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# Hypothalamo-Pituitary-Adrenal Axis Sensitization after Chronic Salt Loading

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**Key Words**
Rehydration · Corticotropin · Corticotropin-releasing hormone · Vasopressin · Paraventricular nucleus · Adrenal steroids

**Abstract**
Hypothalamic parvocellular vasopressin (VP) and corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN) are major secretagogues of corticotropin (ACTH), and central plasticity including their alteration is closely related to hypothalamic-pituitary-adrenal (HPA) axis modulation. Chronic hyperosmotic stress caused by 2% salt lodaing has been known to alter VP and CRH expression. We recently reported that rehydration, a recovery stage from salt loading, induced a prolonged increase in parvocellular VP mRNA expression and suggested that rehydration can modulate HPA axis function without obvious external stress. In the present study, we examined hypothalamic VP and CRH mRNA expression and their responsiveness to acute immobilization stress in control, salt-loaded and rehydrated animals, in order to clarify the precise mechanism of HPA axis regulation during rehydration. The results were further compared with plasma corticosterone and ACTH levels. Plasma corticosterone decreased during salt loading, whereas it increased during rehydration at 1 week. Basal ACTH concentration increased in 1-week-rehydrated animals, with enhanced responsiveness to the acute immobilization stress. In the hypothalamic parvocellular PVN, basal CRH mRNA levels also decreased during salt loading and increased during rehydration. Basal VP mRNA was upregulated during both salt loading and rehydration. VP mRNA responded to additional acute stress during salt loading and rehydration, but CRH mRNA did not. These results indicate that the HPA axis activity of parvocellular neurons is still altered at 1 week of rehydration and that VP plays a dominant role in regulating ACTH release in response to acute stress. This rehydration stage may thus be a good model for analysis of post-stress sensitization of the HPA axis.



## Introduction

Regulation of hypothalamic-pituitary-adrenal (HPA) axis in vivo is complex and its activity is modulated by surrounding conditions such as social stress, immune stress or lactation [1–5]. Repeated stress affects responsiveness of pituitary corticotropes, an effect depending upon the type of stress and determined by corticotropin-releasing hormone (CRH) and vasopressin (VP) release from the paraventricular nucleus (PVN) in the hypothalamus [6]. CRH is known to regulate pituitary ACTH release [7, 8] and production [9]. VP, a strong osmoregulating hormone mainly synthesized in the magnocellular PVN, was found to be

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co-localised with CRH in the same vesicles of PVN parvocellular neurons [10] and in the external layer of the median eminence [11]. In vivo and in vitro studies demonstrated that VP potentiates CRH-induced ACTH secretion [7, 12]. VP [13–17] and/or CRH mRNA [18–21] expression is increased following stress. The expression pattern of VP and CRH is stressor specific and accounts for the diversity of HPA responsiveness to repeated stress.

Chronic osmotic stimulation affects endocrine responsiveness of the HPA axis as does stress. Chronic salt loading or water deprivation has been reported to diminish ACTH release from the anterior pituitary lobe [22–24]. In the PVN, CRH expression shifts from parvocellular to magnocellular neurons after chronic osmotic stimulation [25, 26]. We recently reported that parvocellular VP expression increased in a chronic salt-loading model [1]. This parvocellular VP expression remains increased even after 1 week rehydration, a time when osmotic stimulation is no longer present. As parvocellular VP is considered to regulate ACTH, the activity of the HPA axis in animals rehydrated for 1 week is thought to be still modified without obvious stressful condition. The rehydration procedure, therefore, represents a good model to study poststress modulation of the HPA axis. Little is known however on the extent of HPA axis alteration during rehydration and on the mechanisms involved.

In the present study, we hypothesized that the HPA axis does not recover immediately after osmotic stimulation, but may enter a new status of activity during rehydration. This long-lasting alteration of the HPA axis could result from functional plasticity of hypothalamic VP and CRH neurons. To test this hypothesis, we examined basal and stress-induced expression of parvocellular CRH and VP mRNA during salt loading and rehydration using quantitative in situ hybridization. Plasma corticosterone and ACTH levels were also monitored under the same conditions.

## Materials and Methods

*Animal Treatment*

Adult male Sprague-Dawley rats (weighing 250–300 g, Nihon Animal Co.) housed under 12 h light/12 h dark schedule (5 animals per cage) were used in these experiments. All animal experiments were conducted in accordance with the NIH guidelines for the care and use of laboratory animals. Experiments were also permitted by inspection of the Committee of Animal Research, Kyoto Prefectural University of Medicine. Animals in the salt-loaded group were given 2% NaCl in their drinking water ad libitum for 7 days. Those in the rehydrated group were allowed free access to water, following the initial 7 days of salt loading. Animals in the control group were allowed free access to water throughout the study.

