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Effect of Hyperosmotic Stimulation and Adrenalectomy on Vasopressin mRNA Levels in the Paraventricular and Supraoptic Nuclei of the Hypothalamus:

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Abstract

The effects of salt loading and adrenalectomy on arginine vasopressin (AVP) mRNA levels in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus were studied by semiquantitative in situ hybridization histochemistry, using a synthetic oligonucleotide probe and a computer-assisted image analysis system. Salt loading (2% NaCl) for 7 days produced marked increases in AVP mRNA levels in the magnocellular neurons of the PVN, SON, and accessory nuclei. Adrenalectomy caused an increase in AVP mRNA expression in the magnocellular part of the PVN and the expansion of hybridization signals into its medial parvocellular region, where the cell bodies of corticotropin-releasing hormone (CRH) neurons are located. No apparent alteration of AVP mRNA levels was observed in the SON following adrenalectomy. These results indicate that hyperosmotic stimulation and the loss of circulating glucocorticoids had differential effects on AVP gene expression in the PVN and SON, and that the magnocellular PVN and SON neurons responded in different manners to the loss of feedback signals.

KEYWORDS: in situ hybridization, arginine vasopressin mRNA, hypothalamus, osmotic stimulation, adrenalectomy

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Effects of Hyperosmotic Stimulation and Adrenalectomy on Vasopressin mRNA Levels in the Paraventricular and Supraoptic Nuclei of the Hypothalamus: *In Situ* Hybridization Histochemical Analysis Using a Synthetic Oligonucleotide Probe

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The effects of salt loading and adrenalectomy on arginine vasopressin (AVP) mRNA levels in the paraventricular nucleus (PVN) and the supraoptic neucleus (SON) of the hypothalamus were studied by semiquantitative *in situ* hybridization histochemistry, using a synthetic oligonucleotide probe and a computer-assisted image analysis system. Salt loading (2% NaCl) for 7days produced marked increases in AVP mRNA levels in the magnocellular neurons of the PVN, SON, and accessory nuclei. Adrenalectomy caused an increase in AVP mRNA expression in the magnocellular part of the PVN and the expansion of hybridization signals into its medial parvocellular region, where the cell bodies of corticotropin-releasing hormone (CRH) neurons are located. No apparent alteration of AVP mRNA levels was observed in the SON following adrenalectomy. These results indicate that hyperosmotic stimulation and the loss of circulating glucocoriticoids had differential effects on AVP gene expression in the PVN and SON, and that the magnocellular PVN and SON neurons responded in different manners to the loss of feedback signals.

Key words : ***in situ*** **hybridization, arginine vasopressin mRNA, hypothalamus, osmotic stimulation, adrenalectomy**

The magnocellular neurons in the paraventricular (PVN) and supraoptic (SON) hypothalamic nuclei, projecting their axons to the posterior pituitary via the zona interna of the median eminence, contain the neurohypophyseal hormones, arginine vasopressin (AVP) and oxytocin. It is well known that osmotic stimuli increase the rate of synthesis of the precursor of AVP (1) and the rate of secretion of AVP (2). On the other hand, adrenalectomy produces the co-expression of AVP by corticotropin-releasing hormone (CRH)-containing neurons in the medial parvocellular part of the PVN and an enhancement of AVP immunoreactivity in the zona externa of the median eminence (3-5). There is considerable evidence suggesting that increased secretion of AVP into the hypophyseal portal circulation occurs following adrenalectomy (6-8),

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and that AVP as well as CRH has a substantial role in the ACTH response to adrenalectomy (9, 10).

In situ hybridization was originally developed to investigate the localization of nucleic acids within tissues, nuclei, and chromosomes (11, 12). This method has been applied to the investigation of the localization of mRNAs coding for specific peptides or proteins within the cytoplasm using complementary DNAs (cDNAs). More recently, *in situ* hybridization techniques using synthetic oligonucleotide probes have been developed for a number of neuropeptides, and these have substantial advantages over the techniques using cDNA probes (13, 14). However, methods of quantitative analysis for mRNA levels *in situ* still remain to be improved.

