Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10653–10657, November 1995
Neurobiology

Localization of vasopressin mRNA and immunoreactivity in pituicytes of pituitary stalk-transected rats after osmotic stimulation

(hyperosmotic stress/neuropeptide/astrocytes/*in situ* hybridization/immunocytochemistry)

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Communicated by Susan E. Leeman, Boston University School of Medicine, Boston, MA, July 20, 1995

ABSTRACT **The presence of [arginine] vasopressin (AVP) mRNA and AVP immunoreactivity in pituicytes of the neural lobe (NL) of intact and pituitary stalk-transected rats, with and without osmotic stimulation, was examined. AVP mRNA was analyzed by Northern blotting, as well as by *in situ* hybridization in combination with immunocytochemistry using anti-glial fibrillary acidic protein (GFAP) as a marker for pituicytes. In intact rats, a poly(A) tail-truncated 0.62-kb AVP mRNA was detected in the NL and was found to increase 10-fold with 7 days of continuous salt loading. Morphological analysis of the NL of 7-day salt-loaded rats revealed the presence of AVP mRNA in a significant number of GFAP-positive pituicytes in the NL and in areas most probably containing nerve fibers. Eight days after pituitary stalk transection the NL AVP mRNA diminished in animals given water to drink, whereas in those given 2% saline for 18 h followed by 6 h of water, a treatment repeated on 6 successive days beginning 2 days after surgery, the 0.62-kb AVP mRNA was present. The AVP mRNA in the pituitary stalk-transected, salt-loaded rats showed an exclusive cellular distribution in the NL, indicative of localization in pituicytes. Immunoelectron microscopy showed the presence of AVP immunoreactivity in a subpopulation of pituicytes 7 and 10 days after pituitary stalk transection in salt-loaded animals, when almost all AVP fibers had disappeared from the NL. These data show that a subset of pituicytes in the NL is activated to synthesize AVP mRNA and AVP in response to osmotic stimulation.**

The [arginine] vasopressin (AVP) gene is abundantly expressed in magnocellular neurons of the supraoptic nucleus (SON) and paraventricular nucleus (PVN) in the hypothalamus (1). However, recent studies have shown the presence of AVP mRNA in the neural lobe (NL) of the pituitary as well, where the axons of these neurons terminate. This NL mRNA is shorter than that present in the hypothalamus due to a truncated poly(A) tail and is up-regulated during osmotic stimulation (2–5). After disconnection of the hypothalamo-neurohypophysial tract by electrically damaging the ventromedial hypothalamic area, the AVP mRNA disappeared from the NL (5). This latter observation, together with the detection of AVP mRNA at the electron microscopic level in a subset of axonal swellings in the median eminence and NL (6), has led investigators to propose that the NL AVP mRNA is derived from the hypothalamic magnocellular neurons and is axonally transported to the NL (5, 6). Furthermore, the poly(A) tail of this AVP mRNA appears to be truncated during axonal transport (7). The existing data, however, do not fully exclude the possibility that some of the NL AVP mRNA is expressed in pituicytes.

In this study, we have carried out *in situ* hybridization histochemistry in combination with immunocytochemistry, using a glial fibrillary acidic protein (GFAP) antibody as a marker for the pituicytes (8, 9) to examine the expression of AVP mRNA in these astrocytes in the NL. Studies were conducted in normal and pituitary stalk-transected animals, with and without osmotic stimulation. Immunoelectron microscopy was also carried out to determine the presence of AVP in the pituicytes of these animals.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats weighing 225–250 g and given access to food and water ad lib were used in these studies. For salt-loading experiments, animals were given 2% saline for the times indicated. An intermittent salt-loading protocol in which animals were given 2% saline for 18 h (1600–1000) and then water for 6 h each day was used for pituitary stalk-transected animals. Salt loading began 2 days after pituitary stalk transection and lasted 4–6 days. The volumes of saline and water ingested daily were monitored in all the animals. This paradigm provides a mild osmotic stimulation resulting in a 1.5- to 2-fold increase in hypothalamic AVP mRNA after 6 days of treatment (10). Pituitary stalk-transected and sham-operated animals were purchased from Zivic–Miller. Briefly, a 1.5-cm ventral midline incision was made with the caudal terminus at the level of the clavicle and the bone (i.e., cranium floor) was removed. The pituitary stalk was then transected through the dura with a fine stainless steel probe and the transection was visualized for completeness.

