11]. Specificity tests of immunohistochemistry were also described previously [2, 10]. In this study, primary antisera were used as follows: rabbit anti-AVP (UCB-Bioproducts) was diluted 1:32000 with phosphate buffered saline containing 0.5% bovine serum albumin (PBS-BSA, pH 7.6) and rabbit anti-OXT (a gift from Professor S. Kawashima, Hiroshima

University) was diluted 1:20000 with PBS-BSA. These values for dilution of the antisera, with which tissue sections from normal rats were stained half-maximally, were determined by serial dilution experiments [1].

Intensity of immunohistochemical stainability in

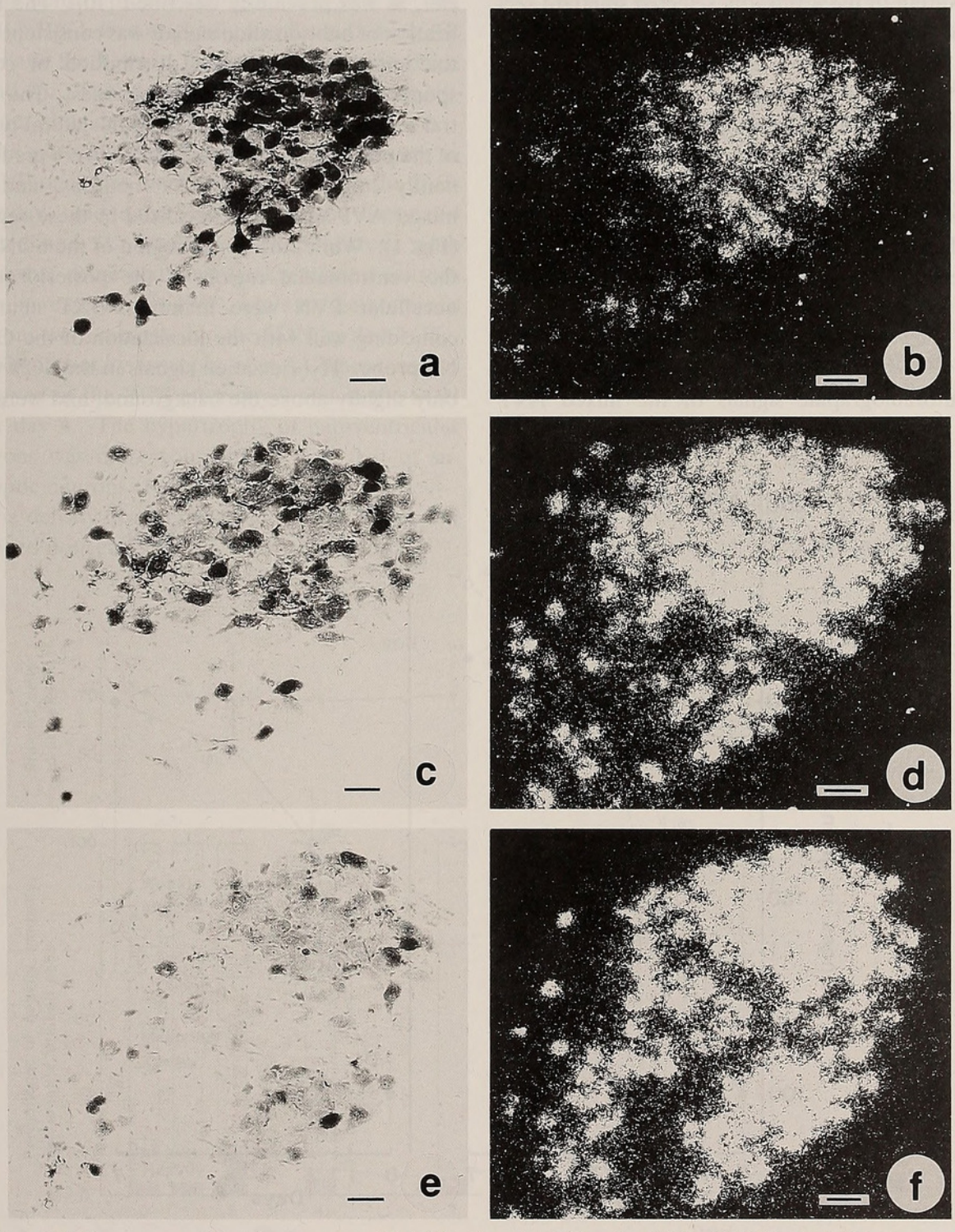

FIG. 1. AVP immunoreactive (ir) neurons and hybridization signals for the AVP mRNA in the PVN of normal (a, b), 4-day (c, d) and 7-day (e, f) water deprived rats in mirror image sections. Note that the density of silver grains over the PVN of the water deprived rats (d, f) is higher than that in the control rat (b). On the contrary, stainability of ir-AVP neurons in the water deprived rats (c, e) is reduced from that in the control rat (a). Scale bar, 50 μm. Dark-field photomicrographs for ISH.

each magnocellular neuron was scored according to the following criteria: not stained, weakly stained, medially stained and heavily stained, by consulting to the arbitrarily selected standard sections. Immunoreactivity in each of the SON and the PVN was shown by the percentage of the number of ir-neurons to that of total magnocellular neurons, and the percentages of weakly, medially and heavily stained neurons in either ir-AVP or ir-OXT neurons. In addition, after depicting the outline of individual ir-neurons with a camera lucida, their cellular areas were determined with a tablet degitizer-microcomputer system.

RESULTS

Autoradiographic signals of the mixed AVP probe and the OXT-NP probe were densely localized over the magnocellular neurons in the SON, the PVN and several accessory magnocellular nuclei, as was previously described [10]. The localization of hybridization signals was consistent with the immunohistochemical distribution of corresponding neurohypophysial hormones. The ventral region of the SON and the dorsolateral region of the posterior magnocellular PVN were predominantly composed of ir-AVP neurons, and the mixed AVP probe was localized in these regions (Fig. 1). While, the dorsal region of the SON and the ventromedial region of the posterior magnocellular PVN were mainly ir-OXT neurons, coinciding well with the localization of the OXT-NP probe. Hybridization signals in the SCN were only slightly above the background, and were not

