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Aggregation of Vasopressin mRNA in a Subset of Axonal Swellings of the Median Eminence and Posterior Pituitary: Light and Electron Microscopic Evidence

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The mRNA encoding vasopressin has recently been documented within the magnocellular hypothalamo-neurohypophyseal projections of the rat such as the median eminence (ME) and the posterior pituitary (PP), suggesting the possibility of its axonal transport. To address the origin of this mRNA and to investigate the functional significance of this unexpected axonal transport of mRNA, we have examined its subcellular localization within both magnocellular perikarya and their axonal projections. For this purpose, we have used nonradioactive *in situ* hybridization techniques in order to localize the vasopressin mRNA with precision at the ultrastructural level in magnocellular perikarya, dendrites, and axons from control, salt-loaded, and lactating rats. This approach permitted us to demonstrate directly the axonal localization of vasopressin mRNA. Moreover, we were able to obtain novel information concerning vasopressin mRNA compartmentation within both perikarya and axons.

At both light and electron microscopic levels, we observed vasopressin mRNA–containing cells in the hypothalamic magnocellular cell body groups, but not in the ME or in the PP. When vasopressin mRNA was detected in medium-size dendrites, it was always associated with the rough endoplasmic reticulum (RER). Within the labeled magnocellular perikarya, the abundant vasopressin mRNA was mainly associated with discrete areas of the RER. However, vasopressin mRNA was never detected in the Golgi apparatus or in association with neurosecretory granules, in perikarya or axons. These data suggest that vasopressin mRNA translation is restricted to certain segments within the RER, and that axonal transport of vasopressin mRNA does not involve the classical neurosecretory pathway, via the Golgi apparatus and the neurosecretory granules, as has been proposed.

Within the magnocellular neuron axons, vasopressin mRNA could be detected only in a subset of axonal swellings, all of which were confined to the internal layer of the ME and the PP. The mRNA–containing swellings were numerous in 7 d salt-loaded animals, less abundant in lactating animals, and almost undetectable in control animals. In all groups of animals, no vasopressin mRNA was detectable in any other region of the magnocellular neuron axons, including undilated axonal segments or varicose swellings. These results strongly suggest that, under physiological activation such as chronic salt loading, axonal vasopressin mRNA is increased and becomes aggregated in a selected subset of swellings of the ME and the PP. Furthermore, these data indicate that along the magnocellular neuron axons, the swellings may differ in their biochemical and functional features. Further analysis focused on the mRNA-accumulating swellings may illuminate the function of RNA within the axonal compartment.

[Key words: vasopressin mRNA, neuropeptide, electron microscopy, axonal transport of mRNA, in situ hybridization, salt loading, lactation, median eminence, pituitary]

The main molecular steps of neuropeptide gene expression (e.g., transcription into mRNA, maturation of mRNA, translation into the prohormone, and posttranslational peptide processing) have been well documented using biochemical approaches (for review, see Sherman et al., 1989). It has been demonstrated that neuropeptide-encoding mRNAs are synthesized and processed in the cell nucleus, transferred into the cytoplasm, and transported to the rough endoplasmic reticulum (RER), where they are translated. Thus, consistent with this view, neuropeptide-encoding mRNAs would be confined within the neuronal perikarya. However, using morphological approaches such as nonradioactive *in situ* hybridization, several neuropeptide-encoding mRNAs have been detected in neuronal processes of various central peptidergic neurons beyond the perikaryon (Bloch et al., 1990). Moreover, the well-characterized rat hypothalamo-neurohypophyseal system has provided additional departures from expectations. This neuronal system is composed of vasopressin- and oxytocin-producing neurons located in hypothalamic nuclei and projecting via the median eminence (ME) to the posterior lobe of the pituitary (PP). Over the past 4 years, several groups have reported biochemical detection of various RNAs including oxytocin and vasopressin mRNAs in the PP and ME (Murphy et al., 1989; Jirikowski et al., 1990; Lehman et al., 1990; Levy et al., 1990; McCabe et al., 1990; Mohr et al., 1990, 1991; Mohr and Richter, 1992).