Tissue Preparation. Control and salt-loaded rats were sacrificed and, within the experimental groups, completeness of the stalk transection was confirmed visually. The neurointermediate lobes were rapidly removed for total RNA preparation. For *in situ* hybridization and immunocytochemistry, at the light microscopic level, pituitaries from different experimental rats were rapidly frozen. Serial sections (12 μm) were cut throughout the whole pituitary in coronal or horizontal planes on a cryostat, thaw-mounted onto gelatin-coated microscope slides, and stored at −70°C until further processing. For immunoelectron microscopy a different protocol was followed (see below).

Northern Blot Analysis. Total RNA was extracted from neurointermediate lobes by using the acid guanidinium thiocyanate/phenol/chloroform method (11). Samples were fractionated on 1.0% agarose/0.6 M formaldehyde gels along with RNA size standards (BRL). For RNase H analysis, RNA was

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Abbreviations: AVP, [arginine] vasopressin; GFAP, glial fibrillary acidic protein; SON, supraoptic nucleus; PVN, paraventricular nucleus; NL, neural lobe.

first annealed with $(dT)_{12-18}$ (BRL) and then digested with RNase H (BRL). Northern blotting was carried out exactly as previously described (12). VPSP, a 48-mer oligonucleotide representing base pairs 1–48 of the rat AVP cDNA (within the signal peptide sequence), that had been 3′ end-labeled by using [α-^{32}P]dATP and terminal deoxynucleotidyltransferase (13) was used as a probe. A 550-bp almost-full-length rat AVP cDNA (gift of J. Battey, National Institutes of Health), which was random primer labeled with [α-^{32}P]dCTP by using the Prime-IT II kit (Stratagene), was also used. After hybridization, the filter was washed several times in 2× SSC/0.1% SDS at 22°C, and then twice in either 1× SSC/0.1% SDS at 60°C for the oligonucleotide probe or 0.1× SSC for the cDNA probe. Filters were exposed to Kodak X-Omat film, and quantitation of the AVP mRNA band was carried out by image analysis of the autoradiograms as described by Birch *et al.* (12). Autoradiographs were normalized to the amount of 28S rRNA in each lane determined from photonegatives of the Northern gels obtained by using the same imaging system.

cRNA *in Situ* Hybridization Histochemistry. pGrVP (gift from W. S. Young, National Institute of Mental Health), a plasmid containing a 232-bp *Pst* I/*Dra* I fragment from rat AVP cDNA encoding the C terminus of proAVP, subcloned into pGEMZ, was used as a probe (14). This probe has no homology with other neuropeptide probes and shows no cross-hybridization with oxytocin mRNA (W. S. Young, personal communication). To synthesize ^{35}S-labeled antisense or sense cRNA probes, pGrVP was linearized with *Hin*dIII or *Eco*RI and then transcribed *in vitro* with T7 or T3 RNA polymerase in the presence of ^{35}S-labeled uridine 5′-[α-thio]triphosphate (DuPont/NEN). cRNA *in situ* hybridization histochemistry was carried out as previously described (15).

Immunocytochemistry. Immunocytochemistry at the light microscopic level for GFAP was performed by a biotin–streptavidin–horseradish peroxidase method as previously described (16). Briefly, tissue sections were fixed in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS), washed in PBS/3% normal goat serum/0.3% Triton X-100, and incubated in the rabbit antiserum to GFAP (1:5000; a gift from D. Dahl, University of Texas, Dallas) (17) overnight at 4°C. After several washes, the sections were treated with biotinylated goat antiserum to rabbit IgG (1:800, Vector Laboratories) for 2 h, followed by a 1-h incubation with the streptavidin-peroxidase conjugate (Vector Laboratories). The antigen–antibody complex was visualized by incubation with 3,3′-diaminobenzidine tetrahydrochloride.