*Experiment 1: Plasma Corticosterone Assay*

Control, salt-loaded, 1-, 2- and 4-week-rehydrated animals (n = 4 for each group) were decapitated immediately and trunk blood was collected. Blood samples were then centrifuged and plasma was collected. Plasma corticosterone levels were measured using corticosterone RIA kit according to the manufacturer's instructions (Peninsula Lab., Calif., USA)

*Experiment 2: Combined Procedure of Salt Loading/Rehydration and Acute Immobilization Stress*

To clarify more precisely the mechanism of HPA axis plasticity under chronic salt loading, we investigated the effect of acute immobilization stress on plasma ACTH level, hypothalamic CRH and VP expression in control, salt loaded and rehydrated animals. Rehydration extent was decided as 1 week because plasma corticosterone levels were increased during 1 week rehydration and then gradually returned to normal levels 4 weeks after the start of rehydration. Figure 1 shows the experimental schedule of immobilization stress and salt-loading/rehydration treatment. Animals in the control (CONT), salt loaded (SALT) and 1-week-rehydrated (REHYD) groups were further divided into stressed (ST; n = 5 for each group) and non-stressed (NS; n = 5 for each group) subgroups. On the final day, animals in the stressed group were exposed to immobilization stress for 90 min by fixing their limbs onto a fixing cage. Stressed and non-stressed animals were decapitated immediately and trunk blood was collected and brains were removed. To examine the extent of plasma osmolality alteration, plasma osmotic pressure was measured in each group. To minimize the circadian variation of plasma corticosterone and ACTH concentration, all procedures were performed between 09.00 and 12.00 h in the morning.

*ACTH RIA Assay*

Three-milliliter blood samples were collected into chilled polypropylene tubes containing ethylenediaminetetraacetic acid (EDTA, 1 mg/ml of blood) and aprotinin (500 kIU/ml of blood). Samples were centrifuged and plasma was extracted. Plasma ACTH was assayed using an ACTH RIA kit (Peninsula).

*Synthesis of Probes for in situ Hybridization*

[$^{35}$S]-CTP labeled cRNA probes were used for in situ hybridization. The antisense RNA probes for prepro-CRH and VP were transcribed from rat prepro-CRH cDNA (765 bp; synthesized by PCR from single-strand complementary DNA from the rat hypothalamus) or VP cDNA (195 bp; a gift from Dr. J.P.H. Burbach) subcloned into pGEM (Promega Co., Wisc., USA) using T7or Sp6 polymerase and 1,000 Ci/mmol [$^{35}$S]-CTP. Control experiments using sense RNA probes synthesized from the same cDNAs yielded no specific hybridization signals on these sections.

*In situ Hybridization*

After fixation with 4% paraformaldehyde in 0.1 *M* phosphate buffer (PB) adjusted to pH 7.4 for 10 h at 4°C, brains were cryoprotected in 20% sucrose in 0.1 *M* PB. Frontal sections (20 μm thick) containing the hypothalamus were cut on a cryostat, and immersed in 4 × SSC. Sections of all experimental groups were processed simultaneously for in situ hybridization to minimize methodological variations. The protocol employed for hybridization was similar to that described previously [27]. Briefly, tissue sections were deproteinized with proteinase K and acetylated prior to hybridization. Then, sections were incubated with 1 μl/ml labeled riboprobe in

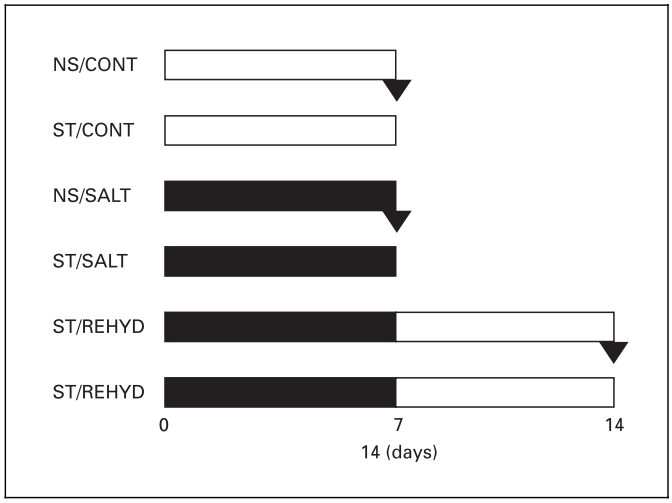
hybridization buffer (10 h at 60°C). Following the hybridization, tissue sections were washed in 2 × SSC containing 50% formamide, RNAase solution and 0.4 × SSC. Sections were mounted on gelatinized slides, and exposed to X-ray film (Fuji Imaging plate, Fuji Photo Co., Japan) for 12 h. Then, sections were dipped in photographic emulsion (K5, Ilford, England, diluted 1:1 with water) and developed after exposure in the dark for 3 days at 4°C.

