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Vasopressin mRNA Regulation in Individual Hypothalamic Nuclei: A Northern and *in situ* Hybridization Analysis

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The present study examines the relative levels of vasopressin (AVP) mRNA within the paraventricular (PVN), supraoptic (SON), and suprachiasmatic (SCN) nuclei of the rat hypothalamus, and details the rates at which these levels change over the course of a 6 d salt-loading regimen. The quantitation of vasopressin mRNA was achieved by using three different procedures: (1) cell-free translation in rabbit reticulocyte lysate or (2) Northern analysis of poly(A)RNAs isolated from micro-punch dissected SON, PVN, and SCN, and (3) *in situ* hybridization histochemistry. The former involved the quantitative immunoprecipitation of the neurophysin precursors containing arginine⁸-vasopressin (AVP) or oxytocin, and the latter two techniques employed a radiolabeled synthetic oligodeoxynucleotide complementary to the 3′ region of the AVP mRNA. Both the cell-free studies and the Northern gel analyses detected a sevenfold increase of AVP mRNA in the SON, a fivefold increase in the PVN, and no significant change in the SCN following 6 d of salt-loading. After the initiation of salt-drinking, these increases were shown to occur between 24 and 48 hr in the SON and between 48 and 72 hr in the PVN. The *in situ* hybridization studies revealed the anatomically correct hybridization of either ^{32}P- or ^{3}H-labeled AVP oligonucleotide to magnocellular perikarya within both the SON and PVN. Autoradiographic grains could be shown to be confined to the cytoplasm of these cells, and could be co-localized with immunoreactivity directed against the carboxy terminus of the AVP percursor. Comparison of x-ray level autoradiograms of control and 6 day salt-loaded SON revealed up to a sevenfold increase in specific signal in the salt-loaded sections. It is concluded that the response of AVP mRNA to osmotic stimuli in the three hypothalamic nuclei is heterogeneous, and that this heterogeneity can be explained by separating AVP neurons into two systems: one responsible for eliciting the antidiuretic actions of AVP via plasma AVP levels, and the other involved in CNS activities not directly involved with antidiuresis.

Neurons containing arginine⁸-vasopressin (AVP) immunoreactivity are principally located within three cellular groups in the rat hypothalamus: the paraventricular (PVN), the supraoptic (SON), and the suprachiasmatic (SCN) nuclei. The PVN and SON, comprising the hypothalamo-neurohypophysial system, are two magnocellular areas that account for greater than 60–70% of the hypothalamic perikaryal AVP, with the remainder scattered in accessory cell groups and non-magnocellular neurons of the SCN (Rhodes et al., 1981). Neuroendocrine axons projecting from the PVN and SON to the posterior pituitary and median eminence are the primary sources of circulating AVP (Sachs et al., 1967). In addition, axons from the SCN, and, to a smaller extent, the SON and PVN, also project to many other extrahypothalamic sites within the brain and spinal cord. Even though the precise roles for these many projections of AVP are, in most cases, unknown, it remains clear that the cardinal physiological functions of AVP are threefold (Valiquette, 1980): (1) to control the renal excretion of water; (2) to regulate hemodynamic parameters dependent on effective blood volume (vasopressor activity); and (3) to regulate the secretion of ACTH. Although evidence for these roles for AVP has been reviewed extensively elsewhere, the following regulatory aspects are germane: (1) The secretory behavior of AVP from the posterior pituitary is tightly regulated by plasma osmolarity and/or plasma [Na+] (McKinley et al., 1978; Vernay, 1947); (2) AVP can be demonstrated to interact centrally with neurogenic mechanisms of blood pressure regulation (Ferrario et al., 1985) and systemically with a good correlation of plasma AVP levels with central venous pressure, with responses to blood volume changes of both emergency (hemorrhage) and nonemergency (assuming an upright posture) magnitude (Claybaugh and Share, 1973; Moses and Miller, 1974); and (3) AVP, along with corticotropin releasing factor (CRF), can stimulate the release of ACTH from the anterior pituitary via its secretion from the median eminence, a pathway also implicated in AVP responses to stress (for a review, see Makara et al., 1981). In a broad sense, these functions are subserved by three AVP efferent systems projecting, respectively, to posterior pituitary, extrahypothalamic CNS, and brain stem (Matsuguchi et al., 1982), and the external layer of the median eminence.

The contributions of the two nonposterior pituitary projection systems of AVP to the endocrine effects of circulating AVP remain essentially unknown, even though the hypothalamo-neurohypophysial system itself has been studied extensively by many biological disciplines. Few studies exist that have carefully investigated the biological responses of the three hypothalamic AVP cell groups to physiological stimuli known to regulate the secretory and/or biosynthetic activity of AVP neurons (i.e., osmotic challenge, hemorrhage, stress, etc.; Burbach et al., 1984; Sherman et al., 1985; Zerbe and Palkovits, 1984).

On the other hand, many biosynthetic studies have been performed on whole hypothalamus or the SON nucleus. It has been clearly shown, for example, in a sequence of studies initiated by the novel Common Precursor proposal of Sachs and Taka-

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batake (1964), that the biogenesis of biologically active AVP proceeds via the proteolytic processing of a high-molecular-weight precursor (Gainer et al., 1977a; Schmale and Richter, 1981). The biosynthesis of this precursor was shown to be greatly increased by chronic osmotic stimulation (Gainer et al., 1977b). With the molecular cloning of the mRNA for the bovine AVP precursor (Land et al., 1982), and subsequent isolation of the rat gene (Schmale et al., 1983), DNA sequence information became available for investigations into the identification and quantitation of AVP mRNA in response to physiological manipulations, using synthetic oligodeoxynucleotide probes. Such studies have shown that hypothalamic levels of AVP mRNA increase 8–10-fold following chronic salt-loading or dehydration (Majzoub et al., 1983; Sherman et al., 1985).

These later studies, utilizing quantitative Northern and dot-blot analyses, provide a level of information to the regulatory neurobiologist not readily available by other techniques. Quantitative RNA studies, for example, have indicated that, under chronic secretory demand, the degrees to which protein processing and translational enzymes can be stimulated are limited, and that increases in specific mRNA pool sizes must be made in order to increase the biosynthetic capacity of a neuron. Unlike peptide levels, specific mRNA pool sizes reflect the long-term biosynthetic preparedness of a cell. Specific mRNA levels, therefore, are an additional regulatory site of peptide cell biology. This reasoning has prompted us, in this paper, to expand on previous physiological studies in the hypothalamus in order to investigate the SON, PVN and SCN nuclei, individually, to determine both the degree to which these areas of the rat hypothalamus are osmotically responsive and the rates at which AVP mRNA levels in these areas increase, thereby determining the relative contributions of individual hypothalamic nuclei to posterior pituitary function, as well as beginning to clarify the responses of the 3 AVP nuclei, portions of which project to targets throughout the CNS, to systemic hyperosmicity.

The anatomical discreteness of the SON, PVN, and SCN was maintained throughout this study by coupling two techniques: (1) Palkovits' punch microdissection with mRNA quantitation (cell-free translation and Northern analysis), which not only allows for the preparation of tissue devoid of contamination by adjacent AVP cellular groups but, when carefully performed, provides for an RNA sample greatly enriched for the nuclei of interest; and (2) *in situ* hybridization histochemistry, an exciting and complex set of anatomical/molecular biological techniques in the early stages of development, which is now beginning to allow meaningful questions to be asked about regulatory molecular neurobiology at the single-cell level.