Combination of Immunocytochemistry and *in Situ* Hybridization Histochemistry. For the combination of immunocytochemistry with *in situ* hybridization, diethyl pyrocarbonate (final concentration, 0.04%) and RNasin (180 units/ml, Promega) were added to antibody, normal serum, and avidin–biotin complex as described elsewhere (18). Chromogenic reagents contained RNasin only. After immunocytochemistry to detect GFAP, *in situ* hybridization histochemistry was performed on these tissue sections as described above.

Immunoelectron Microscopy. Seven pituitary stalk-transected rats were intermittently salt loaded (see above) immediately after surgery for 7 (n = 3) and 10 (n = 4) days. As controls sham-operated rats were kept for 7 (n = 3) and 10 (n = 3) days and given water to drink. After anesthesia with sodium pentobarbital (60 mg/kg, i.p.) the animals were perfused transcardially with 0.9% NaCl (for a short time) and then with 150 ml of freshly prepared 4% (wt/vol) paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4. The pituitaries were subsequently left in the skulls after removal of the brain for overnight immersion fixation at 4°C. After dissection, the pituitaries were transferred into 0.4% paraformaldehyde in a 0.1 M sodium cacodylate, pH 7.4. Thirty-micrometer-thick Vibratome sections of the neurointermediate lobe were collected and rinsed in 0.05 M Tris·HCl/0.9% NaCl, pH 7.6 (TBS). Pre-embedding immunostaining of the Vibratome sections for AVP (using monoclonal antibody D_7) was carried out exactly as described by Boersma *et al.* (19). Subsequently, ultrathin sections were cut for immunoelectron microscopy. As controls, a few sections were processed for light microscopic immunocytochemistry of AVP and GFAP as described by Boersma (20).

RESULTS

Characterization and Osmotic Regulation of NL AVP mRNA in Control Animals. Northern blot analysis of the hypothalamus using the VPSP or the AVP cDNA probe revealed a single band of AVP mRNA which migrated with an apparent size of 750 bases, while the neurointermediate lobe showed an AVP mRNA of 620 bases. After deadenylylation by RNase H digestion of the poly(A) tail, both the messages had a size of 580 bases (data not shown), similar to other reports (2–5).

The effect of osmotic stimulation on the expression of the 0.62-kb AVP mRNA in the NL was examined. Total RNA from neurointermediate lobes of control and experimental animals which were salt loaded for 2, 4, or 7 days were probed with VPSP on Northern blots (Fig. 1). Quantitation of the autoradiographic signal showed 2-, 5-, and 10-fold increases in AVP mRNA, 2, 4, and 7 days after salt loading, respectively. Furthermore, there was no change in the size of NL AVP mRNA [i.e., poly(A) tail length] with salt loading.

In situ hybridization histochemistry was carried out to localize the AVP mRNA in the neurointermediate lobe. When both the pro-AVP cRNA (Fig. 2) and the VPSP probes (data not shown) were used, labeling was seen only in the NL, not in the intermediate or anterior lobe of the pituitary (Fig. 2 *A* and *B*). With 7 days of salt loading, there was a major increase of labeling in the NL (Fig. 2*B*). Examination of the pituicytes by immunocytochemistry, using anti-GFAP, showed a significant increase in the number of pituicytes in the NL after salt loading (compare Fig. 2 *C* and *D*).

To further determine if the NL AVP mRNA was present in pituicytes, colocalization studies using a combination of *in situ* hybridization histochemistry for AVP mRNA and immunocytochemistry with anti-GFAP were carried out on the pituitary of salt-loaded rats. Fig. 3 shows clusters of silver grains over the cell bodies of a subset of GFAP-immunoreactive pituicytes which have been counterstained with methyl green to reveal cell nuclei. In addition, there were also silver grains over areas which were not immunostained with anti-GFAP, most probably reflecting localization within nerve fibers (see *Discussion*).