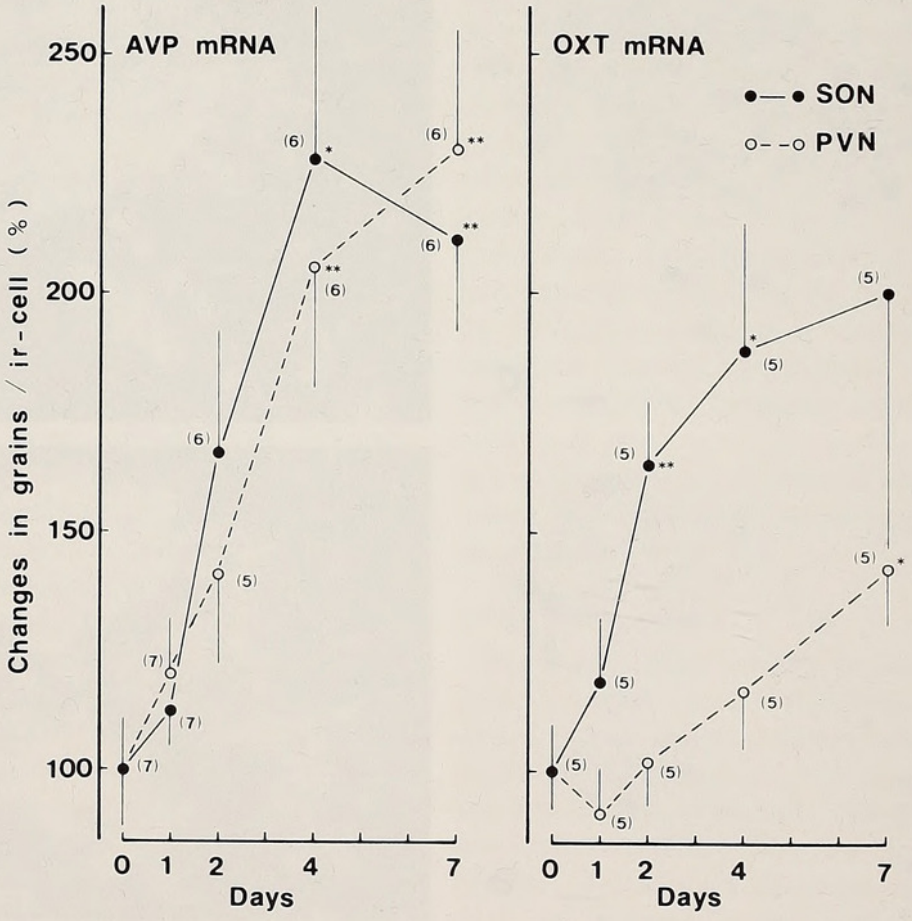

FIG. 2. Effects of water deprivation on the AVP and OXT mRNA levels in the SON and the PVN. The mRNA levels are expressed relatively as percent changes compared to the level of day 0. The numbers of silver grains/ir-cell on day 0 are: AVP mRNA in the SON, 45.8±7.2; AVP mRNA in the PVN, 95.7±10.8; OXT mRNA in the SON, 15.9±1.6; OXT mRNA in the PVN, 42.7±3.7. Each point represents the mean±S.E.. The number of animals is given in parentheses. *, $p<0.05$; **, $p<0.01$; by the t-test compared to day 0.

noticeable in the parvocellular part of the PVN.

AVP neurons

The density of autoradiographic signals for the AVP mRNA was markedly increased in both the SON and the PVN by the water deprivation (Figs. 1 and 2). The increase became statistically significant after day 4 of the treatment.

The percentage of heavily stained neurons in ir-AVP ones was markedly decreased in the PVN after day 1 and in the SON at day 1 and after day 4 (Figs. 1 and 3). Medially stained neurons were somewhat decreased, and weakly stained neurons were increased in the PVN, while medially stained neurons were increased in the SON by day 7 (Fig. 3). Significant hypertrophy of ir-AVP neurons was observed in the PVN after day 2 and in the SON after day 4. The hypertrophy of paraventricular neurons was more conspicuous than that of supraoptic neurons (Fig. 4).

The density of silver grains in the SCN was not changed noticeably by the water deprivation.

OXT neurons

The density of hybridization signals for the OXT mRNA was rapidly increased and attained to a submaximal level in the SON on day 2 of water deprivation (Fig. 2). On the other hand, the increase in the hybridization signals in the PVN was much smaller than that in the SON and was significant only by day 7.

The percentage of the number of ir-OXT neurons to total magnocellular neurons in the SON was decreased after day 4, while medially stained neurons were increased after day 4 (Fig. 5). The percentage of heavily stained neurons was decreased in the PVN after day 4 (Fig. 5). Significant hypertrophy of ir-OXT neurons was observed in the PVN after day 2 and in the SON after day 4 (Fig. 4).

Neurohypophysis

Although immunoreactivity of the neurohypophysis was not analyzed quantitatively, it

