Arch. Biol. Sci., Belgrade, 63 (3), 579-587, 2011 DOI:10.2298/ABS1103579L

VASOPRESSIN MODULATES HYPOTHALAMO-PITUITARY ACTIVITY BY PARACRINE ACTION DURING ACUTE AND CHRONIC IMMOBILIZATION STRESS IN RATS

IVA LAKIC, P. VUJOVIC, N. JASNIC, S. DJURASEVIC and GORDANA CVIJIC

University of Belgrade, Faculty of Biology, Institute of Physiology and Biochemistry, 11000 Belgrade, Serbia

Abstract - The aim of the study was to examine changes in corticotropin releasing hormone (CRH), vasopressin (VP), adrenocorticotropin (ACTH) as well as VP3 receptor protein concentration in the hypothalamus and the pituitary of rats exposed to acute (3 h) and chronically repeated (3 h daily for 7 days) immobilization stress. Our results show that, unlike the increase in ACTH, there were no changes in serum VP despite the significant changes in this hormone and its receptor concentrations in the hypothalamus and pituitary (HP). This suggests that VP regulates HP activity by predominantly acting in a paracrine manner under the examined stress condition.

Key words: CRH, vasopressin, hypothalamus, pituitary, immobilization stress, rat

UDC 612.43:577.17:57.08

INTRODUCTION

Stressful events pose a threat to homeostasis and affect numerous physiological processes throughout the central nervous and endocrine system. The hypothalamic-pituitary-adrenal (HPA) axis is a major part of the neuroendocrine system that controls reactions to stress and regulates many body processes engaged in the process of acclimatization. Corticotropin releasing hormone (CRH), a 41 amino acid peptide, originally discovered and sequenced by Vale et al. (1981), is produced in the medial parvicellular neurons of the hypothalamic paraventricular nucleus. These neurons project to the external zone of the median eminence where CRH releases into the portal vasculature reaching the anterior pituitary. Vasopressin (VP), a nonapeptide, was identified in 1954, and was considered as an important factor in the regulation of ACTH release. Beside magnocellular, VP like CRH is also produced by the parvicellular neurons of the paraventricular nucleus (PVN) and secreted into the pituitary portal system in the external zone of the median eminence (Antoni, 1993). This means that VP-containing cell bodies in the PVN are co-localized with CRH-containing neurons. In control, non-stressed rats, within the pool of CRH neurosecretory cells, 50% of them co-express VP (Whitnall et al., 1987).

In some stress conditions, CRH and vasopressin (VP) seem to act synergistically in the regulation of adrenocorticotropin (ACTH) release from the corticotrophes of the anterior pituitary, which in turn stimulates the secretion of corticosterone from the adrenal cortex (O'Connor et al., 2000). The actions of vasopressin are mediated by plasma membrane receptors belonging to the guanyl-nucleotide-binding-protein (G-protein) family (Jard et al., 1987; Peter et al., 1995). Three major vasopressin receptor subtypes (V1a, V1b and V2) encoded by different genes have been identified and cloned. The V1b receptor or V3 is coupled to phospholipase C (PLC) and can be found in the pituitary gland (Lolait et al., 1992; Morel et al., 1992; Seibold et al., 1992; Lolait et al., 1994; Sumimoto et al., 1994). Regulation of the V3 VP receptors in the pituitary seems to play

an important role in corticotrophe cell responsiveness to stressors, as judged by the good correlation between receptor concentration and ACTH responses in the pituitary (Aguilera, 1994; Aguilera et al., 1994).

In acute stress of any type, PVN has a key role in the activation of HPA, stimulating the release of both CRH and VP (Antoni, 1986) by up-regulation of VP, as well as CRH mRNA production in the hypothalamic neurosecretory system (Bartanusz et al., 1993).