Effect of Pituitary Stalk Transection on NL AVP mRNA. To determine whether the 0.62-kb AVP mRNA was locally synthesized in the pituicytes of the NL, the effect of pituitary stalk transection on the expression of this mRNA was examined. Complete stalk transection was confirmed by increased water intake by these animals. Stalk-transected animals ingested 276–334 ml/kg per day from the fourth day after surgery, while sham-operated animals ingested 134–165 ml/kg per day.

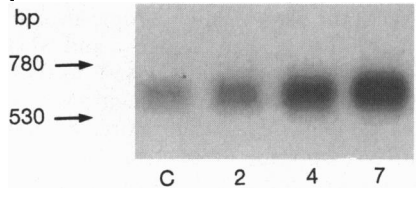

FIG. 1. Northern blot analysis of NL AVP mRNA in salt-loaded rats. Total RNA, 6 μg per lane from neurointermediate lobes of control animals (C) and animals that had been salt loaded for 2, 4, or 7 days, probed with VPSP.

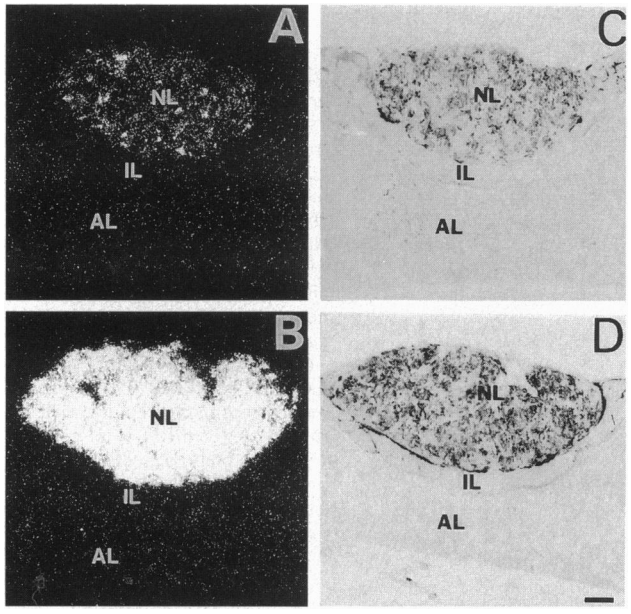

FIG. 2. (*A* and *B*) *In situ* hybridization histochemistry of pituitary sections, showing dark-field views of AVP mRNA expression in NL of control (*A*) and 7-day salt-loaded (*B*) rats. Note that the intermediate lobe (IL) and anterior lobe (AL) are not labeled. (*C* and *D*) GFAP immunoreactive pituicytes in NL of control (*C*) and 7-day salt-loaded (*D*) rats as shown by bright-field microscopy. (Bar = 125 μm.)

Lightman *et al.* (21) had reported that successful stalk transection is indicated by water intake of >200 ml/kg per day, and that is correlated with >95% decrease in NL AVP immunoreactivity relative to intact animals.

Northern blot analysis showed that 8 days after pituitary stalk transection, the 0.62-kb AVP mRNA had significantly decreased in the NL of rats given water to drink (compare lanes 1 and 2 in Fig. 4). However, after osmotic stimulation of stalk-transected animals by intermittent salt-loading for 6 days, the 0.62-kb AVP mRNA was increased in the NL (Fig. 4, lane 3) compared with the control (Fig. 4, lane 2). These results suggest the synthesis of the 0.62-kb AVP mRNA in pituicytes.