Materials and Methods

Salt-loading

Male Sprague-Dawley rats (200–225 gm; Charles River, Kennsington, WI) were housed in individual cages with a 12 hr light–dark cycle (0600 on, 1800 off). Animals were kept for 1 week prior to the start of the experiment and were given free access to water and Purina Lab Rodent Chow (Beynen, 1985). For salt-loading, rats were given 0.34 M (2% wt/vol) sodium chloride ad libitum for their drinking water. Plasma [Na^+] was determined in acidified plasma by a sodium electrode (Beckman Astro-8 Analyzer).

Palkovits' punch microdissection

Cell-free translation studies. Hypothalamic nuclei were punched from 1.0 mm dry-ice frozen brain sections using a 1.0-mm-I.D. punch, essentially as described (Palkovits and Brownstein, 1983). Northern gel analysis studies were as described above, except dissections were carried out using rapidly chilled brain sections instead of frozen ones. Punches were kept at −80°C until use.

RNA isolation

Total RNA was extracted from frozen tissue after a modification of Chirgwin et al. (1979). Punched tissue from one to three animals was homogenized in 0.5 ml, 6.0 M guanidinium-isothiocyanate (Fluka Chemical Corp.) containing 25 mM sodium citrate, pH 7.0, 17 mM *N*-lauroyl sarkosine, and 0.86 M 2-mercaptoethanol. Homogenates were incubated at 65°C for 10 min and centrifuged for 10 min at 4°C in a Fisher 235A microfuge. To each 1.0 ml volume of supernatant, 0.4 gm cesium chloride (CsCl) was added, and this was layered over 1.2 ml of a 5.7 M CsCl, 0.1 M EDTA, pH 7.5, cushion in a Sarstedt polyallomer tube for the Beckman SW 50.1 Ti rotor. Gradients were centrifuged for 16 hr at 20°C at 35,000 rpm. The supernant was removed by aspiration, the tube inverted, and the walls dried with a cotton-tipped applicator. The RNA pellet was resuspended in 0.2 ml, 0.1 M Tris-HCl, pH 7.3, containing 12.5 mM EDTA, 0.15 M NaCl, 0.2% wt/vol SDS, and 0.02 mg/ml proteinase K, and incubated at 37°C for 30 min. The RNA was extracted once with phenol/chloroform, once with chloroform, and precipitated from ethanol.

Poly(A)RNA isolation

Poly(A)RNAs were isolated using a microbatch method employing 3–4 mg of oligo(dT)-cellulose (Type 3; Collaborative Research). Total RNAs were heat denatured at 85°C for 5 min in 100 μl water. One volume of 2× high-salt buffer (2×-HSB: 20 mM sodium citrate, pH 7.5, 2 mM EDTA, 1.0 M NaCl) was added. This was transferred to a tube containing 3–4 mg of oligo(dT)-cellulose pre-equilibrated in 1×-HSB. After vortexing gently 3–4× over a 5 min period at 24°C, the resin was pelleted and the supernatant removed. The pellet was washed twice with 1×-HSB and once with intermediate-salt buffer (containing 0.1 M NaCl). The RNA was eluted in five 50 μl washes of water and co-ethanol precipitated with 1.0 μg *E. coli* tRNA (cell-free translation studies) from 0.25 M potassium acetate, pH 5.2, or 10 μg Baker's yeast tRNA (Northern gel studies) from 0.25 M sodium acetate, pH 5.2.

Poly(A)RNA quantitation

The poly(A) content of samples of total RNA was determined essentially as described (Pfeifer-Ohlsson et al., 1984) using ^{3}H-poly(U) (New England Nuclear).

In vitro *biosynthesis and immunoprecipitation of AVP-RNp I and OXY-RNp II precursors*

Cell-free translations using rabbit reticulocyte lysates (Bethesda Research Labs) and immunoprecipitations using protein A affinity-purified IgG against rat neurophysin (Alan G. Robinson, Pittsburgh, PA) were performed essentially as described (Sherman et al., 1984). All translations were performed in the presence of 1.0 mM diisopropylfluorophosphate (DFP, diisopropyl phosphofluoridate; Sigma Chemical Co.; Sherman et al., 1984) and a ^{35}S-cysteine concentration of 1.0 mCi/ml (1200 Ci/mmol ^{35}S-cysteine; New England Nuclear). Each sample was translated for 60 min at 30°C in a 30 μl volume of lysate. Ribosomes and polysomal complexes were pelleted during a 15 min ultracentrifugation at 100,000 rpm (30 psi in an A-100 rotor) in a Beckman Airfuge. The supernatants were diluted 1:1 with 2× immuno-binding buffer (2×-IBB: 100 mM sodium phosphate, pH 8.0, 0.3 M NaCl, 4% vol/vol Triton X-100, 48 mM sodium deoxycholate, 2.0 mM phenylmethyl sulfonyl fluoride, 0.2% dimethyl sulfoxide, 20 mM cysteine, and 0.62 mM sodium azide). To this was added, at a final concentration of 0.5 mg/ml, anti-rat neurophysin IgG or normal rabbit serum IgG. These were incubated at 22°C for 6 hr. The immunocomplexes were recovered on a 0.1 ml volume protein A Sepharose column equilibrated in 1×-IBB. The resin was washed with 100 column volumes of IBB, and 50 column volumes of PBS, pH 8.0. The immunoprecipitates were collected with 1.0 *N* acetic acid and lyophilized.

Total translational activity was defined as the extent of incorporation of ^{35}S-cysteine into reduced, *S*-carboxymethylated, TCA-precipitable protein (Sherman et al., 1984).

Northern analysis

The poly(A)RNAs were resuspended in 50% deionized formamide, 20 mM morpholinepropanesulfonic acid (MOPS), pH 7.0, 5 mM sodium acetate, 1 mM EDTA, and 2.2 M formaldehyde. The RNA was denatured at 65°C for 10 min and fractionated on a 1.5% agarose gel containing

2.2 M formaldehyde, 20 mM MOPS, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA, using an electrophoresis buffer of the same composition without formaldehyde. The separated RNAs were passively transferred to either nitrocellulose or Nytran membranes (Schleicher & Schuell) in 20×-SSC (3.0 M NaCl, 0.5 M sodium citrate, pH 7.2). Filters were prehybridized overnight at 45°C in a buffer containing 10% deionized formamide, 5×-SSC, 20 mM sodium phosphate, pH 7.5, 5× Denhardt's [1× = 0.2% polyvinylpyrrolidone (M_r = 40,000), 0.2% Ficoll (M_r = 400,000), and 0.2% BSA], 0.1% SDS, and 10 μg/ml sheared, heat-denatured salmon sperm DNA. The hybridization buffer was similar except that it contained 1× Denhardt's and 1.0 μg/ml salmon sperm DNA.