In the present study, we performed a semi-quantitative *in situ* hybridization histochemical analysis, using a synthetic oligonucleotide probe and a computer-assisted image analysis system, to investigate changes in AVP mRNA levels in the PVN and the SON after hyperosmotic stimulation and adrenalectomy.

Materials and Methods

Animals and treatment. Male Wistar rats weighing 235–245 g were housed with free access to food and tap water in the animal room to acclimatize for several days before the experiments. The following two experiments were carried out on different days. Rats were given either hypertonic saline (2% NaCl solution) (osmotically stimulated group, $n = 4$) or tap water (control group, $n = 4$) for 7 days. In another experiment, rats were either adrenalectomized ($n = 4$) or underwent sham operations as a control ($n = 4$). The adrenalectomy group underwent removal of both adrenal glands. After surgery, both groups were given 0.5% NaCl solution to drink for 7 days. During each experiment, the rats were allowed free access to food.

Tissue preparation. Seven days after the start of each experiment, the animals were anesthetized with sodium pentobarbital and perfused transcardially with 4% paraformaldehyde in sodium phosphate buffer. The brains were removed and immersed in 20% sucrose solution overnight. Then coronal frozen sections (with $8\,\mu m$ thickness) of the hypothalamus at the levels of the SON and the PVN were cut on a cryostat at $-20\,^{\circ}C$. The tissue sections were mounted directly from the blade onto autoclaved chrome alum-gelatin-coated slides, and placed on a slide warmer at $45\,^{\circ}C$ for 1–2 h.

Probe. A ^{35}S-labeled synthetic oligonucleotide directed against bases 286–315 of the rat prepro AVP mRNA was purchased from Du Pont Company and used as the probe. With the use of data banks of nucleic acid sequences, the oligomer was compared with all sequences in rodents stored in Gene Bank (15). Almost all the nucleotide sequences were not relevant to the analysis of hypothalamic peptides or neurotransmitters. The computer analysis showed that the oligomer sequences of the AVP probe had no homology with the oligomer sequences of oxytocin. Specific activity was 1.30×10^9 dpm/μg.

In situ hybridization. The tissue sections were denatured at $72\,^{\circ}C$ for 30 min in a solution of $40\,\mu l$ of diethylpyrocarbonate in 200 ml of $2 \times SSC$ (0.3 M sodium chloride, 0.03 M sodium citrate in autoclaved nanopure distilled water) titrated to pH 3.5 with acetic acid, and then were deproteinized at $37\,^{\circ}C$ for 15 min in a solution of $1\,\mu g/ml$ pepsin in $2 \times$ SSC-acetic acid (pH 3.5). The slides were washed twice in $0.2 \times SSC$ for 1 min each, and then washed in 5 mM dithiothreitol in autoclaved nanopure distilled water for 1 min at room temperature. Sections were then dried thoroughly. Prehybridization buffer (PHB) was used to reduce nonspecific binding of the labeled probe to nucleic acids in the tissues. A microcentifuge tube containing PHB was heated to $100\,^{\circ}C$ for 5 min, and then rapidly cooled on ice. The buffer was then diluted with the same volume of formamide. A $20\,\mu l$ aliquot of the PHB plus formamide solution was then applied to each section, and the slides were placed flat on filter paper in a Tupperware container at room temperature for 2 h. After this incubation, the PHB-formamide solution was drained from the slides. The labeled probe was dissolved in a solution of the hybridization buffer (HB) plus formamide, and was heat-denatured at $100\,^{\circ}C$ for 5 min followed by rapid cooling on ice. Twenty microliters of the HB-formamide solution containing the probe (approximately 1×10^5 cpm) was then applied to each section, and hybridization was performed overnight (14–16 h) in a humid Tupperware container at room temperature. After hybridization, the mounted tissue sections were dipped in $2 \times SSC$, washed twice in $2 \times SSC$ at room temperature for 10 min each, and finally washed in $0.5 \times SSC$ containing 5 mM dith-

iothreitol. After washing to remove unhybridized probe, the sections were dehydrated in 50, 70, 90 and 100% ethanol for 1 min each. The 50, 70 and 90% ethanols were diluted with 300 mM ammonium acetate to decrease the chance of loss of hybridization signals. Finally, the slides were air dried in a desicator.