In situ hybridization histochemistry was carried out to examine the distribution of AVP mRNA in the NL of pituitary stalk-transected rats (Fig. 5). Six days after pituitary stalk transection, only a small number of silver grains were seen in

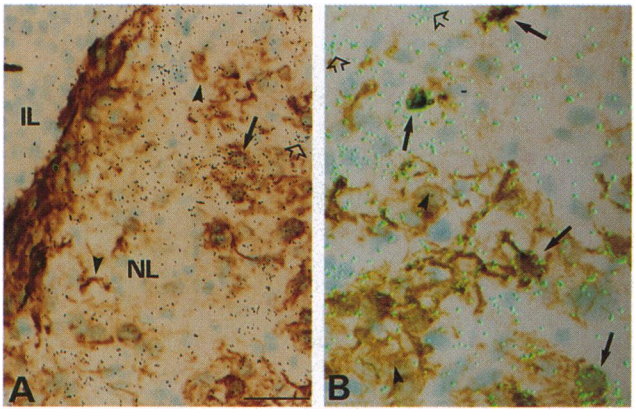

FIG. 3. *In situ* hybridization histochemistry combined with immunocytochemistry, showing bright-field (*A*) and bright-field/dark-field (*B*) views of AVP mRNA expression in a subset of GFAP-immunoreactive pituicytes (see arrows) in the NL of 7-day salt-loaded rats. Note that some GFAP-immunoreactive cells were not labeled for AVP mRNA (see arrowheads). AVP mRNA was also present in some areas not immunoreactive for anti-GFAP (open arrows). [Bar = 25 μm (*A*) and 20 μm (*B*).]

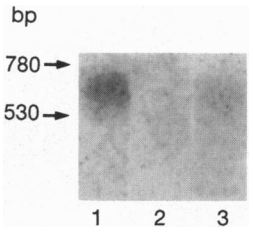

FIG. 4. Northern blot analysis of NL AVP mRNA in pituitary stalk-transected animals. Total RNA (6 μg per lane) from neurointermediate lobes of control sham-operated rats given water (lane 1); stalk-transected (8 days) rats given water (lane 2); and stalk-transected rats, intermittently salt loaded for 6 days, beginning 2 days after surgery (lane 3), probed with VPSP.

the NL of animals given water to drink (Fig. 5*B*), consistent with the Northern blot data in Fig. 4. With intermittent salt loading for 4 days, a larger number of silver grains were seen, most of which were associated specifically with cells in the NL (Fig. 5*A*), indicating localization of AVP mRNA in a subset of pituicytes. A semiquantitative analysis of silver grains distributed over the NL in random areas totaling 17,000 μm^2 for each condition (as described for Fig. 5 *A* and *B*) yielded the following results. With salt loading, 1110 grains were present, of which 827 (75% of total) were directly over 147 of the 199 cell nuclei counted. In the control, only 322 grains were present in an equivalent area, of which 196 (61% of total) were over 39 of 199 nuclei counted. The numbers of grains per nucleus were similar, but there were 3.8-fold (147 vs. 39) more pituicytes induced to synthesize AVP mRNA with osmotic stimulation compared with unstimulated animals. Immunostaining with anti-GFAP showed normal morphology of the pituicytes (Fig. 5 *C* and *D*), although the NLs of these stalk-transected animals were significantly reduced in size compared with intact rats, due to the degeneration of the nerve terminals (see Fig. 6). There was an increase in the number of pituicytes in the NL of pituitary stalk-transected animals (Fig. 5*C*) that were intermittently salt loaded, compared with those given water (Fig. 5*D*). A control using the sense cRNA probe on the NL of the pituitary stalk-transected 4-day salt-loaded rats showed only background labeling (Fig. 5 *E* and *F*).

Immunocytochemistry of NLs of the pituitary stalk-transected/intermittently salt-loaded rats (7 and 10 days) at the light microscopic level (data not shown) showed that AVP immunoreactivity was still present, although the amount was considerably reduced in the stalk-transected salt-loaded group compared with the sham-operated animals given water to drink. The GFAP immunoreactivity was prominent in these groups. Electron microscopic analysis of the NL of the pituitary stalk-transected/intermittently salt-loaded animals (7 and 10 days) showed the AVP immunoreactivity in a subpopulation of pituicytes (Fig. 6).