*Quantitative Analysis of mRNA Signals*

Four anatomically matched sections of the PVN at least 40 μm apart were analyzed bilaterally for each animal. Quantitative analysis of CRH/VP mRNA signal intensities was performed using a computerized Neuroimage analysis system on a Macintosh computer [28]. For CRH mRNA analysis, optical density of autoradiographic signals in the area corresponding to parvo- and magnocellular PVN was measured [29]. The medial parvocellular and magnocelluar subdivisions of the PVN were determined histologically [30] from cresyl violet staining and aligned with corresponding dark field images of in situ signals under ×100 magnification. Signal intensity in the cerebral cortex of each section was taken as background. Quantitative analysis of parvocellular VP mRNA signals were determined by analyzing the intensity of silver grains over individual cells on cresyl violet-counterstained sections. The medial parvocellular PVN [30] was determined histologically under ×100 magnification, then silver grains in all parvocellular neurons in the area with visible nuclei were measured under ×400 magnification [16]. Scattered magnocellular VP neurons in the parvocellular subdivisions were excluded by their relative size (under 25 μm in its diameter for parvocellular neurons while over 25 μm for magnocellular neurons) and grain intensities. Neurons were considered to be positive for VP if the number of silver grains was at least threefold higher than the background. Average densities of neurons were evaluated as the mean density of each animal.

*Statistical Analyses*

All quantitative data were presented as the mean ± SD. One-way analysis of variance (ANOVA) was used in the analysis of plasma corticosterone level and two-way ANOVA was used to evaluate the influence of salt loading and acute immobilization stress. Statistical significance of the differences between experimental groups was determined by Fisher's PSLD posthoc procedure. A $p < 0.01$ was considered significant.

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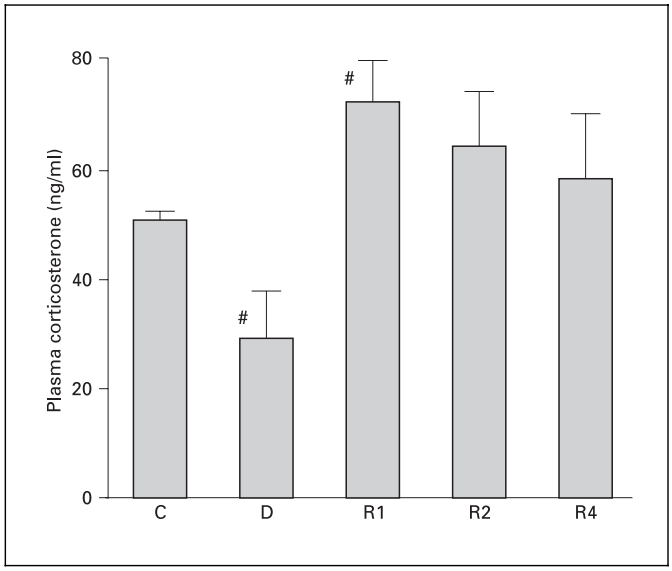


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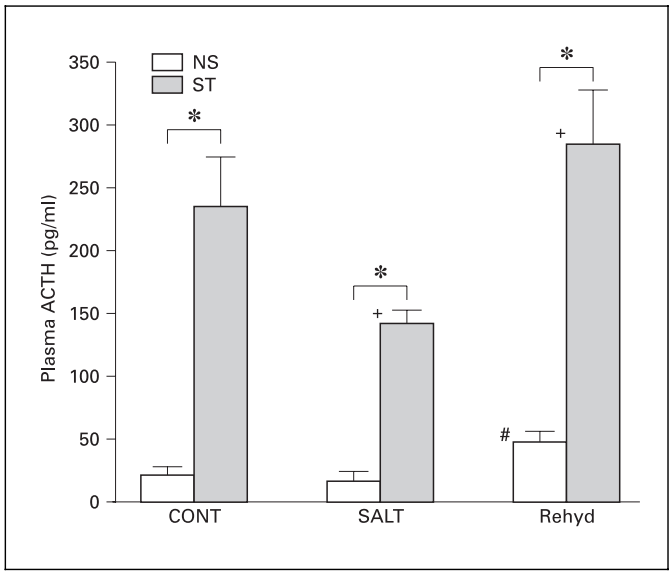


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**Fig. 1.** Procedure of animal treatment in experiment 2. NS/CONT = Nonstressed control animals; ST/CONT = stressed control animals; NS/SALT = nonstressed salt loaded animals; ST/SALT = stressed salt loaded animals; NS/REHYD = nonstressed rehydrated for 1 week animals; ST/REHYD = stressed rehydrated for 1-week animal. Closed bar = Animals were given 2% NaCl in drinking water; open bar = animals were given tap water; arrowhead = immobilization stress for 90 min.