Repeated stress alters basic mechanisms for the maintenance of HPA homeostasis and also suggests the involvement of CRH in the adaptive responses to stressful stimuli (Habib et al., 2001). Chronic stress can be elicited by repeated exposure to short-acting stressors or by action of a sustained or persistent stimulus, e.g., disease. Chronic stress is characterized by the classical symptoms of decreased body and thymus weight and a parallel increase in the size and width of the adrenal cortex, consistent with chronic activation of the HPA axis (Du Ruisseau et al., 1977; Blanchard et al., 1993). The sustained activation of the CRH/VP cells in the PVN is suggested to have a central role during chronic stress-induced HPA axis activation. This hypothesis is also consistent with the changes of CRH and VP mRNA concentration in the PVN of chronically stressed rats (Sawchenko et al., 1993; Aguilera, 1994; Herman et al., 1995; Makino et al., 1995; Ma et al., 1997; Ma and Aguilera, 1999; Pinnock and Herbert, 2001). However, extrapolation from acute to chronic stress, as well as the period of recovery after stress action, requires careful consideration of many factors.

The aim of our study was to monitor changes of CRH, VP, as well as VP3 receptor protein concentration in the hypothalamus and pituitary under the acute (3 h) and chronically repeated (3 h daily for 7 days) effects of immobilization stress which belongs to the psycho-physical type. The anterior pituitary response was determined by measuring ACTH concentration in the peripheral circulation. The effect of the recovery period was evaluated by killing animals at different times after the end of exposure to the stressor – 0 and 3 h.

MATERIALS AND METHODS

Animals

Adult male 11 week old *Wistar* rats, weighing 230 - 310 g, were used in this experiment. Two animals were housed per cage under a controlled temperature (22±1°C) and lighting (a 12 h light/dark regime) with lights on at 06:00 h and off at 18:00 h. All the animals had free access to commercial rat food and tap water.

Animal handling and treatment were carried out in accordance with the Serbian Laboratory Animal Protection Law proposed guidelines and protocols approved by the Ethical Committee of Faculty of Biology, University of Belgrade.

Experimental design – stress procedures

The animals were divided into four groups with each containing six animals. The **first** group consisted of intact controls; the **second** group was acutely exposed to immobilization stress for 3 h and killed immediately after the end of the exposure to the stressor, the **third** group was also acutely exposed to immobilization stress for 3 h but killed 3 h after termination of the immobilization stress, and the **fourth** group was subjected to repeated immobilization stress for 3 h a day for 7 days. Immobilization stress was performed according to Kvetnansky and Mikulaj (1970) by fixing all four limbs to a board with adhesive tape. The heads were also fixed by a metal loop round the neck to limit movement. To avoid the effects of circadian rhythms, all the rats were exposed to acute stress between 8:00 and 11:00 a.m.

Experimental procedures

Animals were decapitated without anesthesia with a guillotine (Harvard-Apparatus, Holliston, MA). The pituitary glands and brains were quickly excised. Hy-

pothalami were removed and then frozen at -80°C until further use.

Western blot

For western blotting, 25 µl of either pituitary or hypothalamic samples (protein concentration 1 mg/ml), were run on a 20% SDS polyacrylamide gel (120 V, Criterion Cell, Bio-Rad, Hercules, CA) and then transferred (overnight, 20 mA per gel, Criterion blotter, Bio-Rad) onto 0.45-mm PVDF membranes. After Ponceau S staining and destaining, membranes were blocked for 3 h in 5% nonfat dry powder milk (Santa Cruz) in Tris-buffered saline containing 0.1% Tween 20 (TBST). After that, the blots were incubated with the primary antibodies against CRH (1:5000 dilution, ab17475), VP (dilution 1:3000, ab39363) and V3 receptor (1:1000 dilution, sc-30026), overnight at 4°C on a shaker. The blots were washed four times for 15 min in TBST before a 1 h incubation with the horseradish peroxidase-conjugated secondary antibody (1:5000 dilution, Abcam). The blots were re-washed four times for 15 min in TBST. Lastly, the ECL+ (Abcam) was added onto the PVDF membranes for 5 min. The quantification of immunoreactive bands was performed with the Image Quant 5.2 (Molecular Dynamics) program.

Data were statistically evaluated by two way analysis of variance (ANOVA). Holm Sidak comparisons were performed when ANOVA was significant ($p<0.05$). Data are presented as mean ± standard error (S.E.).