DISCUSSION

Reports of AVP mRNA in the NL (2–5) prompted studies to determine whether the transcripts are axonally transported from the magnocellular neuron cell bodies to the terminals or locally synthesized in the NL pituicytes. Evidence supporting the axonal transport hypothesis includes presence of AVP mRNA in axons and terminal swellings in the NL as determined by electron microscopic *in situ* hybridization (6), absence of AVP mRNA transcripts in the NL after interruption of the hypothalamoneurohypophysial tracts (5) or colchicine treatment (23), and lack of detection of RNA intron sequences of the AVP gene in the NL (7). Furthermore, our study showing the significant reduction of AVP mRNA in the NL after pituitary stalk transection in rats drinking water suggests that, under normal physiological conditions in intact animals,

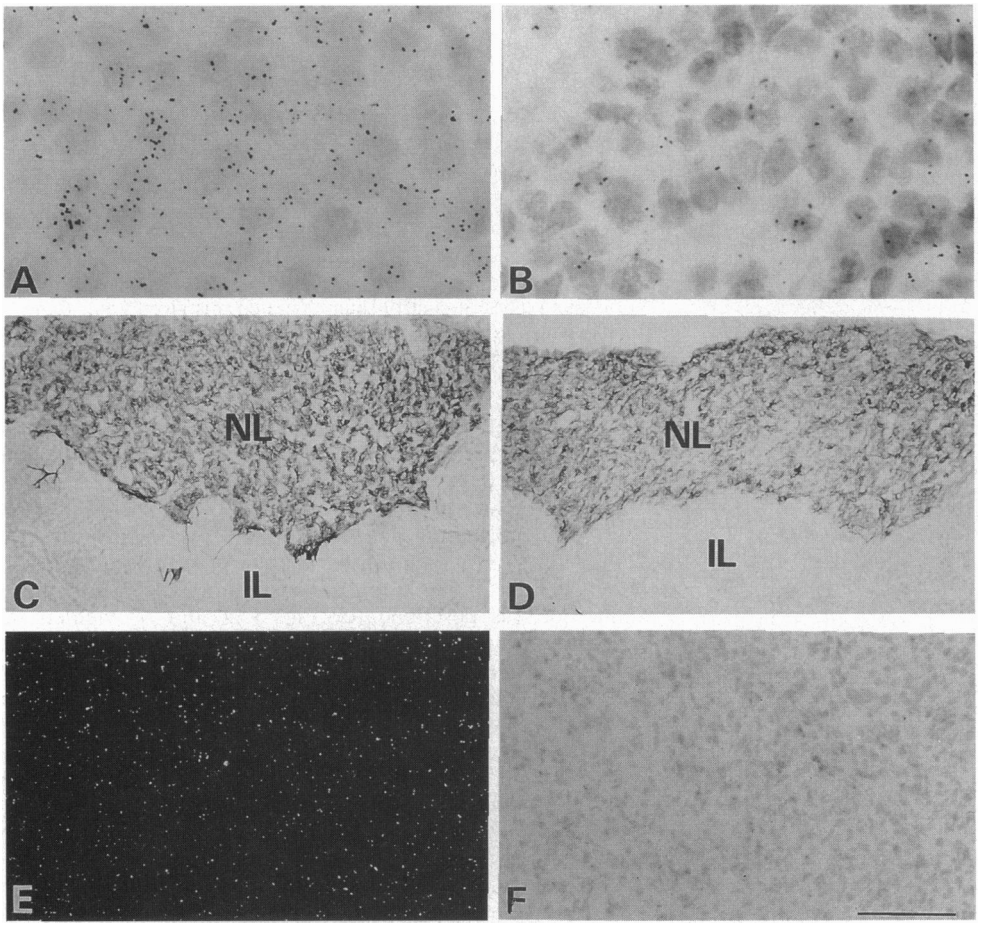

FIG. 5. (*A* and *B*) *In situ* hybridization histochemistry showing bright-field views of AVP mRNA expression in NLs of stalk-transected rats that were intermittently salt loaded for 4 days, beginning 2 days after surgery (*A*), or given water to drink for 6 days after surgery (*B*). Photomicrographs shown are representative of three animals for each experiment. In *B* note the distribution of silver grains over a number of the cells. The few clusters of label not apparently associated with a visible methyl green-stained nucleus likely reflect AVP mRNA in cells in which the plane of section did not, or only partially, hit the nucleus, since NL axon terminals have degenerated and are devoid of AVP in these animals (see Fig. 6). (*C* and *D*) GFAP-immunoreactive pituicytes in NL of pituitary stalk-transected rats that were intermittently salt loaded (*C*) or given water to drink (*D*) as described for *A* and *B*. (*E* and *F*) Dark-field (*E*) and bright-field (*F*) views of NL sections from pituitary stalk-transected, intermittently salt-loaded rats, as described for *A* and *B*, probed with an AVP sense cRNA. Note the lack of labeling in this control experiment. [Bar = 20 μm (*A* and *B*), 250 μm (*C* and *D*), or 50 μm (*E* and *F*).]

most of the AVP mRNAs in the NL are transported from the magnocellular neuron cell bodies. Presence of AVP mRNA primarily in axons of salt-loaded rats (6) indicates that, under osmotic stimulation conditions, the major source of AVP mRNA in the NL also was from the hypothalamus. While axon terminals cannot be discriminated from other cellular structures (e.g., processes of fibrous pituicytes) at the light microscopic level, in our study the presence