A 26-nucleotide-long (26mer) oligodeoxynucleotide was synthesized on an Applied Biosystems Model 380A DNA synthesizer, purified, and ^{32}P-labeled essentially as described (Lewis et al., 1985). Northern hybridizations were carried out at 45°C for 24–48 hr at an oligomer concentration of 2–5 nM and a probe specific activity of approximately 10^9 dpm/nmol. Filters were washed to a stringency of 2×-SSC, 0.1% SDS, at 45°C.

In situ hybridization

All tissue preparation, sectioning, hybridizations, and autoradiography were performed essentially as described by Lewis et al. (1985). When immunocytochemistry was performed prior to *in situ* hybridization, the techniques used were as described previously (Watson et al., 1982, 1983), except that all antibody-containing buffers contained 2.0 mM vanadyl ribonucleoside complex (VRC; Bethesda Research Labs).

Data from salt-loading experiments were compared using unpaired *t* tests.

Results

The relative quantitation of specific mRNA by cell-free translation

RNA-dependent, cell-free translation in rabbit reticulocyte lysate (RRL) is linear with respect to added RNA at only very low concentrations of RNA. With rat hypothalamic poly(A)RNA, deviations from linearity occurred at concentrations greater than 10 μg RNA/ml, even though the observed maximum translational activity was nearly 40 μg RNA/ml (data not shown). For the application of cell-free translation in a quantitative assay of mRNAs coding for specific proteins, it was found necessary, therefore, to limit the amount of poly(A)RNA added to a 30 μl translation volume to less than 0.3 μg. Figure 1 illustrates the linearity of prepro-AVP/RNp I translation obtained with poly(A)RNA isolated from 1 to 6 rat hypothalamic magnocellular nuclei (SON or PVN), even when this poly(A)RNA was added in conjunction with the 1.0 μg of *E. coli* tRNA found necessary for quantitative recovery of the purified poly(A)RNA. The 1.0 μg of *E. coli* tRNA was found to decrease total translational activity by 10–12%. On average, 2 SON punches (a bilateral dissection from one rat using a 1.0-mm-I.D. punch on a 1.0-mm-thick frozen section), weighing approximately 2–4 mg, wet weight, would yield 1.4–2.5 μg of total RNA, from which approximately 0.05 μg of poly(A)-enriched RNA could be recovered. When translated in a RRL system (30 μl) in the presence of 1.0 mCi/ml ^{35}*S*-cysteine (>1200 Ci/mmol), 1.0 mM DFP, and 1.0 μg *E. coli* tRNA, 2200 dpm could be incorporated into immunoprecipitable neurophysin precursor products, approximately 55% of which migrated as an 18,000 Da band corresponding to prepro-AVP/RNp I (Schmale and Richter, 1981; Sherman and McKelvy, 1983). The greatly homologous OXY/RNp II precursor migrated at 16,500 daltons. Because there are 36 cysteine residues divided between these 2 precursors (Ivell and Richter, 1984), the radioactivity associated with neurophysin-like immunoreactivity accounted for greater than 30% of the ^{35}*S*-cysteine incorporated into punched-hypothalamic poly(A)RNA-dependent, reduced, carboxymethylated, TCA-precipitable translation products. When the total translation products were examined with two-dimensional gel electrophoresis, the relative amounts of both high molecular weight forms of neurophysin were increased relative to total protein, but were by no means the only induced proteins (data not shown). Greater than 10% of the visualized proteins were induced to a recognizable degree (Sherman et al., 1984).

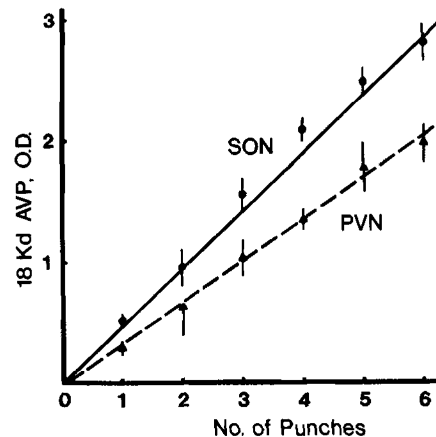

Figure 1. Linearity of punch translations. Supraoptic (*SON*) or paraventricular (*PVN*) nuclei in normal Sprague-Dawley rats were punch-dissected from 1.0 mm frozen sections using a 1.0-mm-I.D. punch. Punches totaling 1, 2, 3, 4, 5, and 6 nuclei were pooled. Total RNA and poly(A)RNAs were isolated and translated *in vitro,* and rat neurophysin immunoprecipitates collected, as described in Materials and Methods. Eluted proteins were fractionated on a 15% SDS-polyacrylamide gel. Gels were prepared for fluorography using EN^{3}HANCE (New England Nuclear), and exposed for 10–12 d. Densitometry on the 18,000 Da prepro-AVP/RNp I band is expressed as 0.1 × total optical density (O.D.; area in pixels × average absorbance) of that band (see legend to Fig. 6). Correlation coefficients, *SON* = 0.987; *PVN* = 0.968.

This assay for translatable vasopressin mRNA was applied to RNAs isolated from bilaterally-punched hypothalamic nuclei dissected from rats which had been salt-loaded for up to 6 days. The ability of SON RNA to direct the translation of prepro-AVP/RNp I and prepro-OXY/RNp II increased sevenfold over the course of 6 days (Fig. 2). The salt induced stimulation observed in the PVN was less pronounced, amounting to 4.3-fold. Within the sensitivity limits of this assay, no significant change was detected in the levels of non-magnocellular vasopressin mRNA in the SCN. These results are summarized in Figure 3. The ratio of translatable AVP mRNA in rat hypothalamic nuclei (SON:PVN:SCN) changes from 12:7:5 in controls to 92:38:5 in 6-day salt-loaded animals. There was no significant change in the ratio of AVP:OXY precursors using this paradigm.

Salt-loading rats with 2% saline as drinking water is well established as a powerful, albeit nonselective, chronic secretagogue for neurohypophyseal AVP. Rats quickly habituate to the salinity, effectively increasing their plasma sodium levels from 141.7 mEq/liter to 150.7 mEq/liter after 24 hr (Table 1). Profound body weight loss is elicited in these animals, however (Table 1). Although no plasma AVP radioimmunoassay data are presented here, this plasma [Na^+] is commensurate with greater than 10-fold increases in plasma AVP (Moses and Miller, 1971).