ACTH determination

ACTH in the plasma was measured by the chemiluminescent method using an automatic analyzer (Immulite Diagnostic Products Corporation, Los Angeles, CA. USA). Blood samples were collected in separate test tubes with EDTA and centrifuged for 10 min at 3500 rpm at +4°C. The samples were placed in an analyzer and covered by a reaction cup. In the cup there was a mixture of anti-ACTH antibody and the buffer. In the refrigerator of the apparatus two reagents were added – LACA (protein buffer/serum) and LACB (alkaline phosphatase). The last reagent to be added was the chemiluminescent substrate. The apparatus read the values that were expressed as pg ACTH/ml plasma.

Vasopressin determination

Vasopressin in the serum was measured using the Peptide Enzyme Immunoassay (EIA) Kit, Peninsula Laboratories, LLC, CA, USA. Blood samples were collected in separate test tubes, centrifuged for 10 min at 3500 rpm at +4°C. For analysis, the coated plate was used. In each well of the immunoplate antiserum was added in EIA buffer, except in the blank wells that contained only EIA buffer. After incubation at room temperature, standards and samples in diluent were added and in the blank well only diluent was added and incubated for two hours. Bt-tracer (EIA buffer + lyophilized biotinylated peptide) was added in every well, and left in the fridge (+4°C) overnight. The immunoplate was washed 5 times, and after that streptavidin-HRP was added. Another round of 5 washings was performed and after adding TMB solution a blue color was obtained. The plate was incubated for 45 min and the reaction was stopped with HCl and the blue color turned to yellow. Absorbance was read at 450 nm. The values were recalculated into ng vasopressin/ml serum.

RESULTS

As is shown in Fig. 1, a significant rise of CRH concentration in the hypothalamus was detected after acute immobilization and when the animals were killed immediately after the treatment (***$p<0.001$), when compared to the controls. The significant increase in CRH was also observed in animals killed 3 h after the end of stress when compared to the controls (*$p<0.05$), however, this increment was significantly lower than the values obtained in rats which did not have a recovery period ($p<0.001$). However, after chronic exposure to immobilization stress there were no changes in CRH concentration when compared to controls (•••$p<0.001$; •••$p<0.001$).

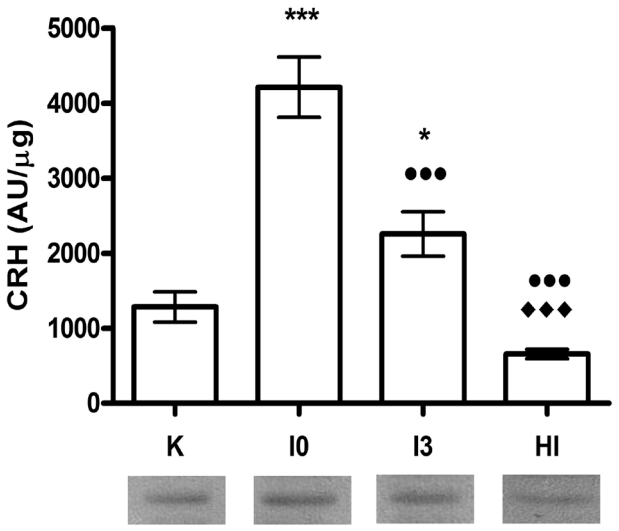


Fig. 1. CRH concentration (AU/μg) in rat hypothalamus: **K** – control non-stressed rats; **I0** – acute immobilization (3 hours) with decapitation performing immediately after the stress termination; **I3** – acute immobilization (3 hours) with decapitation 3 hours after the stress termination; **D** – chronic immobilization (7 days - 3 hours per day). The values are means of six animals ±S.E.M. Statistical significance: K:I 0 - p<0.001; K:I 3 - p<0.05; I 0: I 3 - p<0.001; I 0:HI - p<0.001; I 3:HI - p<0.001.