of clusters of silver grains (AVP mRNA) over areas of the NL not immunostained with anti-GFAP in salt-loaded intact rats (Fig. 3) is consistent with the presence of AVP mRNA in terminals. This interpretation is further supported by the observation of only background silver grains between the cells (Fig. 5*A*) in the NL of pituitary stalk-transected/salt-loaded animals, where the severed axon terminals have degenerated.

There are, however, data suggesting that AVP synthesis can occur in pituicytes. Immunoelectron microscopic studies have detected AVP in a small number of pituicytes in rat NL, although the possibility that it may have originated from nerve fibers cannot be totally excluded (19, 20). In addition, the production of AVP by pituicytes in culture has been reported (24). In the present study, we provide evidence for the synthesis of AVP mRNA and AVP immunoreactivity in a subset of pituicytes after osmotic stimulation. (*i*) *In situ* hybridization histochemistry combined with immunocytochemistry using anti-GFAP has localized AVP mRNA in a significant number of GFAP-immunoreactive pituicytes in the NL of salt-loaded rats. Under these circumstances, enclosure of axons by pituicytes is minimal (25). (*ii*) AVP mRNAs were increased in the NL of pituitary stalk-transected rats that were intermittently salt-loaded for 6 days, compared with those given water; the size of the NL AVP mRNA in pituitary stalk-transected animals was 0.62 kb, similar to the poly(A)-truncated, axonally transported AVP mRNA (Fig. 4). (*iii*) *In situ* hybridization histochemistry showed the distribution of the NL AVP mRNA specifically over cells in pituitary stalk-transected animals (Fig. 5). Moreover, after salt loading there was proliferation of pituicytes in the NL of these animals, and a greater number (3.8-fold more) of these cells expressed AVP mRNA compared to those given water to drink (Figs. 4 and 5). (*iv*) Immunoelectron microscopy of the NL of salt-loaded rats showed the presence of AVP immunoreactivity in pituicytes 7 and 10 days after pituitary stalk transection, when essentially all the axon terminals had degenerated (Fig. 6 and ref. 22), indicating that the 0.62-kb mRNA in pituicytes of stalk-transected animals was translated. This is also consistent with a report that AVP mRNA with a short or absent poly(A) tail is translated by magnocellular neurons (26). These data indicate that AVP mRNA is synthesized locally in pituicytes and is translated and up-regulated in response to osmotic stimulation, although it is not the major source of AVP mRNA in the NL.