The relative quantitation of AVP mRNA by Northern analysis

A 26-nucleotide-long oligodeoxynucleotide complementary to rat vasopressin was chemically synthesized (Fig. 4) using sequence information derived from the published rat prepro-AVP/RNp I gene structure (Schmale et al., 1983). The oligonucleotide corresponds to a 9 amino acid stretch contained within the

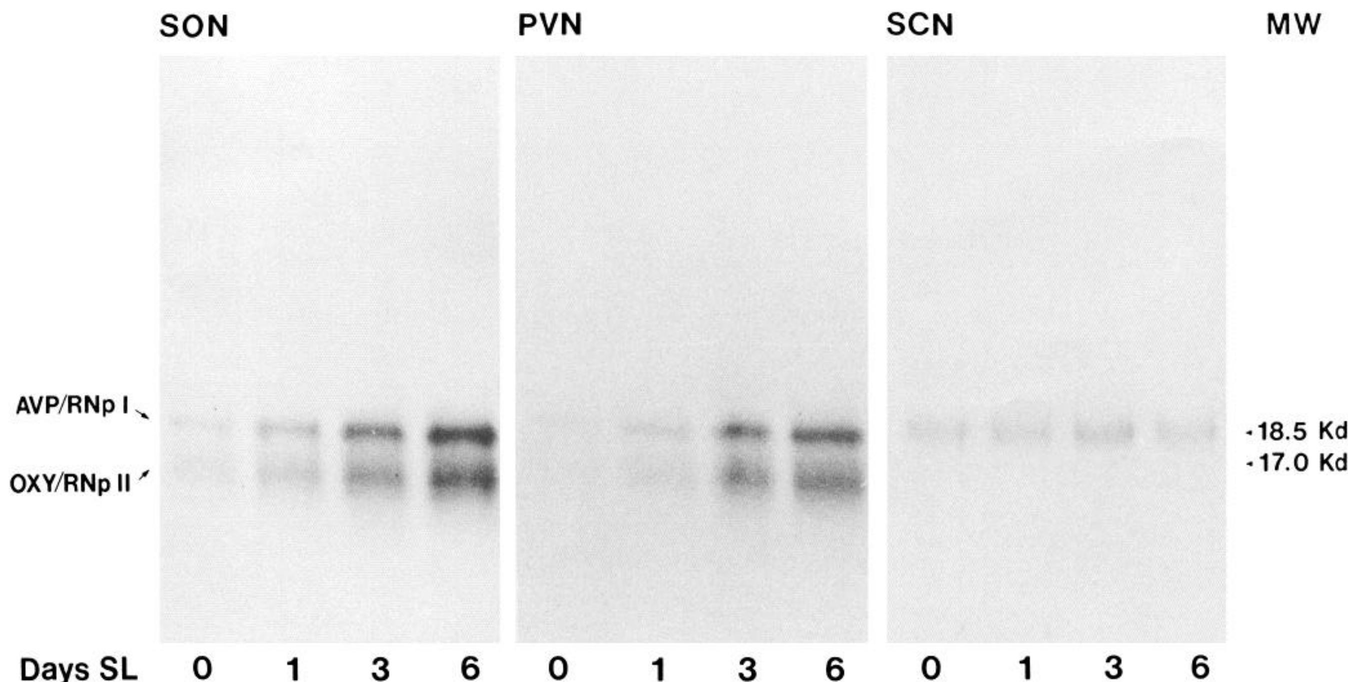

Figure 2. Translation of prepro-AVP/RNp I and prepro-OXY/RNp II in hypothalamic nuclei during salt-loading. Bilateral punches of either *SON, PVN,* or *SCN* were collected from 1.0 mm frozen sections. Poly(A)RNA was isolated from punches from individual animals, translated *in vitro,* and neurophysin-like immunoprecipitates collected, as described in the legend to Figure 1. Immunoprecipitates were fractionated on 15% SDS-polyacrylamide gels and prepared for fluorography. The SON and PVN gels were exposed for 12 d, the SCN gel for 16 d. Each *point* represents the bilateral nuclei from one animal.

amino-terminal half of the carboxy terminal glycoprotein moiety of the AVP precursor. Since this portion of the precursor is absent in the otherwise greatly homologous oxytocin precursor (Ivell and Richter, 1984; Land et al., 1983), no cross hybridization with oxytocin mRNA is possible. The oligonucleotide contains 77% G:C residues and exhibits an experimentally determined T_m of 65°C in 2×-SSC (data not shown).

Because of the greater number of animals involved in these studies, the hypothalamic nuclei dissection was altered to decrease dissection time. The sectioning and punching of fresh, chilled tissue resulted in tissue pieces approximately twice the size obtained with frozen section punches. Table 2 summarizes the yields of total RNA from SON, PVN, and SCN punches in experiments involving 3 groups of 3 animals. To normalize for the amount of poly(A)RNA isolated from these preparations, which were too small for practical quantitation by UV absorbance at 260 nm, total poly(A) content was determined on a 10% aliquot by a solution phase ^{3}H-poly(U) hybridization assay (Table 2). The two magnocellular nuclei (SON and PVN) demonstrated a trend towards greater recovery of total RNA with salt-loading which was not statistically significant; paradoxically, these RNAs reproducibly contained a greater poly(A) content with salt-loading which, when expressed per microgram of total RNA, was decreased compared to controls (Table 2). While

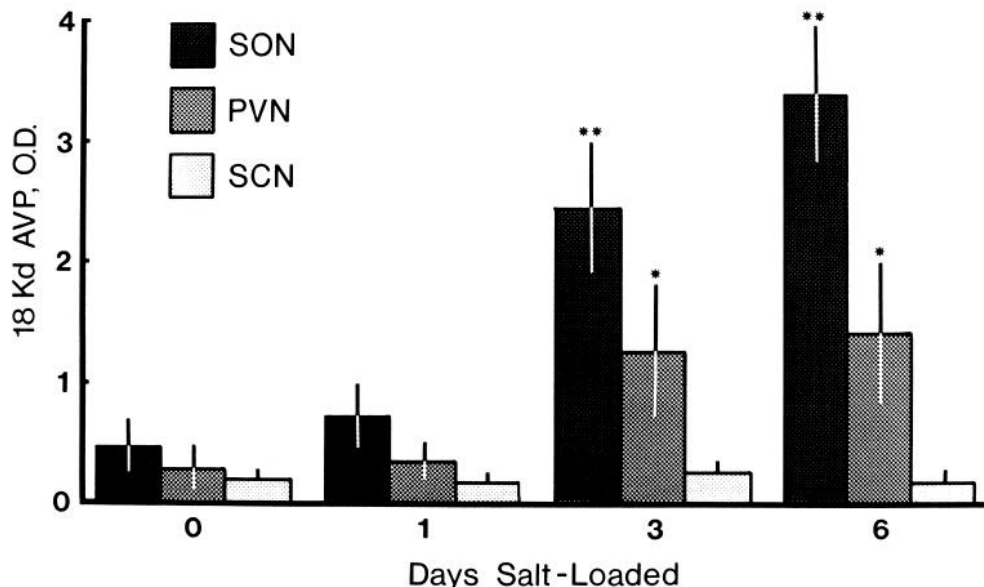

Figure 3. The relative expression of AVP mRNA in hypothalamic nuclei during salt-loading, as determined by a cell-free translation assay. The 18,000 Da prepro-AVP/RNp I bands in Figure 2 were quantitated by digital densitometry on a Loats autoradiography system. The data are expressed as 0.1 × total optical density (see Fig. 6). *SL0–SL6* denote days salt-loaded, from 0 to 6. *$p \leq 0.025$; **$p \leq 0.005$.