The presence of vasopressin and oxytocin mRNA in the PP and ME was initially surprising because these structures contain no neuropeptide-producing neurons. Subsequently, Jirikowski et al. (1990) demonstrated that oxytocin mRNA was of axonal origin by direct *in situ* detection. However, the presence of va-

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sopressin mRNA in axons has previously only been inferred by indirect evidence obtained using polymerase chain reaction, Northern blotting, and radioactive *in situ* hybridization (Murphy et al., 1989; Lehman et al., 1990; Mohr et al., 1991), as well as by light microscopic evidence of the uptake and expression of exogenous vasopressin mRNA injected in the lateral hypothalamus of the vasopressin-deficient Brattleboro rat (Jirikowski et al., 1992; Maciejewski-Lenoir et al., 1993).

Since direct evidence for the presence of vasopressin mRNA in axons of normal rats was lacking, we have attempted in this study to analyze the subcellular compartmentalization of vasopressin-encoding mRNA using nonradioactive *in situ* hybridization and electron microscopy techniques. Although light microscopic *in situ* hybridization on neuronal systems using both radioactive and nonradioactive probes has been widely applied, there have been few extensions to the electron microscopic level (Trembleau et al., 1988, 1990; Guitteny and Bloch, 1989; Jirikowski et al., 1990) and limitations exist in terms of detection sensitivity, resolution, and cytological preservation. Techniques using *in situ* hybridization with a radiolabeled probe and autoradiography at the electron microscopic level (Trembleau et al., 1988, 1990; Guitteny and Bloch et al., 1989) present major disadvantages including long time exposures and poor resolution, though this approach allows an optimal morphology. A recent alternative approach using biotinylated probes (Trembleau et al., 1991; Trembleau, 1993) was similarly limited by its sensitivity and the lack of preserved morphology.

Thus, we developed new *in situ* hybridization techniques (Trembleau et al., 1992) that are sufficiently sensitive to detect vasopressin mRNA and compatible with morphological preservation. Using these approaches, we were able to define the subcellular compartmentation of vasopressin mRNA within the magnocellular perikarya and their axons. Our data are discussed in relation to the mechanisms and functional significance of the axonal transport of this mRNA.

Materials and Methods

Animals and tissue preparation. Adult Sprague–Dawley rats (200–350 gm) were used. Control animals were fed with standard rat chow and water ad libitum. Some male rats were osmotically stimulated, providing 2% NaCl as the exclusive water source for 6, 24, 48, and 72 hr and 7 d ("salt-loaded animals"). Lactating female rats (2–17 d lactation) were also studied. Animals were anesthetized using chloral hydrate (300 mg/kg, i.p.), perfused through the ascending aorta with 30 ml of sterile saline solution (0.9% NaCl, warmed at 37°C), followed by an ice-cooled fixative solution (100 ml/100 gm body weight), containing 4% paraformaldehyde and 0.05–0.1% glutaraladehyde in 0.1 M sterile phosphate buffer (PB). The brain was dissected and postfixed in 4% paraformaldehyde in PB (2 hr, 4°C), rinsed in sterile phosphate-buffered saline (PBS), and immersed overnight at 4°C in 15% sucrose. For the experiments performed on vibratome sections, 50 μm coronal sections of the hypothalamus and 100 μm sections of the pituitary were transferred into sterile PBS. After three washes in PBS, 3 min each, the tissue was permeabilized either by 1 hr of incubation in PBS containing 0.1% Triton X-100, or using the following freeze–thaw protocol. Vibratome sections were immersed in sterile Eppendorf tubes containing a cryoprotective buffer (10% glycerol and 25% sucrose in sterile PBS) for at least 2 hr at 4°C. The samples were then frozen by dropping the tubes into liquid nitrogen. The tubes were rapidly removed from the liquid nitrogen and brought to room temperature. Finally, vibratome sections were transferred to sterile culture dishes containing PBS, and rinsed in three baths of PBS (10 min each). In the experiments in which the double detection of vasopressin peptide and mRNA was performed, cryostat sections were used. In this case, after the overnight incubation of the fixed brains in sucrose, they were frozen in liquid nitrogen vapors. Then, 16 μm sections were obtained on a cryostat, rinsed in sterile PBS, and processed for *in situ* hybridization and immunohistochemistry.