**Fig. 2.** Changes in plasma corticosterone level in control (C), salt-loaded (S) and 1, 2 and 4 weeks rehydrated (R1, R2 and R4, respectively) animals. # $p < 0.01$ vs. CONT. n = 4 for each subgroup. Values are expressed as means ± SD.

**Fig. 3.** Changes in plasma ACTH levels in experiment 2. * $p < 0.01$ vs. NS; # $p < 0.01$ vs. CONT/NS; + $p < 0.01$ vs. CONT/ST. n = 10 for each subgroup. Values are expressed as means ± SD.

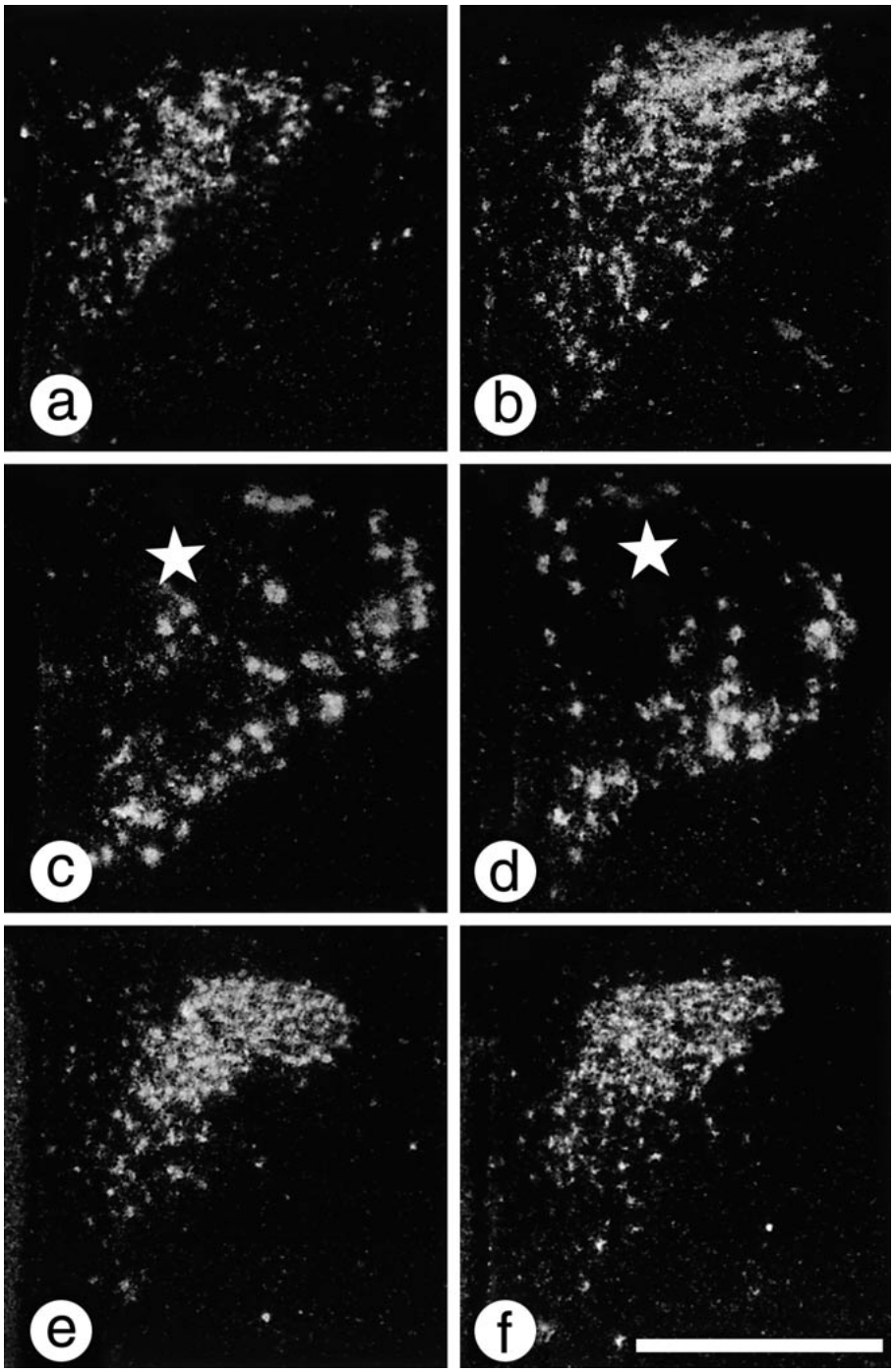


**Fig. 4.** Dark-field photomicrograph of CRH mRNA expression in the PVN. **a** NS/CONT. **b** ST/CONT. **c** NS/SALT. **d** ST/SALT. **e** NS/REHYD. **f** ST/REHYD. CRH mRNA signals (silver grains) were found within parvocellular PVN in CONT (**a, b**). Signals of CRH mRNA were decreased in the parvocellular PVN (star), but increased in the magnocellular PVN in SALT (**c, d**). In REHYD, CRH mRNA expression was again localized in the parvocellular PVN (**e, f**). Acute immobilization stress induced increases in parvocellular CRH mRNA expression in CONT (**b**). In SALT and REHYD, however, no obvious changes were observed between NS and ST animals (**d, f**). Bar = 200 μm.