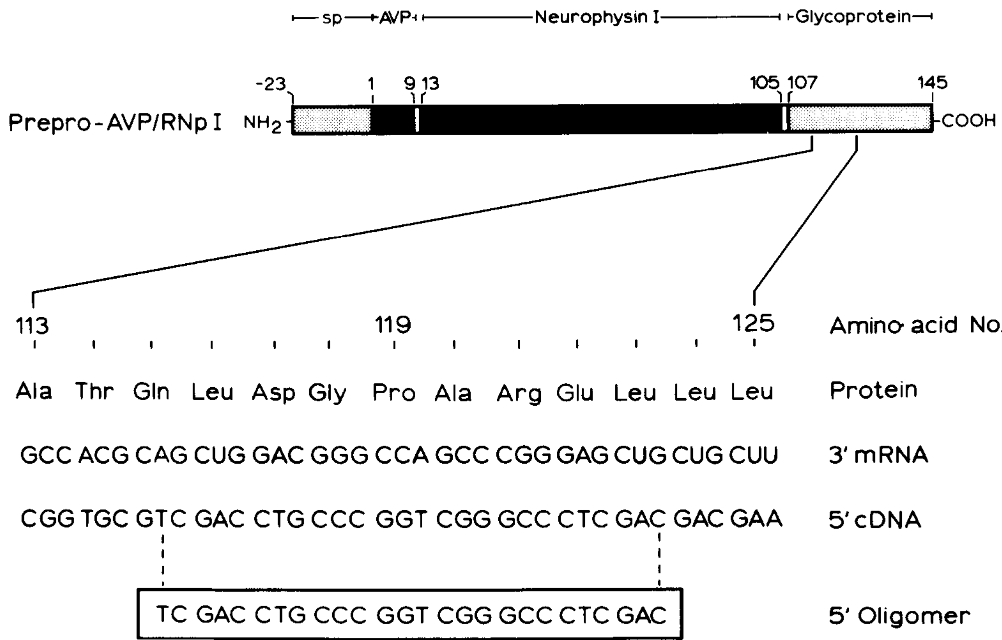

Figure 4. Sequence, orientation, and region of complementarity for synthetic AVP oligonucleotide. The AVP precursor structure and RNA sequence was derived from Schmale et al. (1983). The amino acids encoded by the probe sequence are immediately adjacent to the sole glycosylation site on the AVP precursor (AA 112–114).

this phenomenon is still under investigation, one probable explanation is that, as part of the overall stimulation of protein biosynthesis occurring in these nuclei due to hyperosmotic challenge, even though poly(A)RNA levels are elevated, ribosomal RNA levels are elevated even further to accommodate the greater translational activity.

Hybridization of the ^{32}P-kinased oligomer to a nitrocellulose membrane containing fractionated poly(A)RNAs isolated from either SON, PVN, or SCN throughout a 6 d salt-loading study revealed a single band (Fig. 5) migrating at 740 nucleotides (Schmale et al., 1983). In RNAs isolated from the SON, the intensity of the AVP mRNA band increased 7.3-fold over 6 d. Vasopressin mRNA increased 5.1-fold over the same period in the PVN, and by approximately 60% in the SCN. These results, normalized for poly(A) content, are summarized in Figure 6.

As was shown in Figure 3, these data depict the increase of AVP mRNA in the magnocellular hypothalamus in response to hyperosmotic stimuli. Furthermore, in the SON, a 24–48 hr delay in this response was observed, whereas, in the PVN, this lag was between 48 and 72 hr. Each, however, plateaued on approximately the fourth day.

The relative anatomical quantitation of AVP mRNA by in situ *hybridization*

The magnocellular neurons of the rat hypothalamus lend themselves well to the technique of *in situ* hybridization histochemistry by virtue of their large, closely packed perikarya and their possession of relatively abundant peptide hormone mRNAs. While strategies and technical controls for this technique are adequately discussed elsewhere (Lewis et al., 1985), we present evidence here for the applicability of this procedure to the visualization and relative quantitation of specific mRNA changes in brain.

Within the SON and PVN, AVP immunoreactivity has been clearly shown to reside in large-diameter neurons (25–35 μm) with characteristic distributions (Dierickx, 1980; Rhodes et al., 1981; Swaab et al., 1975). Figure 7 demonstrates that these same anatomical distributions for AVP perikarya can be reproduced autoradiographically following *in situ* hybridization with a radiolabeled AVP oligonucleotide. Figure 7*C* illustrates the distribution of magnocellular AVP neurons in the PVN, using a 5′-^{32}P-kinased oligomer identical to that used in Figure 5 for

Table 1. Changes in body weight and plasma [Na+] with salt-loading

Group	Weight (% starting wt)	*n*	Total intake (ml, 2% saline)	*n*	Plasma [Na+] (mEq/liter)	*n*
SL0	100.00 + 7.87	48	0.0 + 0.0	9	141.7 + 2.7	3
SL1	92.76 + 7.75	45	43.0 + 8.7	9	150.7 + 1.7	3
SL2	86.94 + 5.35	42	113.0 + 23.6	9	152.3 + 2.4	3
SL3	84.85 + 6.47	39	201.7 + 27.3	9	155.7 + 2.8	3
SL4	82.94 + 7.72	36	312.3 + 37.4	9	155.0 + 2.1	3
SL5	80.30 + 8.11	33	460.7 + 47.2	9	155.5 + 2.4	3
SL6	78.10 + 8.14	30	627.3 + 52.5	9	154.1 + 3.3	3

Male Sprague-Dawley rats, 200–225 gm, were individually housed for 1 week prior to salt-loading. Rats were provided with 200 ml of fresh 2% NaCl each day at 1000 hours, at which time body weights were recorded. Salt-loading was timed such that all animals were killed on the same day. Plasma sodium concentrations were determined as described in Materials and Methods. SL0–SL6 denote days salt-loaded, from 0 to 6.

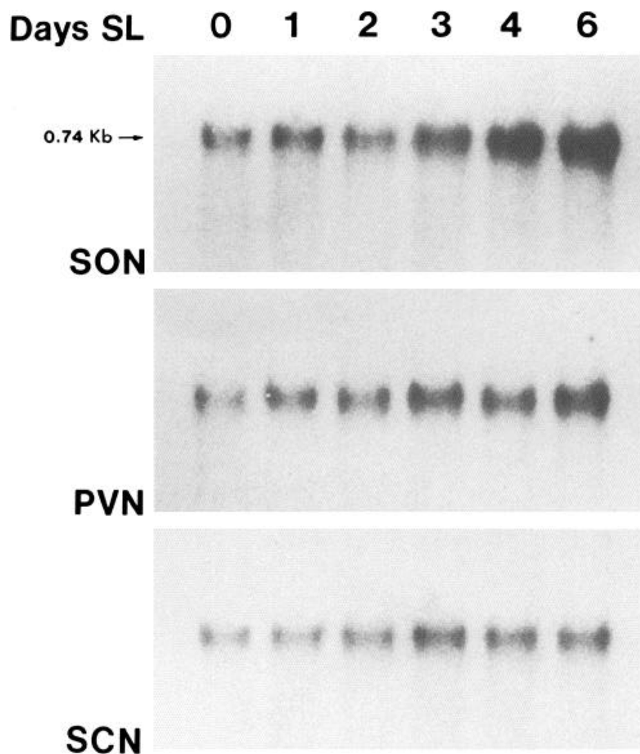

Figure 5. Northern gel analysis of AVP mRNA in hypothalamic nuclei during salt-loading. Poly(A)RNAs isolated from bilaterally punch-dissected *SON, PVN,* and *SCN* from 3 animals were fractionated on 1.5% formaldehyde-agarose gels and passively transferred to Nytran membranes, as described in Materials and Methods. On average, poly(A)RNA, isolated from 90% of the amount of RNA depicted in Table 1 for each nuclei, is loaded per lane. Hybridization with ^{32}P-kinased AVP oligonucleotide and stringency of washes are as described in Materials and Methods. The air-dried, washed Northerns were exposed at 22°C for 85 hr for SON and PVN, and 120 hr for SCN.