Probes. Synthetic oligonucleotide probes were used in this study. The sensitivity of the methods was improved by use of multiple nonoverlapping oligonucleotide probes. Four oligonucleotides were used for detection of vasopressin mRNA: VP1 (nucleotides 1024–1059), VP2 (nucleotides 985–1014), VP3 (nucleotides 942–971), VP4 (nucleotides 904–936). These oligonucleotides were designed from the vasopressin mRNA sequence (Ivell and Richter, 1984), in regions where homologies between vasopressin and oxytocin mRNAs are very low. In some control experiments, a 37-mer oligonucleotide specific for oxytocin mRNA, OX3′B (Jirikowski et al., 1990), and a 30-mer oligonucleotide specific for pro-opiomelanocortin (POMC) mRNA (Boehringer-Mannheim, Mannheim, Germany; for reference, see Larsson et al., 1988) were used. These oligonucleotides were labeled by tailing the 3′ end using terminal transferase (Boehringer-Mannheim). Two labeled deoxynucleotides were used in this study, either digoxigenin-11-dUTP (Boehringer-Mannheim) or biotin-7-dUTP (Boehringer-Mannheim). The digoxigenin labeling reaction was performed using a protocol previously described (Trembleau et al., 1993). Briefly, 100 pmol of oligonucleotide, 1 nmol of digoxigenin-11-dUTP, 9 nmol of dATP, 55 units of terminal transferase, 4 μl of 5× terminal transferase labeling buffer (Boehringer-Mannheim), and 2 μl of 10× $CoCl_2$ (Boehringer-Mannheim) were incubated at 37°C for 45 min in a final volume of 20 μl before ethanol precipitation as previously described (Schmitz et al., 1991) and stored at −20°C. The biotin labeling was performed identically except that 4.5 nmol of biotin-7-dUPT and 4.5 nmol dATP were used.

In situ *hybridization.* The prehybridization step was carried out by incubating the floating sections in 4× SSC (1× SSC:0.15 M NaCl, 0.015 M trisodium citrate) buffer containing 1× Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% ficoll) and 10 μg/ml wheat germ tRNA for 1 hr at 37°C. Thereafter, the sections were immersed overnight at 37°C in the hybridization buffer modified from Young (1989) (50% formamide, 600 mM NaCl, 80 mM Tris-HCl, pH 7.5, 4 mM EDTA, 0.1% sodium pyrophosphate, 10 μg/ml wheat germ tRNA) containing 10 nM concentration of the nonradioactive probe. In some experiments, several probes were mixed in the hybridization buffer. The final concentration of each probe was 10 nM. Following the hybridization step, the sections were washed three times in 2× SSC (30 min each) and in three baths of 0.1× SSC (45 min each) at 37°C, and then immersed in PBS.

Immunohistochemical detection of digoxigenin and biotin for electron microscopic studies. The immunocytochemical detection of digoxigenin-labeled probes was performed as follows. The sections were first preincubated in PBS containing 1% BSA (PBS-BSA) (1 hr at room temperature) and then incubated overnight at 4°C with a sheep anti-digoxigenin antibody (Boehringer-Mannheim) diluted (1:1000) in PBS-BSA. After three washes in PBS (10 min each), the sections were incubated with a biotinylated anti-sheep antibody (Vector) (1:200 in PBS-BSA, 2 hr, room temperature) and an avidin-biotin-peroxidase complex (ABC, Vector) diluted in PBS, according to the provided instructions (2 hr, room temperature). These two latter steps were followed by three rinses in PBS (10 min each). Finally, peroxidase activity was detected by incubating the sections in 50 mM Tris buffer (pH 7.6) containing 0.025% 3,3′-diaminobenzidine tetrachloride (DAB) and 0.006% hydrogen peroxide. In some experiments, nickel chloride was added in the DAB (DAB-Ni) (32 mg/100 ml) to intensify the final reaction product. The reaction was monitored under a light microscope and stopped by transfer into PBS.