## Results

*Plasma Corticosterone Level*

Figure 2 indicates basal plasma corticosterone levels in animals of control (50.5 ± 1.6 ng/ml) and salt loading (29.1 ± 8.6) and after 1 (72.2 ± 6.9), 2 (63.9 ± 9.7), and 4 (58.4 ± 11.3) weeks of rehydration. Plasma corticosterone decreased in salt-loaded animals whereas it increased significantly in 1-week-rehydrated animals. The level of corticosterone concentration gradually decreased 2 weeks after rehydration and returned to normal 4 weeks after rehydration.

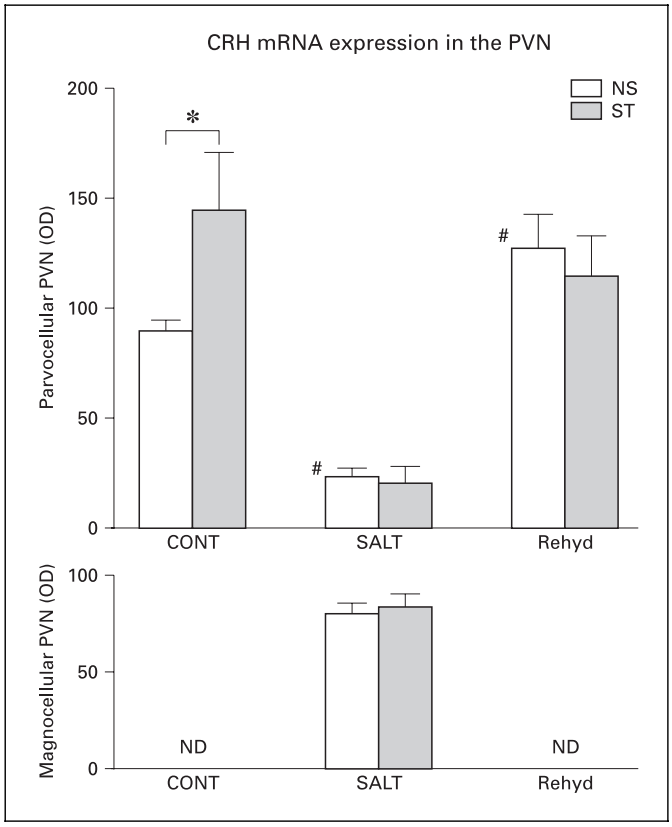


**Fig. 5.** Aeration of parvo- and magnocellular CRH mRNA expression in the PVN in experiment 2. ND: not detected. * $p < 0.01$ vs. NS; # $p < 0.01$ vs. CONT/NS. n = 5 for each subgroup. Values are expressed as means ± SD.

*Alteration of Plasma Osmotic Pressure*

Plasma osmotic pressure was significantly increased in the salt-loading group compared with the control group. Plasma osmotic pressure returned to the control level after 1 week of rehydration. Acute immobilization stress did not affect osmotic pressure in any of the three experimental groups (table 1).

*Plasma ACTH Concentration*

Acute immobilization stress induced an increase in the ACTH concentration in control (243.4 ± 33.9 ng/ml), salt-loading (143.0 ± 13.6) and rehydration groups (288.4 ± 37.8). The magnitude of this increase was not as large in the salt-loading group as in the controls, although it was augmented in rehydrated animals (fig. 3). Basal plasma ACTH concentration did not change between the control (23.0 ± 1.6) and salt-loading groups (16.2 ± 6.8), whereas a significant increase was found in the rehydration group (52.6 ± 3.6).

**Table 1.** Plasma osmotic pressure (mm Hg)

| | NS | ST |
|---|---|---|
| CONT | 279 ± 15 | 281 ± 17 |
| SALT | 294 ± 16[a] | 298 ± 20[b] |
| REHYD | 284 ± 15 | 287 ± 19 |

Values indicate mean ± SD.
[a] $p < 0.01$ vs. CONT/NS.
[b] $p < 0.01$ vs. CONT/ST.