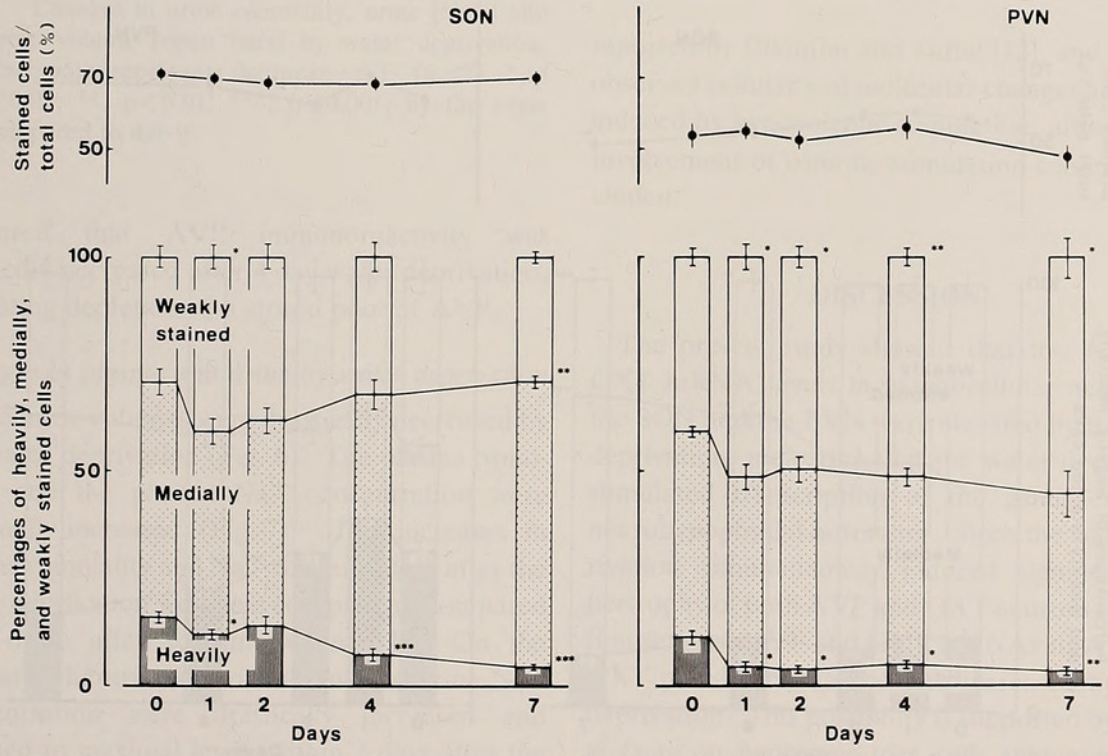

FIG. 3. Effects of water deprivation on the percentages of immunoreactive (ir) AVP neurons to total magnocellular neurons, and the percentages of heavily stained, medially stained and weakly stained AVP neurons per total ir-AVP neurons in the SON and the PVN. Each point represents the mean ± S.E. (n=7). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; by the t-test compared to day 0.