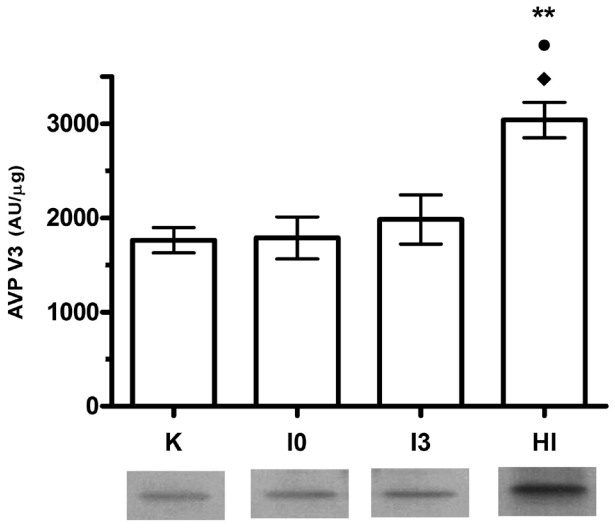


Fig. 3. Vasopressin (V3) receptor concentration (AU/μg) in rat hypothalamus. **K** – control non-stressed rats; **I0** – acute immobilization (3 hours) with decapitation performing immediately after the stress termination; **I3** – acute immobilization (3 hours) with decapitation 3 hours after the stress termination; **D** – chronic immobilization (7 days - 3 hours per day). The values are means of six animals ±S.E.M.Statistical significance: K:HI - p<0.01.

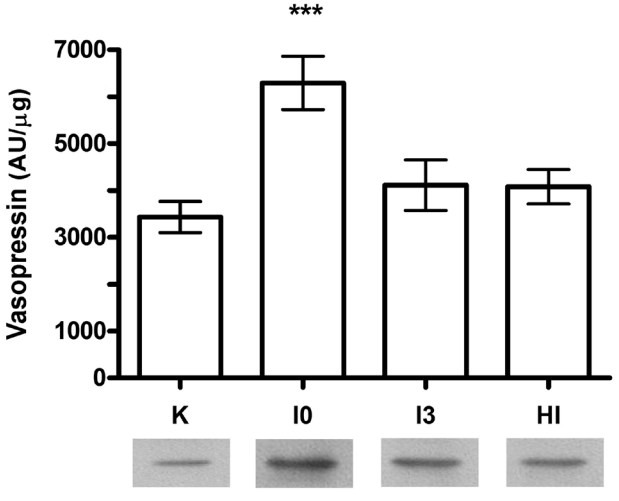


Fig. 2. Vasopressin concentration (AU/μg) in the rat hypothalamus. **K** – control non-stressed rats; **I0** – acute immobilization (3 hours) with decapitation performing immediately after the stress termination; **I3** – acute immobilization (3 hours) with decapitation 3 hours after the stress termination; **D** – chronic immobilization (7 days - 3 hours per day). The values are means of six animals ±S.E.M.Statistical significance: K:I 0 - p<0.001; I 0:I 3 - p<0.05; I 0:HI - p<0.05.

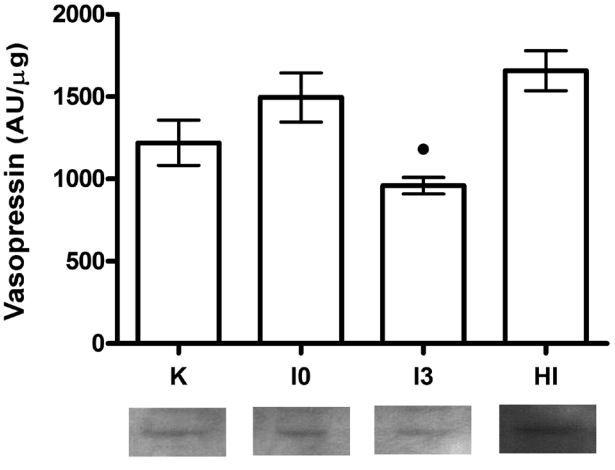


Fig. 4. Vasopressin concentration (AU/μg) in rat pituitary. **K** – control non-stressed rats; **I0** – acute immobilization (3 hours) with decapitation performing immediately after the stress termination; **I3** – acute immobilization (3 hours) with decapitation 3 hours after the stress termination; **D** – chronic immobilization (7 days - 3 hours per day). The values are means of six animals ±S.E.M.Statistical significance: I 0:I 3 - p<0.05; I 3:HI - p<0.001.