*CRH mRNA Expression in the Paraventricular Nucleus*

Figure 4 shows the CRH mRNA distribution before (fig. 4a, c, e) and after immobilization stress (fig. 4b, d, f) in the control (fig. 4a, b), salt-loading (fig. 4c, d) and rehydration groups (fig. 4e, f). The quantified amount of this transcript within parvo- and magnocellular PVN is indicated in figure 5. CRH mRNA was observed in the parvocellular neurons of the PVN in the control and rehydration groups. In the salt-loading group, the CRH mRNA was reduced in the parvocellular neurons, and was now detectable in the magnocellular neurons. CRH mRNA expression was restored in the parvocellular neurons in the rehydration group. The amount of CRH mRNA in nonstressed animals of the rehydration group was significantly increased compared with nonstressed animals in the control group. In the control group, acute immobilization stress increased the level of CRH mRNA expression in the parvocellular PVN. However, the level of CRH mRNA expression in the salt-loading and rehydration groups did not change significantly between before and after immobilization stress.

*VP mRNA Expression in the Paraventricular Nucleus*

Figure 6 shows the distribution of VP mRNA in both magno- and parvocellular PVN. Mean quantified signal intensities of VP mRNA in parvocellular neurons are shown in figure 7. In the control group, VP mRNA expressing neurons were localized in the magnocellular neurons of the PVN, and only a few VP mRNA-positive parvocellular neurons were detected (fig. 6a, b). In the salt-loading (fig. 6c, d) and 1-week-rehydration groups (fig. 6e, f), VP mRNA was expressed in the parvocellular neurons as well, in addition to prominent expression in the magnocellular neurons. There was no significant difference in the VP mRNA expression pattern/level be-