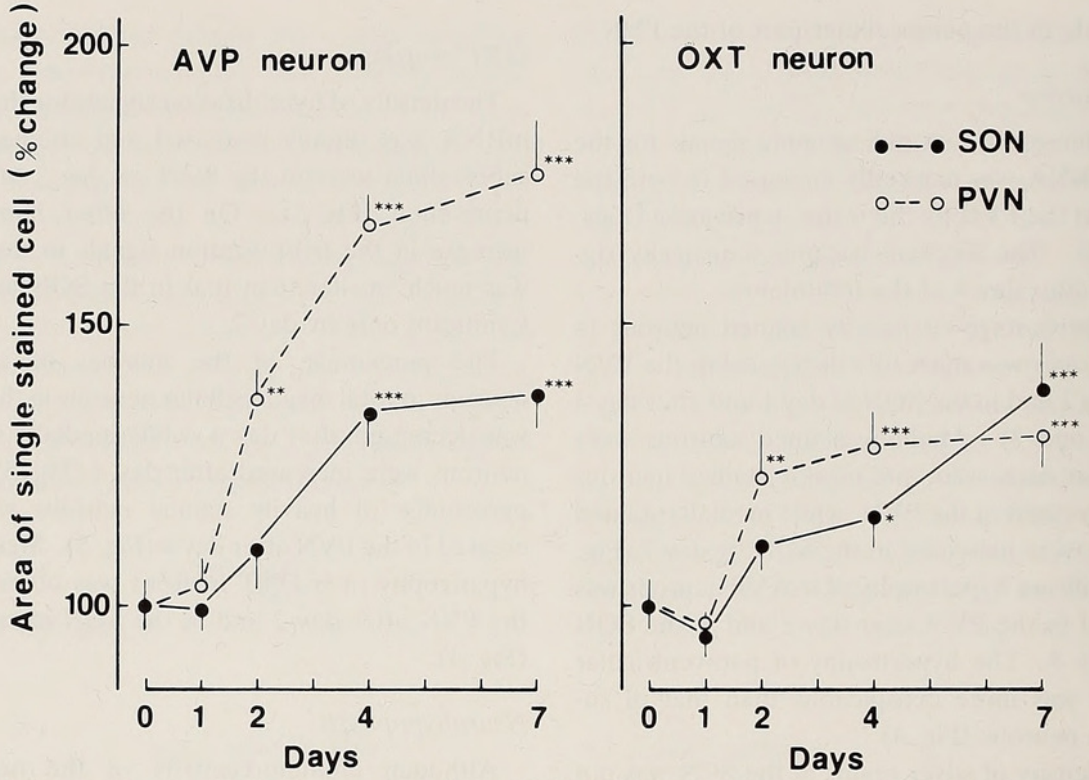

FIG. 4. Effects of water deprivation on sizes of the AVP and OXT immunoreactive neurons in the SON and the PVN. The values of cell areas ($\times 10^{-4}$mm^2) on day 0 are: AVP neurons in the SON, 3.16±0.14; AVP neurons in the PVN, 3.25±0.16; OXT neurons in the SON, 3.00±0.14; OXT neurons in the PVN, 3.04±0.13. Each point represents the mean±S.E. (n=7). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; by the t-test compared to day 0.