Biotinylated probes were detected using an immunogold–silver enhancement–gold toning protocol. The sections were first preincubated in PBS containing 1% BSA (PBS-BSA) (1 hr at room temperature) and then incubated overnight at 4°C with a rabbit anti-biotin antibody (Enzo, New York, NY) (1:1000 in PBS-BSA). After three washes in PBS, the sections were incubated in PBS containing 0.8% BSA and 0.1% gelatin (PBS-BSA-G) for 1 hr at room temperature. Then, the sections were incubated in the same buffer with a 1 nm gold–labeled goat anti-rabbit (Amersham) (1:50) for 24 hr at 4°C. The sections were washed three times in PBS-BSA-G (15 min each) and three times in PBS (10 min each), and then in 2% sodium acetate buffer (SAB) (3 min each). The silver enhancement was performed using the IntenSE kit (Amersham) according to the provided instructions. Sections were then washed three times in SAB (5 min each), immersed first in 0.05% gold chloride diluted in water (10 min at 4°C), then in 0.3% sodium thiosulfate diluted in water (twice, 10 min each at 4°C), and, finally, rinsed several times in PBS.

Following the immunodetection of digoxigenin or biotin, sections were examined by light microscopy, before being postfixed with osmium

tetroxide (1% in PB, 30 min) and embedded in TAAB resin. Semithin sections were prepared and observed with phase-contrast microscopy. Ultrathin sections were contrasted with uranyl acetate and lead citrate before being observed on a Zeiss electron microscope.

Colocalization of vasopressin mRNA and peptide by double immunofluorescence detection. Cryostat sections were prehybridized and hybridized with the four digoxigenin-labeled oligonucleotides at a final concentration of 3 nM for each probe, as described above. Following the posthybridization washes, the sections were incubated overnight at 4°C in buffer A (0.1 M Tris, pH 7.5, 1 M NaCl, 2 mM $MgCl_2$) containing an alkaline phosphatase–labeled anti-digoxigenin Fab fragment (Boehringer-Mannheim; 1:5000) and a monoclonal mouse anti-vasopressin antibody (1:1000; Burlet et al., 1991). Then, the sections were rinsed three times in buffer A (10 min each), incubated for 2 hr at room temperature with a fluorescein-labeled anti-mouse antibody (Jackson; 1:100 in buffer A), and followed by three rinses in buffer A (10 min each). Finally, after the alkaline phosphatase activity was revealed using a fast red TR/Naphthol AS-MX kit (Sigma), the sections were mounted on slides and observed under a Zeiss Axiophot microscope.

Results

Light microscopy

Pattern of localization of vasopressin mRNA

Perikarya. When using vasopressin oligonucleotides, regardless of experimental category of animal studied (control, salt-loaded, or lactating animals), the nonradioactive *in situ* hybridization protocols readily labeled magnocellular neurons in the paraventricular nucleus (PVN) and in the supraoptic nucleus (SON) (Figs. 1*A,E*; 2*A*; see also Fig. 4*A*). Many other positive magnocellular neurons were also scattered throughout the hypothalamus. A significant but weaker labeling was also observed in parvocellular neurons located in the dorsomedial part of the suprachiasmatic nucleus (Fig. 1*B*). These results were consistent across all techniques used here. The use of one digoxigenin-labeled oligonucleotide detected with the ABC-Elite-DAB-Ni technique (Fig. 2*A*) or of a mixture of four digoxigenin-labeled oligonucleotides detected with the ABC-DAB technique (Fig. 1*A*) led to similar patterns and intensity of label. The use of a mixture of four biotin-labeled vasopressin oligonucleotides detected with the immunogold technique also gave similar results (Fig. 1*E*). Within the positive magnocellular cell bodies, the labeling was distributed in the cytoplasm only, and no labeling was observed in the nucleus (Figs. 1*A*, 2*A*; see also Fig. 4*A*). Within the cytoplasm, the labeling was more abundant in discrete areas located more often in the periphery of the cell (see Fig. 4*A*). Perinuclear and other cytoplasmic areas were clearly and consistently devoid of labeling. In some cases, labeled processes, presumably dendrites, were also observed (Fig. 1*A*).