The buffers used in prehybridization and hybridization contained the following reagents per 5 ml of autoclaved nanopure distilled water: 1.2 ml of 5.0 M sodium chloride; 100 μl of 1.0 M Tris (pH 7.6); 16.7 μl of 6% (w/v) bovine serum albumin; 40 μl of 250 mM EDTA; 50 μl of 5% sodium pyrophosphate; 33 μl of 6% polyvinylpyrrolidone; 10 μl of 50 mg/ml yeast tRNA; 250 μl (PHB) or 50 μl (HB) of 20 mg/ml yeast total RNA (type III); 500 μl (PHB) or 100 μl (HB) of 10 mg/ml herring sperm DNA (type IV); and 5 μl of 20 mg/ml dithiothreitol. The HB also contained 1 g/5 ml of dextran sulfate and 33 μl of 15 mg/ml polyadenylic acid (16).

Autoradiography. In the dark room, the dried slides were coated with a smooth layer of Kodak NTB-3 autoradiography emulsion (melted at 42°C in a water bath and not diluted) by dipping them individually in a glass vial. Slides were placed vertically in light-tight slide boxes containing desiccant. The slide boxes were stored at 4°C, and the slides were exposed for 2 weeks. After exposure, the slides were developed in Kodak D-19 at 20°C for 3 min, rinsed in water twice for 2 min each, and fixed in Kodak fixer for 5 min. After fixing, slides were rinsed in water. And then, the sections on the slides were counterstained with cresylviolet or hematoxylin, dehydrated through an ethanol series and xylene, and coverslipped.

Image analysis. Quantitative comparison of the density and distribution of silver grains between the treated and control groups was carried out using a TV Image Processor EXEL (TVIP-4100) (Nippon Avionics Co. Ltd., Tokyo, Japan) and a TV Image Processor EXCEL Image Command 4198 (Ratoc System Engineering Co. Ltd., Tokyo, Japan). The light microscopic images of the PVN and SON in each section were converted to the densography by the computer-controlled system. The edges of the two nuclei were determined in each section, and then the total area of each nucleus was calculated by the computer. The areas of the hybridization signals that were over a certain threshold level of silver grain density were then summated within the nuclei in each section. A certain threshold level of silver grain density was defined in each experiment as a level a little bit over the background to be subtracted. The ratio of the summated supra-threshold area to the totoal area of each nucleus was calculated, and these values were compared between the treated and control groups.

Results

Effect of salt loading on AVP mRNA levels. Seven-day salt loading caused a marked increase in the density of silver grains indicating the location of oligonucleotide AVP mRNA hybrids in the magnocellular part of the PVN (Figs. 1-a, b), as well as in the SON, predominantly the ventral SON where the majority of AVP cells exist (Figs. 2-a, b), and in the accessory nuclei scattered between the PVN and the SON (Fig. 3). In control rats, the accessory nuclei were hard to identify because of scattering of the cells in the nuclei. Image analysis revealed that 7-day salt loading caused approximately a 170-fold and 200-fold increase, respectively, in the ratio of the hybridized area over the threshold density to the total area of the PVN and SON (Figs. 1-c, d, e, f; Table 1). A similar result was found in all the sections from each salt-loaded animal.

Effect of adrenalectomy on AVP mRNA levels. The density of silver grains was significantly increased in the magnocellular part of the PVN and an expansion of their distribution into the medial parvocellular part of the PVN was also noted following adrenalectomy (Figs. 4-a, b, c, d). Image analysis showed that adrenalectomy caused an approximately 3-fold increase in the ratio of the hybridized area over the threshold

Table 1 Percentage ratios of the summated hybridized area over the threshold density to the total area of the paraventricular nucleus (PVN) or the supraoptic nucleus (SON) in representative sections from the treated and control groups of rats.

Treatment	PVN(%)	SON(%)
1) Salt loading	33.3	39.3
Control	0.2	0.2
2) Adrenalectomy	3.3	2.0
Sham operation	1.2	2.4

Data were obtained using the image analysis procedure shown in Fig. 1 from representative sections for the PVN and the SON of one animal in each group, of which photomicrographs are shown in all figures.