Northern analysis. Even with the pronounced autoradiographic scatter obtained with ^{32}P-labeled probes due to the high-energy beta emission, the characteristic clustering of hybrid-positive neurons was recognizable. Individual cells could be visualized in the medial PVN along the third ventricle wall, where the perimeter clustering of grains suggested cytoplasmic localization of AVP mRNAs.

When the AVP oligomer was 3′-labeled with ^{3}H using terminal deoxynucleotidyl transferase (Lewis et al., 1985), the positive identification of individual perikarya was greatly improved. Figure 7*A* shows AVP-positive hybridization in the medial SON just lateral to the optic tract (OT). In many neurons, the grain localizations were distinctly cytoplasmic and were confined exclusively to the cell body, with no evidence for either axonal or dendritic localization. Beyond the demonstration of anatomically correct hybridization, other controls for the oligomer's AVP mRNA specificity included its exhibition of a sharp melting curve for homogeneous hybridization (T_m = 65°C) and the co-localization of autoradiographic grains with AVP peptide immunocytochemistry. Figure 7*B* illustrates this latter control with a high-magnification view of a magnocellular neuron in the rat nucleus circularis. The 10 μm hypothalamic section at the level of the medial SON was first immunostained with antisera directed against the carboxy-terminal glycoprotein proportion of the AVP/RNp I precursor (CPP; Watson et al., 1982) with PAP detection, followed with *in situ* hybridization with the ^{3}H-labeled oligonucleotide complementary to this region of the gene. The strictly cytoplasmic section through this neuron shown in Figure 7*B* clearly displays a relatively homogeneous distribution of CPP-positive immunoreactivity punctated with autoradiographic grains.

Figure 7*D* demonstrates that analyses such as those discussed above can be employed in the relative quantitation of specific mRNA changes in discrete hypothalamic nuclei. Serial 10 μm sections through the medial SON from either control or 6 d salt-loaded rats were subjected, in parallel, to *in situ* hybridization with a ^{32}P-labeled AVP oligomer. Figures $7D_1$ and $7D_2$ clearly demonstrate the increased labeling of the SON at the x-ray film level, following chronic osmotic challenge. Comparative densitometry of these nuclei has yielded several-fold increases, with salt-loading ranging from 3.5- to 7.1-fold in the SON, and from 2.0- to 6.1-fold in the PVN, depending on the study and/or the section. There still exist many variables in these studies, such as possible sampling artifacts and intranucleus heterogeneity, which have yet to be accounted for. We recognize the many problems associated with studies such as these, yet we also recognize that many of the original, and seemingly insurmountable, problems are now being adequately addressed. These points will be considered in the following section.

Discussion

Three distinct, yet complementary, quantitative assessments of AVP mRNA levels clearly indicate, in the present results, that AVP cell groups within the hypothalamus respond to chronic osmotic stimulation by increasing their levels of AVP mRNA. Using a cell-free translation assay, the levels of translatable AVP and OXY mRNAs within the hypothalamus were increased significantly over the course of a 6 d salt-loading study. The total hypothalamic increase in AVP mRNA could be accounted for by increases in 2 of the 3 AVP cell groups in this brain region: a 7.4-fold increase in the SON and a 4.9-fold increase

Table 2. Yields of RNA and poly(A) contents of RNA from hypothalamic nuclei

Group	Total SON RNA/3 rats (μg)	Poly(A) (ng)/μg SON RNA	Total PVN RNA/3 rats (μg)	Poly(A) (ng)/μg PVN RNA	Total SCN RNA/3 rats (μg)	Poly(A) (ng)/μg SCN RNA
SL0	12.6 + 3.1	2.01 + 0.08	12.4 + 2.7	1.69 + 0.14	18.1 + 1.3	1.71 + 0.26
SL1	13.7 + 2.4	1.99 + 0.09	11.9 + 2.4	1.53 + 0.03	23.9 + 2.2	1.59 + 0.12
SL2	15.1 + 3.0	1.90 + 0.08	12.9 + 3.4	1.52 + 0.05	17.2 + 2.8	1.60 + 0.11
SL3	15.6 + 2.9	1.88 + 0.08	13.4 + 2.1	1.48 + 0.07*	20.2 + 4.1	1.71 + 0.09
SL4	17.2 + 1.5	1.85 + 0.09*	13.8 + 3.1	1.48 + 0.01*	19.3 + 2.1	1.76 + 0.11
SL6	17.4 + 2.3	1.80 + 0.08*	14.2 + 2.5	1.46 + 0.07*	16.7 + 3.0	1.82 + 0.06

Each point of total RNA yield represents the average of 3 groups of 3 animals. Poly(A) content measurements were done in triplicate for each group. Ten percent of the total RNA sample from each group was used for poly(A) content assays; that 10% was divided into thirds for triplicate hybridizations against 12.61 ng poly(U) (249,750 dpm). Procedures are as described in Materials and Methods. SL0–SL6 denote days salt-loaded, from 0 to 6.

* $p \leq 0.05$.