Axons. In salt-loaded animals, numerous labeled swellings were observed in the internal layer of the ME (Figs. 1*F,G*; 2*C*) and in the PP (Fig. 1*C,H,I*). These structures, 3–5 μm in diameter, are termed swellings according to the terminology of Morris (1976), who defined them in ultrastructural analyses as axonal dilatations that, by opposition to terminals, are only rarely observed in contact with the basal membrane and contain few or no microvesicles (synaptic vesicles). In 3 d as well as 17 d lactating animals, a few positively labeled swellings by *in situ* hybridization (positive swellings) were also observed in the same areas (Fig. 2*F*). In control animals, the labeling was close to the background levels, and only rare swellings were labeled in the ME or in the PP (Fig. 2*E*). The positively labeled swellings could be observed with either digoxigenin or biotin-labeled oligonucleotides. However, the signal-to-noise ratio in the ME and PP was higher with the biotin-immunogold and with the digoxigenin-ABC-DAB techniques, compared to the digoxigenin-ABC-Elite-DAB-Ni technique, which gave more background, especially in Triton-treated pituitaries. No labeling was observed in the external layer of the ME or in the anterior or intermediate lobes of the pituitary.

In all the animals studied (salt-loaded, lactating, or control animals), no undilated axons or other swelling was labeled in any other areas of the hypothalamo-hypophyseal system, such as the lateral border of the PVN, the lateral hypothalamus between the PVN and SON, or along the retrochiasmatic part of the SON.