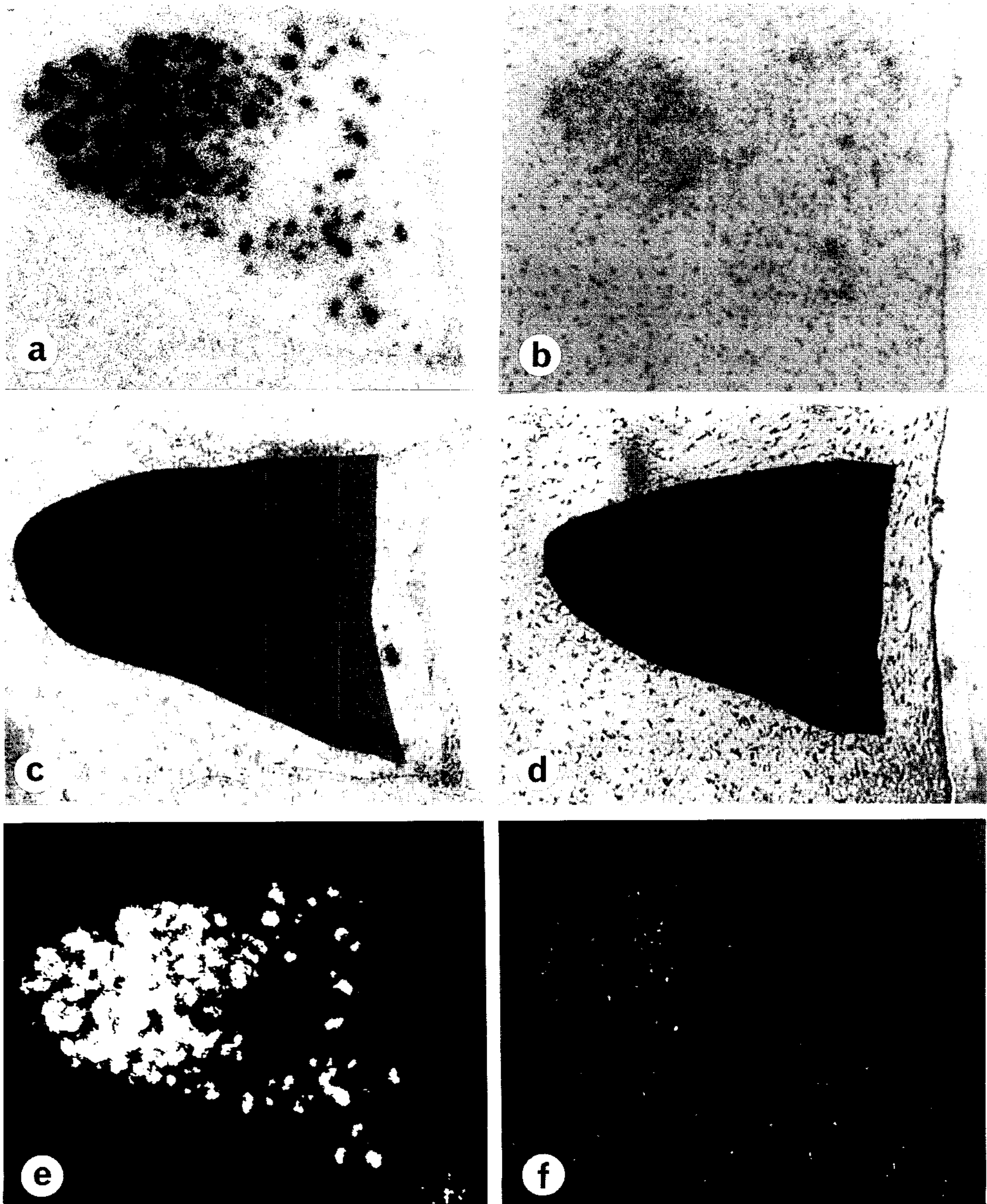

Fig. 1 Arginine vasopressin mRNA expression in the paraventricular nucleus (PVN) in salt-loaded and control rats. The bright field photomicrographs of representative sections counterstained with hematoxylin show that 7 days of salt loading (a) caused a marked increase in silver grains labeling the magnocellular neurons of the PVN in contrast to the control specimen (b). Magnification × 40. Image analysis was performed with a computer-assisted system. The dark areas (c, salt loading; d, control) indicate the whole of each PVN, and the white regions (e, salt loading; f, control) indicate the hybridized areas obtained after subtracting those below the threshold density. The ratios of the summated hybridized areas obtained after this subtraction process to the total of PVN area are shown in Table 1.