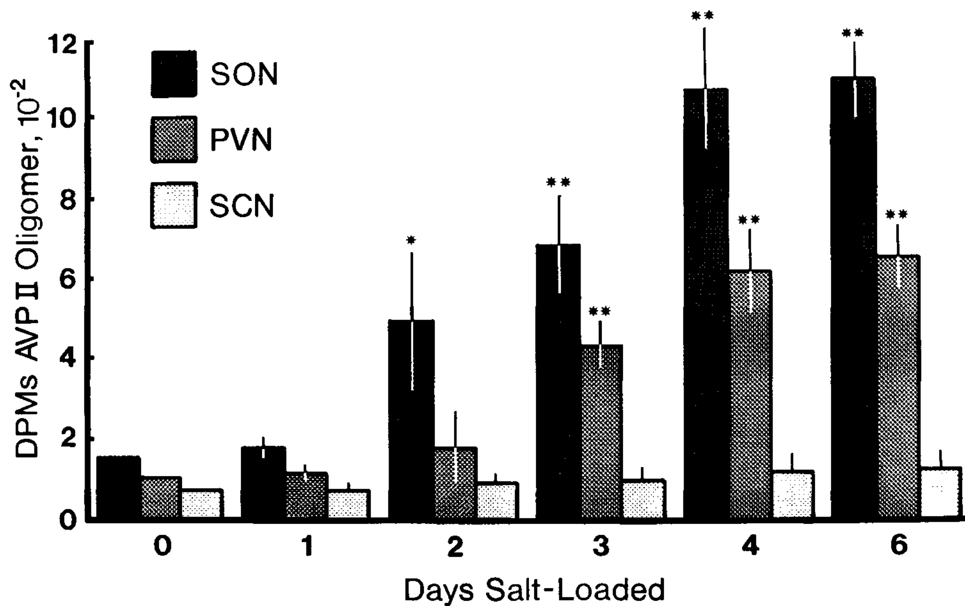

Figure 6. Quantitation of AVP mRNA in hypothalamic nuclei during salt-loading, using Northern gel analysis. The data are normalized for average poly(A) content in the SLO groups, as shown in Table 2. Hybridization-positive bands on Northern filters corresponding to AVP mRNA were quantitated using digital densitometry on a Loats autoradiography system. Total optical density was converted to dpm of ^{32}P using a standard curve constructed by the dot-blot application of ^{32}P-labeled AVP oligomer to nitrocellulose. Triplicates of dot-blotted samples were either counted in a toluene-based scintillation fluid in a Beckman beta-counter or exposed simultaneously with Northern gels. Analysis revealed a film linearity of exposure (correlation coefficient = 0.993) up to an average absorbance of 0.83 ± 0.02, or 209 ± 10 dpm/mm^2 at 15 hr of exposure (22°C). For the Northern gel analyses, 100 units of optical density (area in pixels × average optical density) equaled 180.2 ± 13.8 dpm for the 85 hr exposure at 22°C. *SL0–SL6* denote days salt-loaded, from 0 to 6. **p* ≤ 0.025; ***p* ≤ 0.005.

in the PVN. The only non-magnocellular cell group of the three, the SCN, displayed no significant change over the 6 d salt-loading period, although the amounts of immunoprecipitable AVP or OXY precursor within this nuclei were sufficiently small that truly accurate estimates were difficult to obtain. The use in these immunoprecipitation experiments of an anti-rat neurophysin IgG with high affinity for both AVP and OXY precursors gave the added information, shown in Figure 2, that the translational capacity for both of these precursors was increased in poly(A)RNAs isolated from salt-loaded PVN and SON. This result is substantiated by earlier secretory and biosynthetic studies that indicated that salt-loading, as a neurohypophyseal stimulus, is equally effective for both AVP and OXY peptide secretion (Gainer et al., 1977b; Seif et al., 1977) and for stimulation of AVP and OXY's respective mRNAs (Majzoub, 1985; Majzoub et al., 1983).

Similar results were determined by Northern gel analysis. The hybridization of a 26-nucleotide-long radiolabeled AVP oligomer to a 0.74 kilobase RNA was greatly increased by salt-loading. The calculated increases in the SON and PVN, 7.1-fold and 5.3-fold, respectively, are similar in magnitude to those of the translation assays, indicating that the percentage of total AVP RNA that is translatable remains relatively constant with salt-loading. Studies are currently in progress to determine what percentage this actually represents.

In sum, thus far, it is clearly established that chronic administration of a secretory stimulus results in the eventual increase in secretory product mRNAs. The differential responses of the SON, PVN, and SCN to a single stimulus, salt-loading, and the magnitudes of these responses can be partially predicted by anatomical and physiological data on the distribution and functions of AVP. As was stated in the introduction, the three main physiological functions of AVP, simply stated, are its antidiuretic, vasopressor, and CRF-like activities. The regulation of AVP secretion is complex and multipolar, with the principal regulatory stimuli appearing to be plasma osmolarity, blood volume, and some types of stress, as detected via osmoreceptors, sodium receptors, atrial stretch receptors, arterial baroreceptors, and glucocorticoid-related mechanisms (Travis and Share, 1971). Ultimately, however, some portions of this information are funneled to hypothalamic AVP neurons, whose cell bodies within the SON, PVN, and SCN possess many projections, including: (1) a massive (and perhaps, sole) neuroendocrine projection from the SON to the posterior pituitary (Rasmussen, 1940; Swanson and Sawchenko, 1983); (2) a major projection to the posterior pituitary from the PVN (Swanson and Kuypers, 1980); (3) projections from the periventricular, medial (dorsal half), and anterior parts of the PVN to the external layer of the median eminence (Dierickx et al., 1976); (4) a projection to the nucleus tractus solitarius (NTS) from a caudal division of the PVN (Swanson and Kuypers, 1980); and (5) projections from the SCN to the lateral habenular nucleus, the periventricular nucleus, and the organum vasculosum of the lamina terminalis (Buijs, 1978).

The main question germane to the studies presented here remains, however: Do those projections other than to the posterior pituitary contribute to the neuroendocrine effects of AVP during osmotic challenge? Do subsets of AVP neurons in each area respond differently to osmotic stimulation? If, in fact, AVP projections to regions other than the neural lobe do not respond to salt-loading, then this may partially explain the lessened response of PVN AVP mRNA compared to that of SON. Several reports have begun to address this question by using anatomically discrete biochemical assays, either to assess AVP level changes in the hypothalamus and selected terminal fields following osmotic challenge (Negro-Vilar and Samson, 1982; Zerbe and Palkovits, 1984), or to determine the relative changes in AVP mRNA in hypothalamic nuclei after chronic salt-loading (Burbach et al., 1984; Sherman et al., 1985). Radioimmunoassay data of AVP in terminal fields of the PVN and SCN (Zerbe and Palkovits, 1984) have demonstrated that some AVP neurons respond to salt-loading, as evidenced by either decreased (posterior pituitary and NTS) or increased (median eminence,