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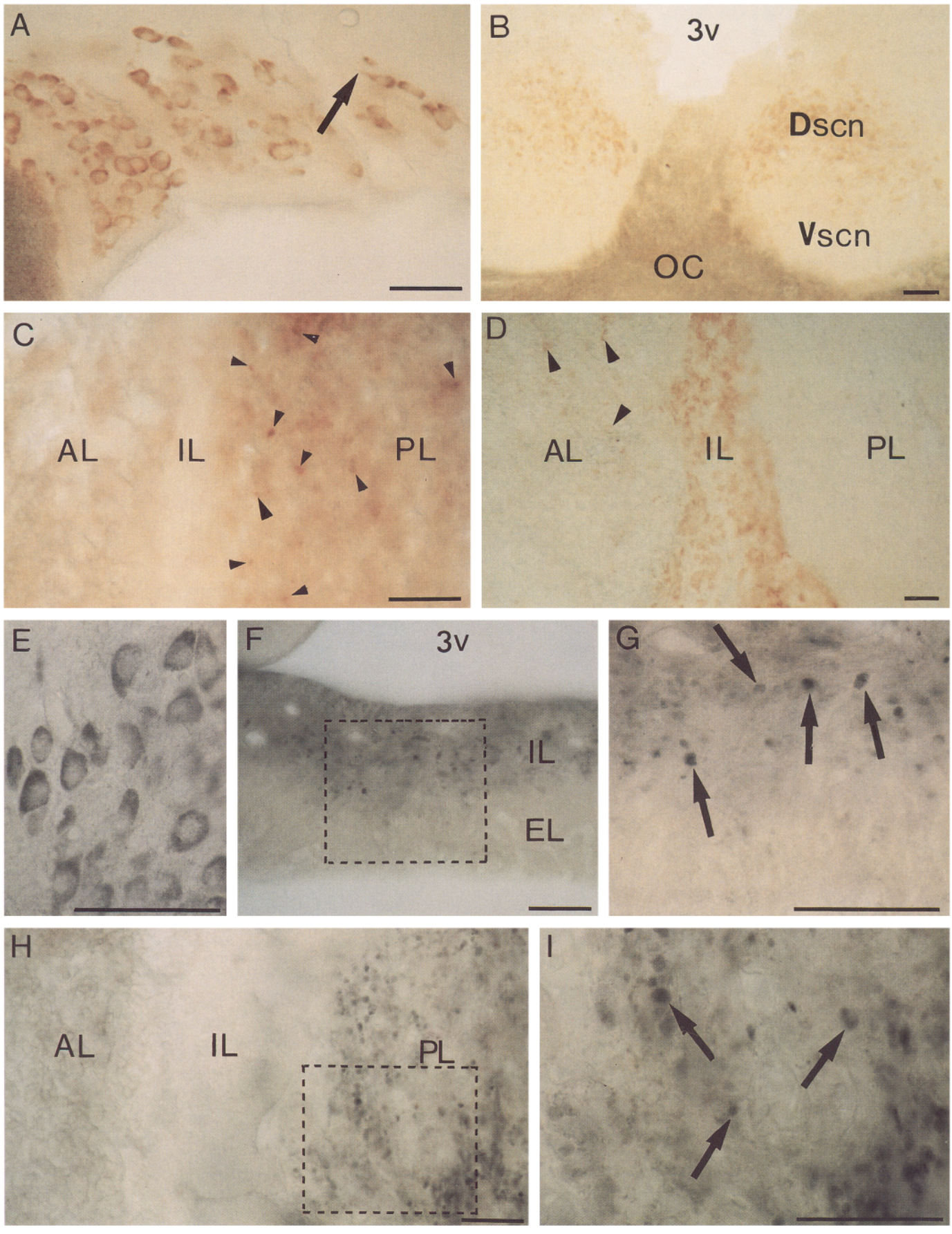
Figure 1. Light microscopic detection of vasopressin mRNA in hypothalamic nuclei, median eminence, and posterior lobe. *A* and *B*, Detection of vasopressin mRNA in the supraoptic (*SON*) (*A*) and suprachiasmatic (*SCN*) (*B*) nuclei of a control animal. The 50 μm vibratome sections were hybridized with a mixture of four digoxigenin-labeled oligonucleotides (VP1–VP4) complementary to vasopressin mRNA. Digoxigenin was detected using the ABC peroxidase-DAB technique. The vibratome section was permeabilized using Triton. The specificity of the labeling is demonstrated by the pattern of distribution of the positive cells within these nuclei (see, e.g., the dorsomedian localization of the labeled cells in the SCN). The labeling of suprachiasmatic parvocellular neurons, which contain low levels of vasopressin mRNA, demonstrates the high sensitivity of the method. Some magnocellular neurons display labeled processes (*A, arrow*). *OC,* optic chiasma; *Dscn,* dorsal part of the SCN; *Vscn,* ventral part of the SCN; *3v,* third ventricle. *C* and *D,* Two 100 μm pituitary sections from a 7 d salt-loaded animal, permeabilized with Triton, were hybridized with four digoxigenin-labeled oligonucleotides (VP1–VP4) (*C*) or a digoxigenin-labeled oligonucleotide complementary to POMC mRNA (*D*). The total concentration of probes was the same for both sections. The vasopressin probes label some axonal swellings in the PP (*C, arrowheads*). However, the POMC probe instead labels many cells in the intermediate lobe, a few cells in the anterior lobe (*D, arrowheads*), but does not label any specific structures in the PP. This control demonstrates that the swellings labeled by the vasopressin probes are likely to be due to specific hybridization. *PL,* posterior lobe; *IL,* intermediate lobe; *AL,* anterior lobe. *E–I,* Localization of vasopressin mRNA in the SON (*E*), ME (*F, G*), and pituitary (*H, I*) of a 7 d salt-loaded animal using a mixture of four biotinylated oligonucleotides (VP1–VP4). Biotin was detected using the immunogold–silver enhancement method. A significant labeling is observed in magnocellular neurons of the SON (*E*), as well as in swellings of the ME (*F, G*) and PP (*H, I*). *Arrows* show some of the positive swellings. *G* and *I* are higher magnifications of *F* and *H,* respectively. The permeabilization was performed using Triton. *IL,* internal layer of the median eminence; *EL,* external layer of the median eminence. Abbreviations for *H* are the same as those for *D.* Scale bars, 50 μm.