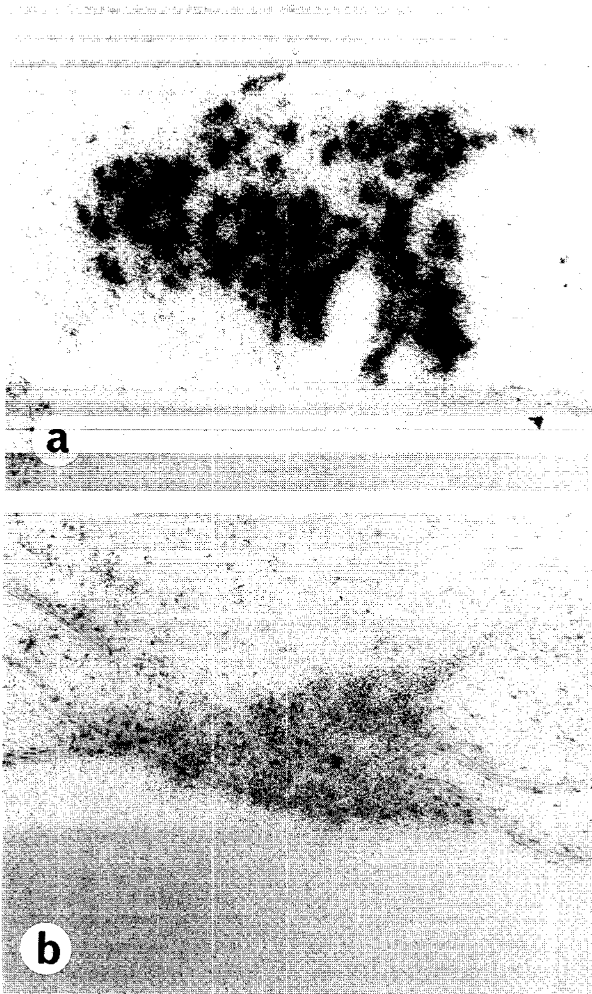

Fig. 2 Arginine vasopressin mRNA expression in the supraoptic nucleus (SON) in salt-loaded (a) and control (b) rats. The bright field photomicrographs of representative sections through the SON show that 7 days of salt loading caused a marked increase in silver grains labeling the SON, and that this change was predominantly in the ventral SON. Magnification $\times 40$.

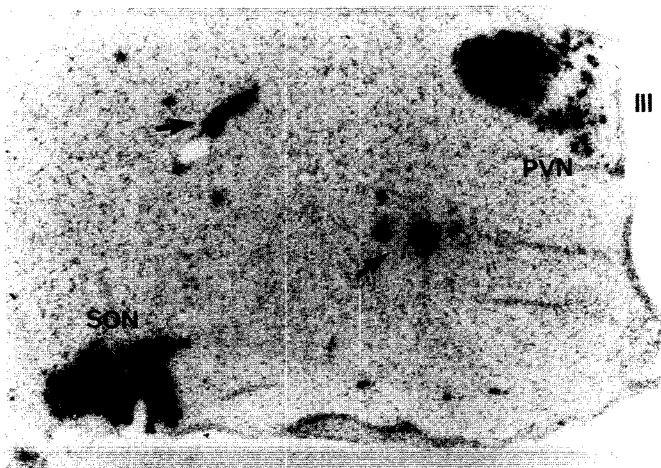

Fig. 3 Arginine vasopressin mRNA expression in the accessory nuclei scattered between the paraventricular (PVN) and supraoptic (SON) nuclei in salt-loaded rats. The bright field photomicrograph shows that 7 days of salt loading produced cells in the accessory nuclei labled clearly with a high density of silver grains. Arrows indicate the accessory nuclei. Magnification $\times 13.2$.