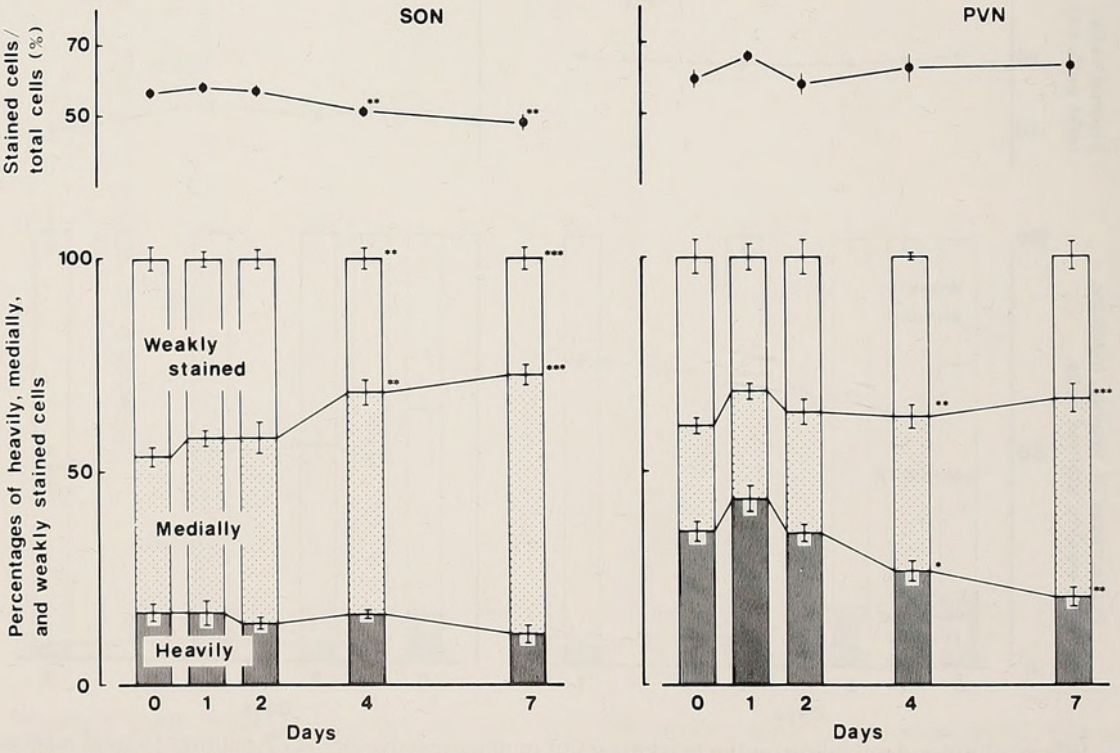

FIG. 5. Effects of water deprivation on the percentages of immunoreactive (ir) OXT neurons to total magnocellular neurons, and the percentages of heavily stained, medially stained and weakly stained OXT neurons per total ir-OXT neurons in the SON and the PVN. Each point represents the mean±S.E. (n=7). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; by the t-test compared to day 0.