AVP mRNA has not been detected in pituicytes by *in situ* hybridization at the electron microscopic level (6). This may be due to the relatively low sensitivity of the technique, since there is less AVP mRNA in these cells than in axons, and detection in axons was possible only after salt loading. Likewise, the lack of detection of intron-containing AVP mRNA in the NL reported by Mohr *et al.* (7) may be due to the overwhelming abundance of axonally transported message present after salt loading. They also reported no increase in NL AVP mRNA with salt loading after electrical deafferentation of SON and PVN neurons. This difference between our results and theirs may be due to the method of causing lesions in the hypothalamoneurohypophysial tract. In our surgical pituitary stalk-transection procedure, the neurosecretory fibers of the PVN and SON neurons are physically transected at the level of the median eminence, closer to the pituitary, resulting in

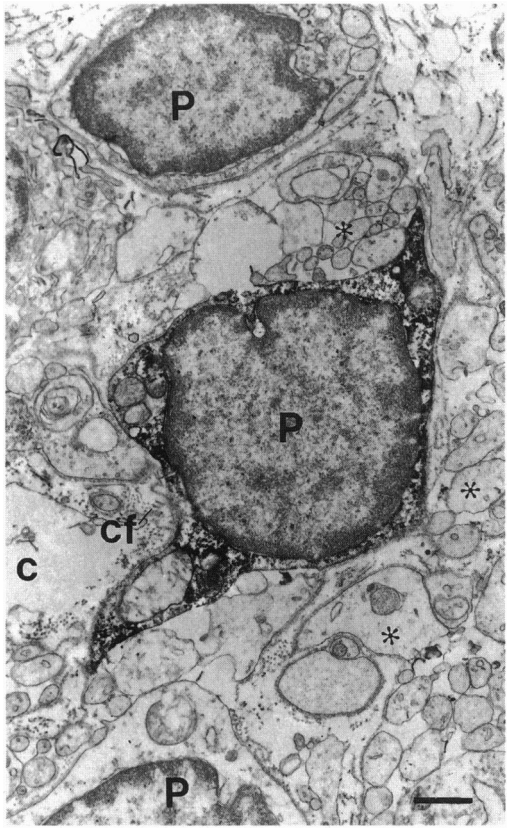

FIG. 6. Ultrathin sections of the NL of a pituitary stalk-transected (for 7 days) intermittently salt-loaded rat. Note the degenerated empty nerve fibers (*) and enlarged extracellular spaces. Collagen fibers (cf) in the perivascular space are present near a capillary (c). Pituicytes (P) can be seen, a subpopulation of which is immunoreactive for AVP. After pituitary stalk sectioning the lipid droplets within the pituicytes gradually disappear as reported before (22). (Bar = 1 μm.)

alterations in AVP biosynthesis (27) and complete degeneration of the severed nerve terminals in the NL by 6 days after transection (22). In contrast, the electrical procedure carried out by Mohr *et al.* (5) produces diabetes insipidus by ablating an area within the ventromedial hypothalamus to interrupt the hypothalamoneurohypophysial tracts closer to the PVN/SON cell bodies, leaving the pituitary stalk intact (28, 29). While the pituicytes showed normal morphology and proliferated with salt loading after surgical stalk transection (Fig. 5 *C* and *D*), similar to intact animals (Fig. 2 *C* and *D*), histological analyses of the NL after electrical damage were not reported (5). The reported diuresis might also be caused by other mechanisms (e.g., inhibition of synthesis of AVP by magnocellular neurons within the SON and PVN, stimulation of drinking behavior by lesions in the periventricular area). Pituicytes may well respond differently to osmotic stimulation under different microenvironments resulting from different sites of lesions of the hypothalamoneurohypophysial tract.

The activation of AVP mRNA and AVP synthesis in pituicytes may be due to osmosensitivity of these cells (30), or the stimulus may come from blood-borne factors secreted by other nerve cells in response to salt loading. Proenkephalin, prosomatostatin, and proangiotensinogen mRNAs and enkephalin peptides have recently been shown to be present in brain astrocytes, *in vivo* and in culture, upon various stimulations (31–34). Such findings, together with the present study demonstrating the activation of pituicytes to synthesize AVP mRNA and AVP upon osmotic stimulation, suggest that glial cell-derived neuropeptides or their mRNAs may play an important physiological role in cellular communication within the nervous system.

We thank Drs. M. Brightman and D. Cool for helpful discussions and critical reading of this manuscript, Mr. G. vander Meulen for the photography, and Ms. M. S. Mood for typing.

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