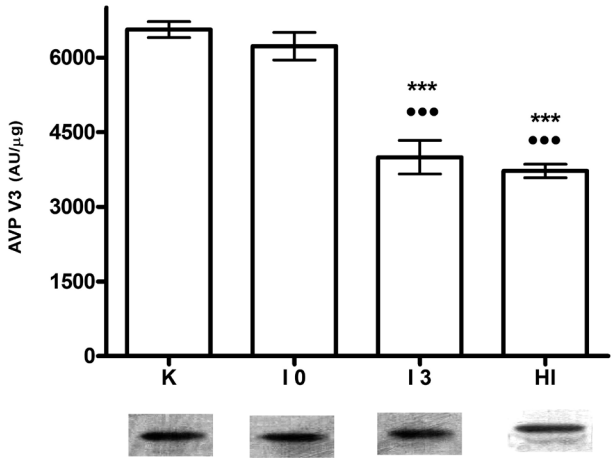


Fig. 5. Vasopressin (V3) receptor concentration (AU/µg) in rat pituitary. **K** – control non-stressed rats; **I0** – acute immobilization (3 hours) with decapitation performing immediately after the stress termination; **I3** – acute immobilization (3 hours) with decapitation 3 hours after the stress termination; **D** – chronic immobilization (7 days - 3 hours per day). The values are means of six animals ±S.E.M.Statistical significance: K:I 3 - p<0.001; K:HI - p<0.001; I 0:I 3 - p<0.001; I 0:HI -p<0.001.

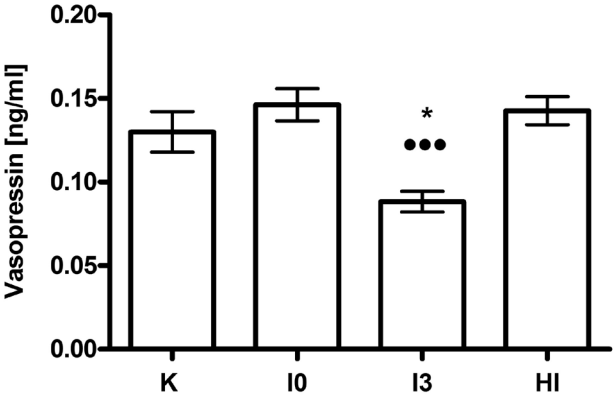
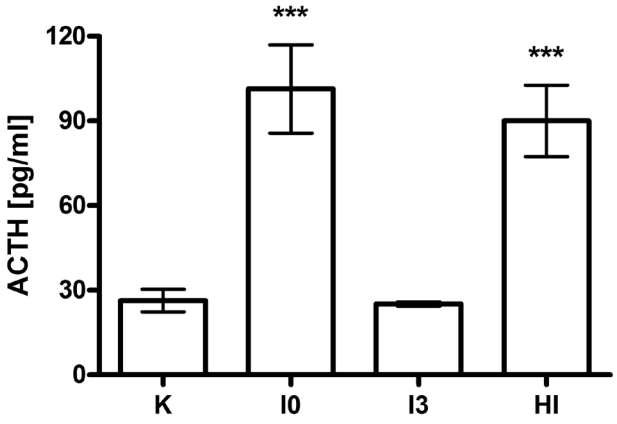


Fig. 6. Vasopressin serum concentation (ng/ml) in rats. **K** – control non-stressed rats; **I0** – acute immobilization (3 hours) with decapitation performing immediately after the stress termination; **I3** – acute immobilization (3 hours) with decapitation 3 hours after the stress termination; **D** – chronic immobilization (7 days - 3 hours per day). The values are means of six animals ±S.E.M.Statistical significance: K:I3 - p<0.05; I 0:I 3 - p<0.001; I 3: HI - p<0.01.