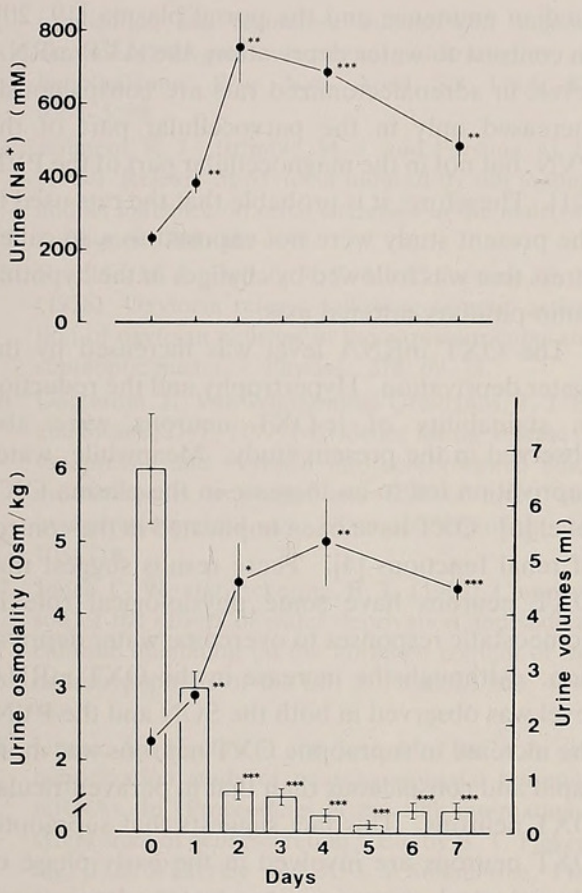

FIG. 6. Changes in urine osmolality, urine [Na^+] and urine volume (open bars) by water deprivation. Each point represents the mean ± S.E. (n=7). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; by the t-test compared to day 0.

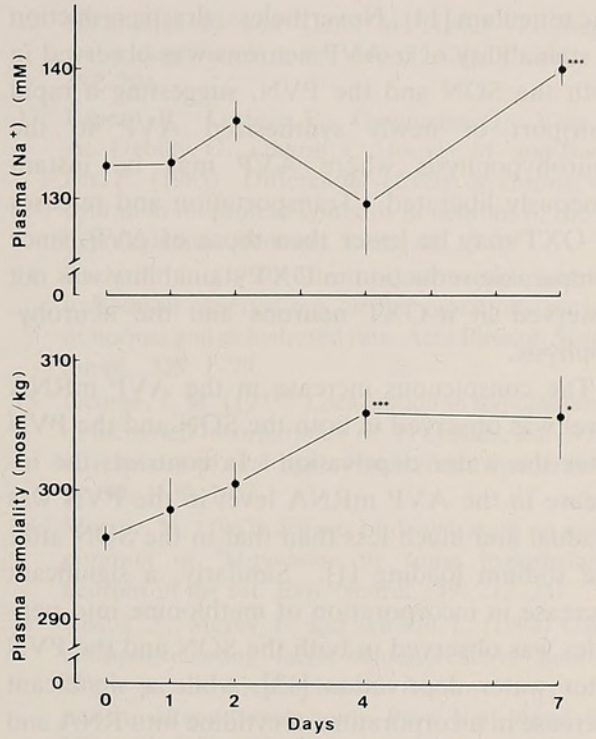

FIG. 7. Changes in plasma osmolality and plasma [Na^+] by water deprivation. Each point represents the mean ± S.E. (n=7). *, $p<0.05$; **, $p<0.001$; by the t-test compared to day 0.

appeared that AVP immunoreactivity was markedly decreased after 4 day water deprivation, indicating depletion of a stored pool of AVP.