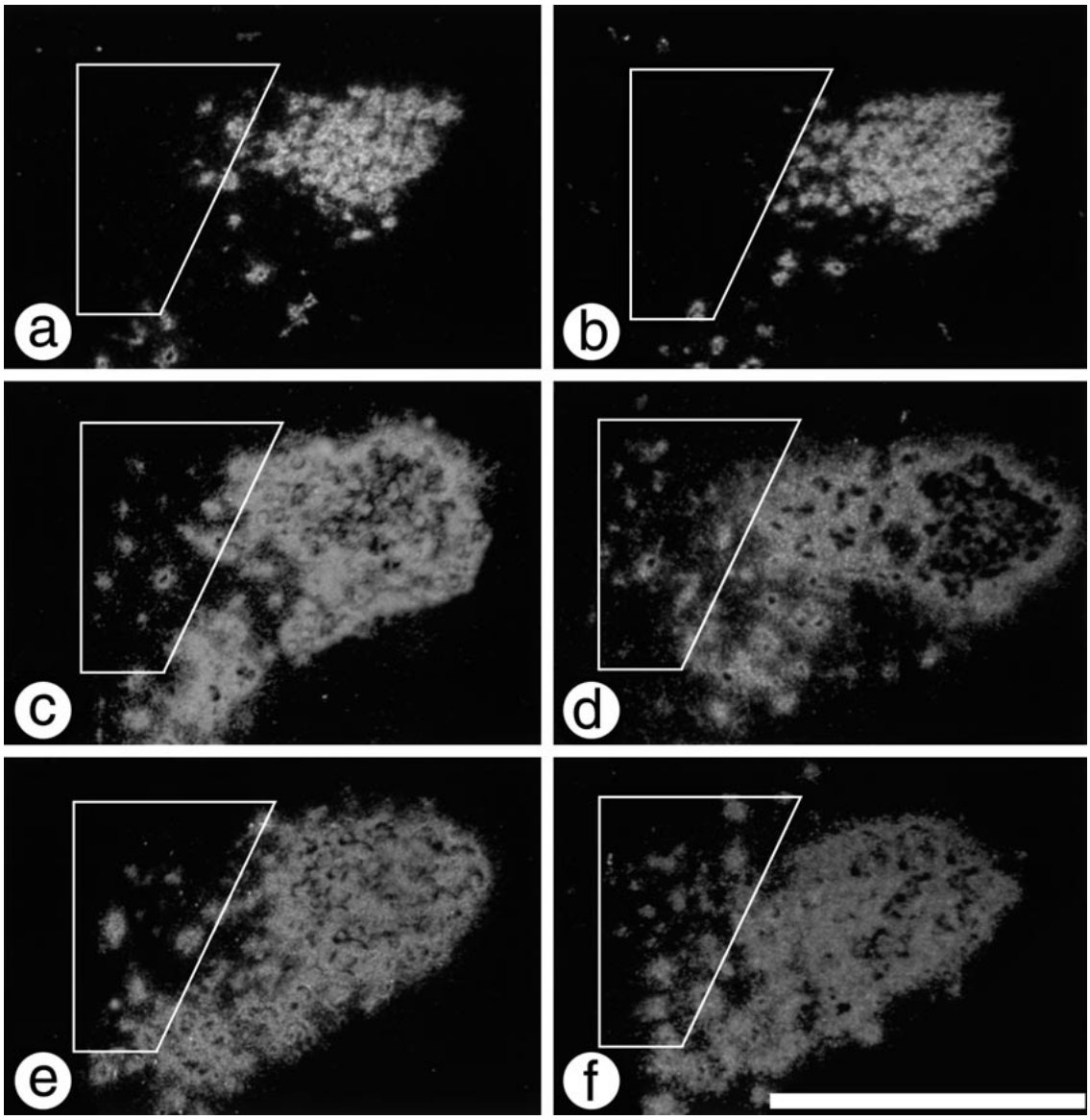


**Fig. 6.** Dark-field photomicrographs of VP mRNA expression in the PVN (**a–f**, bar = 200 μm). The outlined area depicts the dorsomedial parvocellular portion. **a** NS/CONT. **b** ST/CONT. **c** NS/SALT. **d** ST/SALT. **e** NS/REHYD. **f** ST/REHYD. In the CONT group, VP mRNA was expressed predominantly in the magnocellular neurons, and a few parvocellular VP mRNA-expressing neurons were also found (**a**). In CONT, there were no obvious changes in parvocellular VP mRNA expression after stress (**b**). In the SALT and REHYD groups, considerable numbers of VP mRNA-expressing parvocellular neurons were seen, with a marked increase in number of magnocellular VP mRNA-positive neurons (**c, e**). Parvocellular VP mRNA expression increased after stress in the SALT and REHYD groups (**d, f**).

tween stressed and nonstressed animals in the control group. However, the mean signal intensity of VP mRNA in the parvocellular neurons was increased after immobilization stress in the salt-loading and 1-week-rehydration groups.

## Discussion

*Activity of the HPA Axis in Rehydrated Animals*

In terms of plasma corticosterone concentration, the present findings are consistent with previous studies on downregulation of the HPA axis during salt loading [23]. In contrast, plasma corticosterone levels were increased in

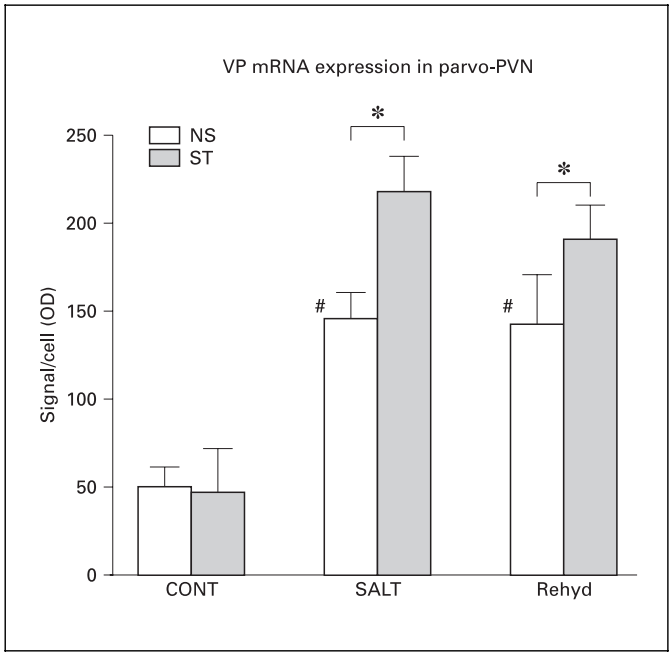


**Fig. 7.** Alteration of mean signal intensity of parvocellular VP mRNA in experiment 2. * $p < 0.01$ vs. NS; # $p < 0.01$ vs. CONT/NS. n = 5. Values are expressed as mean ± SD.

1-week-rehydrated animals as compared with those of controls. Increased plasma corticosterone returned to normal levels 4 weeks after cessation of osmotic stimulation. To ascertain whether the increased corticosterone level in animals rehydrated for 1 week is centrally regulated, we also examined plasma ACTH levels. In the rehydration group, we found that both basal and stress-induced ACTH release was upregulated. Previous studies have revealed that chronic salt loading elicits a desensitization of the corticosterone level and blunted ACTH responses [22]. Taken together, modulation of the HPA axis by chronic osmotic stress consisted of biphasic (desensitized salt-loading and sensitized rehydration) periods. The HPA axis activation continued up to at least one week after the cessation of osmotic stimulation. Although no apparent external stimuli existed and plasma osmotic pressure returned to normal levels, the HPA axis did not normalize for 1 week as if rehydrated animals were receiving some kind of chronic stress. Prolonged treatment with the glucocorticoid agonist dexamethasone also attenuates the HPA axis response. In contrast to salt loading treatment, however, ACTH response are normalized within 7 days after discontinuation of dexamethasone treatment [31]. The reason for the activation of the HPA axis in 1-week-rehydrated animals is unclear.

*VP Predominantly Regulates ACTH during Rehydration*

To assess mechanisms involved in central HPA axis regulation during rehydration, we investigated VP and CRH expression and its response to acute immobilization stress in salt-loaded and 1-week-rehydrated animals.