As can be seen on Fig. 2, the VP hypothalamic concentration, when compared to the controls, was significantly elevated only in the animals which are acutely immobilized once and immediately killed



Fig, 7. Adrenocorticotrophin (ACTH) plasma concentration (pg/ml). **K** – control non-stressed rats; **I0** – acute immobilization (3 hours) with decapitation performing immediately after the stress termination; **I3** – acute immobilization (3 hours) with decapitation 3 hours after the stress termination; **D** – chronic immobilization (7 days - 3 hours per day). The values are means of six animals ±S.E.M.Statistical significance: K:I0 - p<0.001; K:HI - p<0.001.

(***p<0.001), whereas in rats that rested for 3 h and in those that were chronically stressed the concentration of VP did not change.

As far as the VP V3 receptors are concerned, only in the animals that were chronically immobilized was the significant elevation of hypothalamic VP V3 receptor concentration observed when compared to controls (**p<0.01).

As we can see from Fig. 4, the 3 h recovery period after the effect of acute immobilization stress induced the reduction of VP in the pituitary when compared to the controls, as well as to the acutely treated animals that were killed immediately after the treatment (•p<0.05).

The concentration of VP V3 receptors in the pituitary gland (Fig. 5) decreased in all the treated groups of animals as compared to the intact control, but was significant only in the acutely stressed group of rats that were killed 3 h after the termination of the treatment and in the chronically treated group (***p<0.001). These two groups showed an important decline in receptor concentration as com-

pared to both the controls and I0 group of animals (•••p<0.001).

A significant reduction of serum VP concentration (Fig. 6) was detected in the animals that were decapitated 3 h after the end of the acute IMO compared to the control rats (*p<0.05) and rats that were killed immediately after the end of the immobilization (•••p<0.001). The chronic treatment did not alter the VP serum concentration in comparison to the control rats.

At the same time, circulating ACTH (Fig. 7) significantly increased after both acute and chronic exposure to IMO (***p<0.001) compared to the non-stressed rats, unlike the animals that where killed 3 h after the offset of the exposure and whose ACTH values remained at the control level.

DISCUSSION

As expected, the present data confirm that a single exposure to a severe stressor such as IMO dramatically evokes the HPA response. Our findings show that acute 3 h IMO significantly increases the CRH concentration in the hypothalamus, suggesting its intensive synthesis (Fig. 1). During the recovery period after stress termination, the CRH concentration decreased toward the control level, but after 3 h it still remained significantly above these values (Fig. 1). However, the chronic-repetition of exposure to IMO during 7 days causes a strong diminution of CRH concentration, suggesting the desensitization of the HPA axis during the response to the applied stressor (Fig.1). This is in agreement with the reported results which show that repeated IMO for 7 days may attenuate the subsequent acute stress-induced expression of the immediate early genes (c-fos, fosB and junB in PVN), a key structure for CRH secretion (Imaki et al., 1995; Umemoto et al., 1997). The observed diminution of the HPA axis response to IMO stress in rats may result from desensitization of the neurotransmitter and neuropeptide systems involved in the regulation of HPA axis activity. Numerous data indicate the involvement of CRH in the enhancement of almost the entire cascade of the stress response. This neuropeptide influences every cell in the body due to a broad interaction with the endocrine and autonomic system. It was shown that a single exposure to immobilization (IMO) causes long-term desensitization of HPA further response to the same (homotypic) stressor, which affected both peripheral and central, (PVN) components of the HPA axis (Marti et al., 2001; Dal-Zotto et al., 2002; Dal-Zotto et al., 2003; Valles et al., 2003). Interestingly, this homotypic desensitization progressively developed over time, affecting first the release of HPA hormones and later the transcriptional activity of the PVN neurons (Marti, Garcia, Valles, Harbuz and Armario, 2001; Valles, Marti and Armario, 2003).