Changes in plasma and urine by water deprivation

The urine volumes were drastically decreased by the water deprivation (Fig. 6). The plasma osmolality and the plasma Na^+ concentration were gradually increased (Fig. 7). The increases in plasma osmolality and Na^+ concentration after the water deprivation were less conspicuous compared with those after sodium loading [1]. On the contrary, the urine osmolality and the urine Na^+ concentration were drastically increased and attained to maximal levels within 2 days after the onset of the water deprivation (Fig. 6). These facts indicate that the water deprived animals were exposed to severe hypovolemic stimulation, as was reported by Elkinton and Taffel [12], and that the observed cellular and molecular changes are partly induced by hypovolemic stimulation, although the involvement of osmotic stimulation cannot be excluded.

DISCUSSION

The present study showed that the AVP and OXT mRNA levels in magnocellular neurons in the SON and the PVN were elevated by the water deprivation, indicating that the water deprivation stimulated transcription of the genes encoding neurohypophysial hormones. Since the water deprivation simultaneously induced significant hypertrophy of both AVP and OXT neurons, translation rates of AVP and OXT mRNAs to AVP and OXT precursors may be also increased by water deprivation. This possibility is supported by several facts on neurosecretory cells that a few day water deprivation elicited an increase in amino acid incorporation [13], and increases in nuclear and nucleolar diameters and dilation of endoplas-

mic reticulum [14]. Nevertheless, drastic reduction in stainability of ir-AVP neurons was observed in both the SON and the PVN, suggesting a rapid transport of newly synthesized AVP to the neurohypophysis where AVP may be instantaneously liberated. Transportation and releases of OXT may be lesser than those of AVP, since comparable reduction in OXT stainability was not observed in ir-OXT neurons and the neurohypophysis.

The conspicuous increase in the AVP mRNA level was observed in both the SON and the PVN after the water deprivation. In contrast, the increase in the AVP mRNA level in the PVN was gradual and much less than that in the SON after the sodium loading [1]. Similarly, a significant increase in incorporation of methionine into peptides was observed in both the SON and the PVN after water deprivation [13], while a significant increase in incorporation of cytidine into RNA and that of tyrosine into peptides in response to sodium loading were demonstrated in the SON, but not in the PVN [15, 16]. These results suggest that AVP neurons in both the SON and the PVN are sensitive to hypovolemic stimulation, and that supraoptic AVP neurons are more responsible for osmotic or sodium regulation than paraventricular ones.

Although the increase in the AVP mRNA level were similar in the SON and the PVN, the hypertrophy of paraventricular AVP neurons was more conspicuous than that of supraoptic AVP neurons. Furthermore, the reduction in stainability of ir-AVP neurons was also marked in the PVN. These results show the possibility that the release of AVP from axon terminals of paraventricular AVP neurons are more active than that from axon terminals of supraoptic AVP neurons.

In the present study, the noticeably dense localization of signals for the AVP mRNA and the change in the density were observed in the magnocellular part of the PVN, but not in the parvocellular part of the PVN. AVP and corticotropin-releasing factor coexist in parvocellular neurosecretory neurons in the PVN which project to the external zone of the median eminence [17]. They act synergistically to release corticotropin from the anterior pituitary [18]. Adrenalectomy resulted in increases in the AVP levels in the median eminence and the portal plasma [19, 20]. In contrast to water deprivation, the AVP mRNA levels in adrenalectomized rats are conspicuously increased only in the parvocellular part of the PVN, but not in the magnocellular part of the PVN [21]. Therefore, it is probable that the rats used in the present study were not exposed to a so-called stress that was followed by changes in the hypothalamo-pituitary-adrenal axis.

The OXT mRNA level was increased by the water deprivation. Hypertrophy and the reduction in stainability of ir-OXT neurons were also observed in the present study. Meanwhile, water deprivation led to an increase in the plasma OXT level [6]. OXT have been implicated in the control of renal functions [4]. These results suggest that OXT neurons have some physiological role in homeostatic responses to overcome water deprivation. Although the increase in the OXT mRNA level was observed in both the SON and the PVN, the increase in supraoptic OXT neurons was more rapid and conspicuous than that in paraventricular OXT neurons. This fact suggests that supraoptic OXT neurons are involved in the early phase of physiological responses to water deprivation. However, the reason for the above difference between paraventricular and supraoptic OXT neurons remains to be clarified.

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