Expression of parvocellular VP mRNA in both salt-loaded and 1-week-rehydrated animals increased after acute immobilization stress. This is unlikely due to alteration of osmotic induced parvocellular VP, because osmotic pressure showed no difference during immobilization stress. We previously reported increased parvocellular VP transcripts coupled with increased VP-peptide expression in the external zone of the median eminence during salt loading and 1 week rehydration [1]. In the external zone of the median eminence, parvocellular VP is secreted into the portal circulation. Therefore, the present findings suggest that immobilization stress-induced augmentation of parvocellular VP production leads to increased VP release into the pituitary portal circulation during salt loading and 1 week rehydration. Such parvocellular VP hyperfunction was also observed after repeated immobilization [32] or recurrent intraperitoneal injection of hypertonic saline [29].

Under these chronic stresses, VP has an essential role for sustaining HPA axis responsiveness [33]. Administration of interleukin-1β induced a long-lasting ACTH hypersensitivity as well as increased VP stores in CRH terminals in the external zone of the median eminence [34]. In contrast to VP, levels of CRH mRNA, increased in the magnocellular PVN during salt loading and restored in parvocellular neurons after rehydration, were not affected by acute immobilization stress. Taken altogether, this data indicates that salt loading elicits functional parvocellular neuronal plasticity and induces VP, but not CRH, to respond to acute stress stimulation. This plasticity was maintained during rehydration. Therefore, VP can be considered as a dominant ACTH regulator in salt-loaded and 1-week-rehydrated animals.

*CRH Alterations during Rehydration*

CRH mRNA was induced in magnocellular PVN during salt loading, but diminished during the rehydration stage. Magnocellular induction of CRH by chronic salt loading is consistent with previous reports [25, 26, 35].

In contrast, parvocellular CRH mRNA expression was decreased during salt loading and returned to normal after 1 week of rehydration. The basal expression level of CRH mRNA without immobilization stress in the rehydrated group was greater than that of nonstressed animals in the

control group. However, acute stress did not increase the level of CRH mRNA in salt-loaded and rehydrated animals, while marked increase was observed in the controls after immobilization. Similar desensitization of CRH to acute stress was also reported in a model of repeated immobilization stress [36, 37]. Inhibition of CRH expression during repeated stress was interpreted as an adaptation to homotypic stress, and CRH responsiveness to the heterotypic stress was preserved or increased [21, 38]. In the current study, since acute immobilization was a novel stressor to the animals, habituation cannot account for the blunted CRH responsiveness seen in rehydrated animals.

The reason why immobilization stress no longer stimulates CRH transcription during rehydration is unknown. When colocalized VP transcripts in CRH neurons are upregulated, CRH transcription in the parvocellular neurons may not respond to acute extracellular signals from additional homotypic or heterotypic stress. Alternately, there may be a ceiling effect on CRH mRNA expression in the parvocellular region during rehydration, since basal CRH mRNA levels are already high, approaching levels observed after immobilization.

Up-regulation of basal CRH mRNA expression in rehydrated animals suggests that parvocellular neurons are still presumably affected by a rebound effect of intense hyperosmotic stress. Since plasma corticosterone is also increased during the rehydration period, CRH neurons must be relatively insensitive to glucocorticoid negative feedback during that stage [32].

Secretion of ACTH under acute immobilization stress, decreased during salt loading and increased after rehydration, corresponds well to the activity of parvocellular CRH mRNA expression. This correlation suggests that parvocellular CRH plays a role as a tonic stimulator of ACTH synthesis during salt loading and rehydration.

Both CRH and VP receptors are expressed in ACTH-producing cells of the anterior pituitary, but have distinct intracellular signal pathways [6]. Activation of CRH receptors leads to cAMP production and has a marked stimulatory effect on ACTH synthesis and secretion [7, 39, 40]. Pituitary V1b VP receptors [41], in contrast, stimulate protein kinase C [42]. Effects of CRH on ACTH secretion are enhanced by VP, but VP itself has only a weak effect on ACTH synthesis and secretion [7]. Decreased ACTH response during salt loading is due to lack of VP facilitation on ACTH synthesis; the increased ACTH response to acute stress observed in rehydrated animals may be due to a joint action of VP and CRH. Such modulation of the HPA axis during rehydration, involving parvocellular up-regulation of both CRH and VP mRNA while levels of ACTH and corticosterone remain high, appears to be a novel, hitherto unreported condition.

In conclusion, the current study shows that modulation of the HPA axis induced by the stress of salt loading is maintained after 1 week of rehydration under elevated basal ACTH levels and enhanced responsiveness to additional acute stresses. This modulation may reflect a central plasticity of paraventricular parvocellular neurons involving altered expressions of CRH and VP.

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