As far as the VP concentration in the hypothalamus is concerned, our results show that this neurohormone responds to acute IMO stress in the same way as CRH does, given the significant increment of its concentration. However, VP reaches the control level after a recovery period of 3 h. Chronically applied IMO stress brought VP hypothalamic concentration to the control level (Fig. 2), which correlates with the results confirming that prolonged stress specifically affects the alleviation of VP expression in the parvocellular VP neurons. That can be the consequence of the inhibitory effect of glucocorticoids on the expression on both CRH and VP in the parvocellular neurosecretory system (Sawchenko, 1987). On the other hand, the decreased concentration of VP in the hypothalamus might be the consequence of VP reaching the pituitary where we detected its slight increment (Fig. 4).

There are also data showing that acute stress stimulates the release of CRH and VP from the median eminence into the pituitary portal circulation and increases the expression of both peptides in the parvocellular neurons of the PVN (Berkenbosch et al., 1989; Plotsky, 1991). In many studies it was shown that an increase in CRH and VP primary transcripts is followed by a slower increase in mRNA, which reaches significant levels 1 or 2 h after the initiation of the stress (Imaki et al., 1995; Kovacs and Sawchenko, 1996; Ma et al., 1997; Ma and Aguilera, 1999). The increase in VP expression

is due to increases in the density of transcripts, as well as the number of parvocellular cells containing VP hnRNA and mRNA (MaLevy and Lightman, 1997; Ma et al., 1999). It seems that repeated stress, as well as long-term adrenalectomy, is associated with a predominant expression of VP rather than CRH. It has been shown that parvocellular VP mRNA rises significantly after repeated immobilization or restraint (Bartanusz et al., 1993; Aguilera et al., 2000), foot shock (Sawchenko et al., 1993), or i.p. hypertonic saline injection (Ma and Aguilera, 1999), while CRH mRNA levels are elevated only in stress paradigms which show preserved plasma hormone responses to the repeated stimulus, such as repeated i.p. hypertonic saline injection or foot shock (Imaki et al., 1991; Ma and Aguilera, 1999). Similarly, daily repeated immobilization does not affect irCRH stores but causes a progressive increase in VP stores and in the number of CRH nerve endings containing VP in the external zone of the median eminence (de Goeij et al., 1991). In addition, *in vivo* and *in vitro* studies have shown that the rate of release of immunoreactive VP from median eminence terminals increases in response to repeated or chronic stress (de Goeij et al., 1991; Aguilera et al., 1993). Even a transient activation of hypothalamic CRH neurons by a single stressor can cause long-lasting increases in VP co-expression, irrespective of the nature of the stressor, which in most cases is not accompanied by changes of CRH, which is in agreement with our present results (Schmidt et al., 1997).

Analysis of VP V3-receptor mRNA levels by Northern blot shows that VP V3- mRNA levels increase during 4 h following IMO, which demonstrates why there are no changes in the VP V3 concentration in the hypothalamus under the acute (3 h) effect of IMO as compared to the intact control observed in our experiments (Fig. 3) (Ma et al., 1997). However, there are data showing that VP V3 receptor mRNA levels are normal during chronic stimulation, when the VP receptor number is markedly decreased (Aguilera, 1994; Rabadan-Diehl et al., 1995). This suggests that there is probably receptor downregulation in these conditions which is due to the inhibition of mRNA translation, or to post-translational events such as receptor internalization and desensitization rather than transcriptional inhibition (Terada et al., 1993; Young et al., 1993). The decrease in VP V3-receptors in the pituitary after repeated stress, together with significant elevation in the VP concentration, once again confirms the possible downregulation.

According to our results it seems that both CRH and VP respond to IMO which correlates with the results of authors who reported that IMO functions as a stressor that stimulates the various systems that respond to it, including CRH and vasopressin (Holst, 1998). There is some evidence that in rodents and humans VP participates in adaptation of the HPA axis by potentiating the stimulatory effect of CRH, especially in chronically repeated stress when it becomes a predominant regulator.

In conclusion, unlike the increase in ACTH, there were no changes in circulating VP, even though significant changes in this hormone concentration occurred both in the hypothalamus and pituitary. This suggests that VP regulates HP activity by predominantly acting in a paracrine manner during stress caused by acute or repeated IMO.

Acknowledgments - This work was supported by the Ministry of Education and Science, Republic of Serbia, Grant No 173023.

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