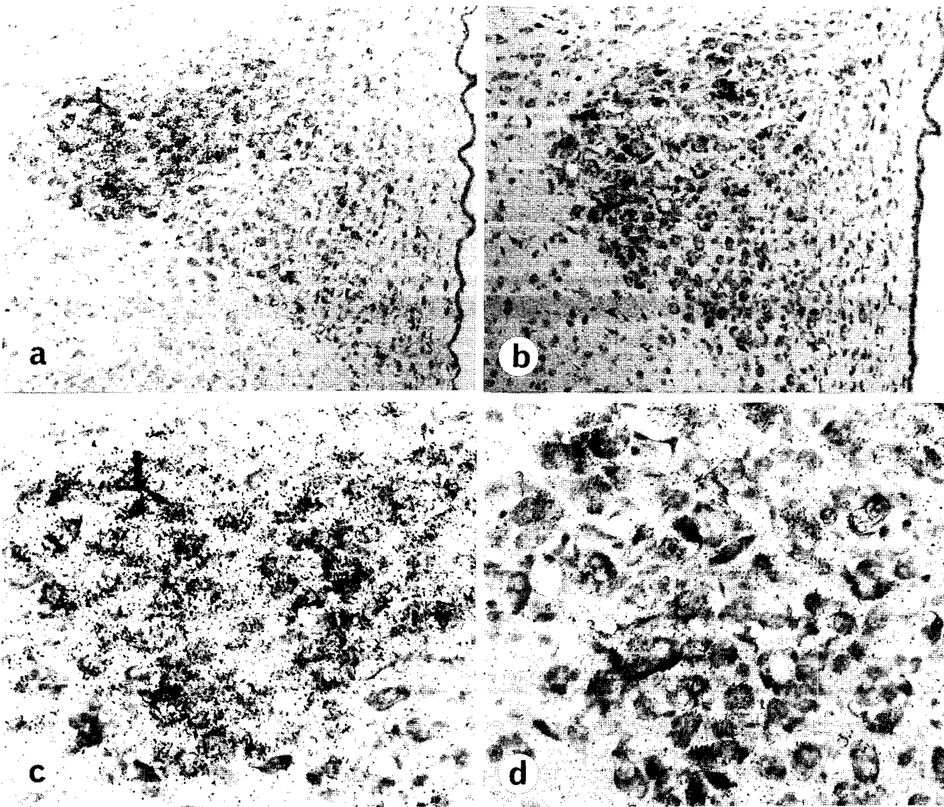

Fig. 4 Arginine vasopressin mRNA expression in the paraventricular nucleus (PVN) in adrenalectomized (a, c) and sham-operated (b, d) rats. The bright field photomicrographs of representative sections counterstained with cresyl violet show that adrenalectomy caused an increase in the number of silver grains labeling the magnocellular neurons of the PVN, and also expanded the distribution of silver grains medially into the parvocellular part of the PVN. (a, b) Magnification × 40. (c, d) High magnification photmicrographs of the magnocellular part of the PVN. Magnification × 100.

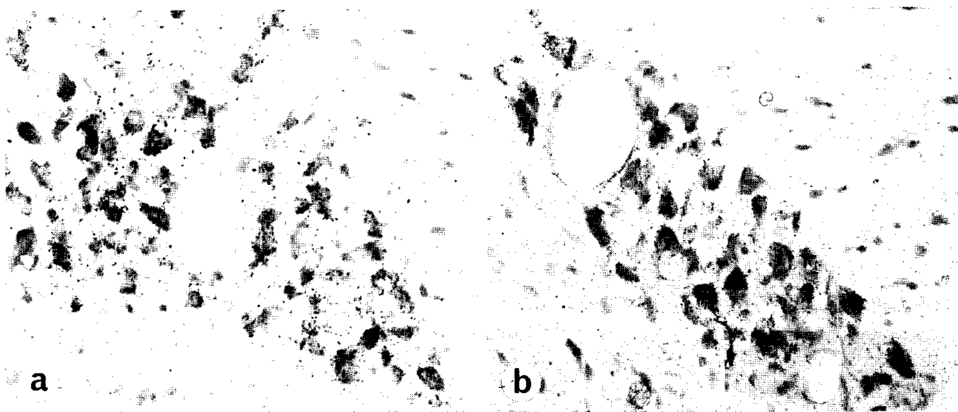

Fig. 5 Arginine vasopressin mRNA expression in the supraoptic nucleus (SON) in adrenalectomized (a) and sham-operated (b) rats. No apparent alteration in the density of silver grains was observed following adrenalectomy. Magnification × 100.

level to the total area of the PVN (Table 1). On the other hand, in the SON adrenalectomy did not cause any detectable increase in hybridization (Figs. 5-a, b; Table 1). All sections from each adrenalectomized animal showed similar findings.