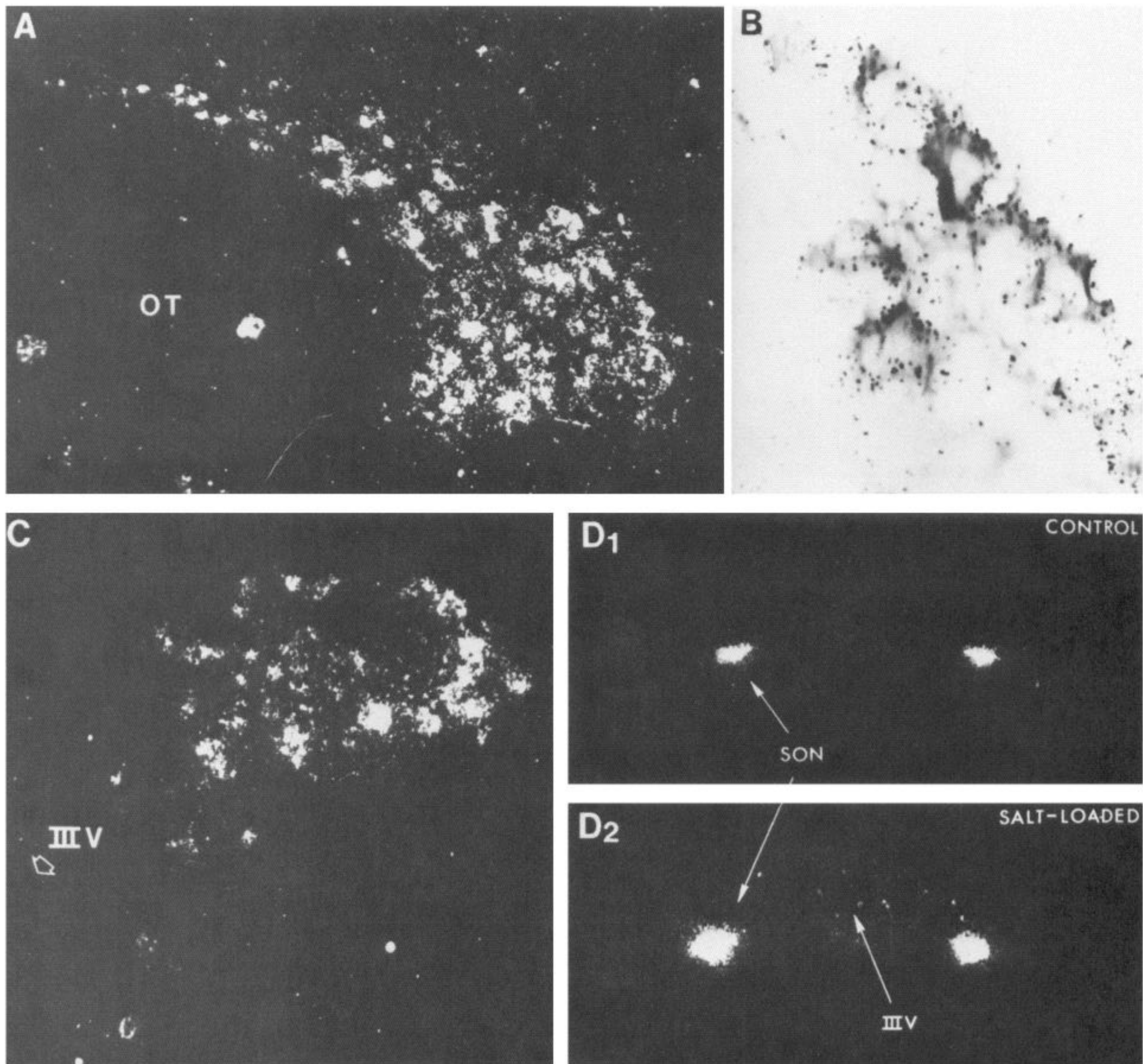

Figure 7. *In situ* hybridization histochemistry of SON and PVN using ^{32}P- and ^{3}H-labeled AVP oligonucleotides. All sections shown here were 10 μm formaldehyde-fixed. General *in situ* hybridization procedures were as described in Materials and Methods. *A*, Dark-field photomicrograph of a section through the medial SON of a normal rat hybridized with a 3′-^{3}H-labeled AVP oligonucleotide. Hybridization was conducted at 37°C with 100,000 dpm of oligomer. The stringency of wash was 45°C at 0.5×-SSC. The section was dipped in Kodak NTB2 emulsion (42°C, diluted 1:1 with 1.0 mg/ml sonicated Dreft), and exposed for 11 weeks at 4°C in the presence of Drierite desiccant. Slides were developed in Kodak D-19 for 2 min at 16°C. *OT*, Optic tract. *B*, Light-field photomicrograph of the co-localization of AVP/CPP immunoreactivity with ^{3}H autoradiographic grains from AVP oligomer hybridization in the nucleus circularis. Prior to *in situ* hybridization, the section was immunostained for the presence of the carboxy-terminal peptide of the AVP/RNp I precursor (CPP), as described in Materials and Methods. All hybridization conditions as in *A*. *C*, Dark-field photomicrograph of a section through the mediocaudal PVN of a 6 d salt-loaded rat hybridized with a 5′-^{32}P-labeled AVP oligomer. Hybridization was conducted at 45°C with 500,000 dpm of probe. The stringency of wash was 45°C at 0.5×-SSC. Autoradiography, as described in *A*. Ten day exposure. *IIIV*, Third ventricle. D_1 and D_2, Dark-field print of *in situ* hybridization in *control* versus 6 d *salt-loaded SON* with a ^{32}P-labeled AVP oligomer. Hybridizations and wash stringency, as described in *C*. Air-dried sections were exposed for 45 hr against Kodak XAR-5 x-ray film at 22°C. Panels shown are representative segments of 6 serial sections through the medial SON from control or salt-loaded animals. *IIIV*, Third ventricle.

subfornical organ, and amygdala) AVP levels. Although the AVP peptide changes, other than those in the posterior pituitary, were modest, it remains difficult to infer from these data whether or not this degree of neuronal responsiveness is sufficient to induce AVP mRNAs, and, therefore, contribute to an overall induction in AVP message in the PVN. The PVN projection to the NTS, for example, is a good case in point. Depletion of AVP from terminals in the NTS with salt-loading suggests a positive response of these neurons to osmolarity (Zerbe and Palkovits, 1984), perhaps as part of a plasma osmolarity/cardiovascular function relationship (Ferrario et al, 1985; Matsuguchi et al., 1982). It can be argued, however, that the amounts of peptide hormone required to convey information in synaptic secretory activity, such as seen here, are much smaller than that required for neuroendocrine secretory activity; consequently, the cell's biosynthetic capacity is not exceeded, no increases in mRNA pools are required, and no contribution to osmotically induced PVN AVP mRNA would be observed. Although this argument

is speculative, the evidence presented here suggests that the magnitude of AVP mRNA increases in the SON, PVN, and SCN is correlated with the extent to which each of these nuclei projects to the neural lobe. The biosynthetic requirements for neuroendocrine (hormonal) secretion, therefore, may rely more heavily on inducible mRNA pools. Clearly, however, the roles for PVN AVP are multifaceted, and this, perhaps, is reflected in this nuclei's muted response to a single stimulus (salt-loading).

The introduction of *in situ* hybridization histochemistry may permit the type of sub-nuclear or cellular analyses necessary to properly examine the probable heterogenic responsiveness of the PVN (Swanson and Sawchenko, 1983; Wolfson et al., 1985). The use of ^{3}H-labeled oligomers, as was shown in Figure *7A*, allows visualization of cytoplasmic mRNA within single magnocellular neurons. Examination of nuclear heterogeneity, such as that seen in the PVN, by *in situ* hybridization, should resolve those questions not answerable by even the best dissections. A recent report, for example, has confirmed the immunocytochemical evidence for AVP expression within CRF cells of the parvocellular PVN following adrenalectomy (Sawchenko et al., 1984), by using *in situ* hybridization with AVP oligonucleotides (Wolfson et al., 1985). At a more quantitative level, we are confident that whole-nuclear averaging quantitations, such as those shown in Figure *7D*, will provide consistent results, perhaps similar in magnitude to those from punched Northern gel analysis; however, many problems remain with quantitative studies at the autoradiographic level. Some of these difficulties are unique to *in situ* hybridization, while others are peculiar to autoradiographic quantitations in general. Of the former, we are particularly concerned about ribosomal loading: Whether, during periods of increased translational activity, efficient probe hybridization to mRNA is blocked by the increased ribosome loading known to occur (Palmiter, 1973), effectively underestimating the real mRNA increase. Similarly, questions of mRNA stability, hybridization efficiency (probe size), and the absence of adequate autoradiographic standards are paramount.

To summarize, we have reported the heterogeneity of response of AVP hypothalamic nuclei to osmotic stimuli. The total extent of this response occurs within the two magnocellular cell groups of the rat hypothalamus, the SON and PVN, and the extent of the increase in translatable AVP mRNA is proportional to that of total hybridizable AVP mRNA in both nuclei. Future directions for these studies involve the qualitative and quantitative assessment of osmotic response heterogeneity in the PVN by *in situ* hybridization.

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