Discussion

In this report, we described a semiquantitative analyzing method of *in situ* hybridization histochemistry using a synthetic oligonucleotide probe. This method was used to investigate stimulus-induced changes in AVP mRNA levels in the hypothalamic nuclei. The *in situ* hybridization histochemistry has the substantial advantage of providing increased anatomical accuracy as compared with Northern blotting analysis (17). Various methods for the quantification of hybridization signals have been reported previously (15, 16, 18–21), but almost all of them seem to have had problems with regard to processing and accuracy. The present method of semi-quantitative analysis also has some problems, including the inability to quantify the absolute mRNA level within a nucleus and difficulty in defining the threshold level for image analysis. However, it is easy to perform and provides a useful comparison of changes in the distribution and density of hybridization signals between experimental groups.

Our study revealed that hyperosmotic stimulation with 2% NaCl for 7 days induced a marked increase in AVP mRNA levels in the magnocellular neurons of the PVN, SON and accessory nuclei in the rostral hypothalamus (situated between the PVN and SON). This result is consistent with the reports by Lightman and Young (22) and Kawata *et al.* (23). As we have reported elsewhere, one-week loading of 2% NaCl solution induced a 5-fold increase in the plasma AVP level and a marked reduction of the immunoreactive content in the neurointermediate lobe of the pituitary (one twentieth of the control level), while the AVP content of the PVN was not changed (24). Together with these observations, the present results suggest that salt loading produces a progressive increase in AVP mRNA levels in the magnocellular neurons, while simultaneously stimulating axonal transport and inducing excess release of AVP from the neurointermediate lobe of the pituitary. As a consequence, the AVP content in the PVN may not change. Thus, the stimulus-induced changes in the level of gene transcripts are not in parallel with the changes in content in this situation.

Adrenalectomy produced an increase in AVP mRNA levels in the magnocellular neurons of the PVN and also caused an expansion of the hybridization signals into the medial parvocellular subdivision of the PVN, indicating the co-expression of AVP mRNA by CRH-containing parvocellular neurons. The finding is in accordance with the established notion that immunoreactive AVP and CRH are co-expressed by parvocellular neurosecretary neurons in the adrenalectomized rat (3, 4), and confirms the previous results by Wolfson *et al.* (25) and Davis *et al.* (20). Very recently, Simmons and Swanson have reported that vasopressin mRNA levels in magnocellular neurosecretory neurons of the PVN and SON did not change following adrenalectomy or in response to high circulating corticosterone levels, and they thus concluded that the effects of corticosterone on AVP mRNA levels in the PVN and SON were restricted to parvocellular neurons (26). In the present study, no apparent alteration of AVP mRNA levels in the SON was observed following adrenalectomy. With regard to the magnocellular neurosecretory neurons of the PVN, however, our finding of an increase in AVP mRNA levels was contrary to their results, but was consistent with the results of Davis *et al.* (20). It may be possible that magnocellular SON and PVN neurons respond differently to stress or to a lack of circulating corticosterone, as Herman *et al.* have demonstrated with chronic electroconvulsive shock treatment (19). In addition, there is a difference of the corticosteroid receptor type between the PVN and SON: the PVN contains

both Type I corticosterone-prefering receptors and Type II glucocorticoid receptors, whereas the SON contains only Type II glucocorticoid receptors (27). Kovács and Makara (28) showed that the inhibitory effect of dexamethasone implants around the PVN on the adrenalectomy-induced enhancement of AVP and CRH immunostaining in the PVN seemed to be mediated via Type II glucocorticoid receptors. The mechanism by which the AVP gene is expressed in a different manner by the magnocellular neurosecretory neurons of the SON and the PVN still remains to be clarified.

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