Figure 2. Detection of vasopressin mRNA (*A, C, E, F*) and oxytocin mRNA (*B, D*) in the SON (*A, B*) and ME (*C–F*) of either a 7 d salt-loaded animal (*A–D*), a control animal (*E*), or a 17 d lactating animal (*F*). The hybridization was performed using either VP1 probe (*A, C, E, F*) or an oligonucleotide specific for oxytocin mRNA (*B, D*) labeled with digoxigenin. Digoxigenin was detected using the Elite-ABC technique, revealed with DAB-Ni. The permeabilization was performed using the freeze–thaw method. In the SON vasopressin mRNA–expressing neurons are abundant in the ventral part (*A*) while oxytocin mRNA–expressing neurons are mainly located in the dorsal part (*B*). In the ME of salt-loaded animals, numerous axonal swellings located in the internal layer contain vasopressin mRNA (*C, arrows*). In contrast, the oxytocin probe does not label such structures in the ME (*D*). In the control animal (*E*), little or no significant labeling is detected in the internal layer of the ME. In the lactating animal (*F*), a few significantly labeled swellings are observed in the internal layer of the ME (*arrows*). *OC,* optic chiasma; *3v,* third ventricle. Scale bars, 50 μm.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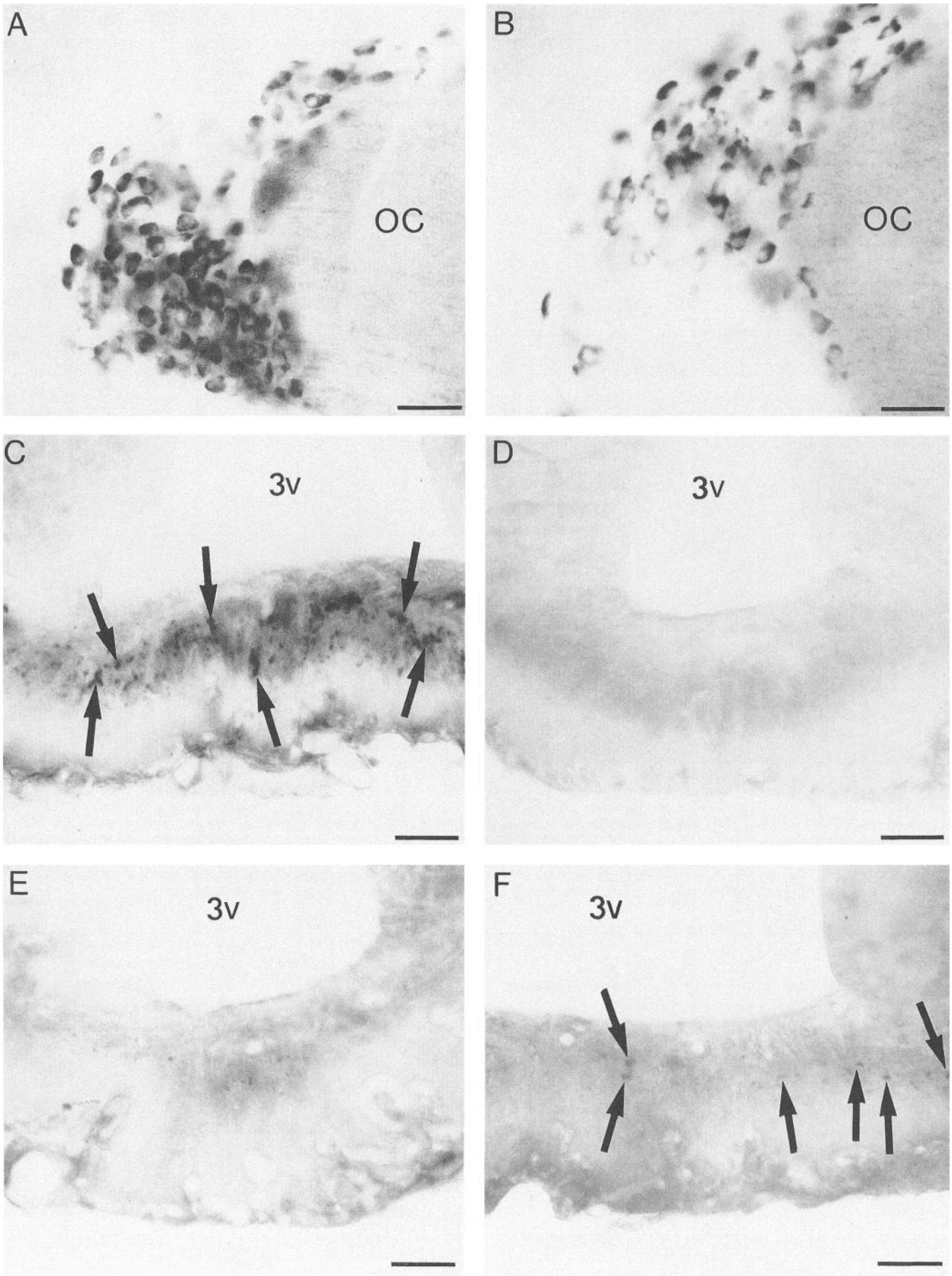
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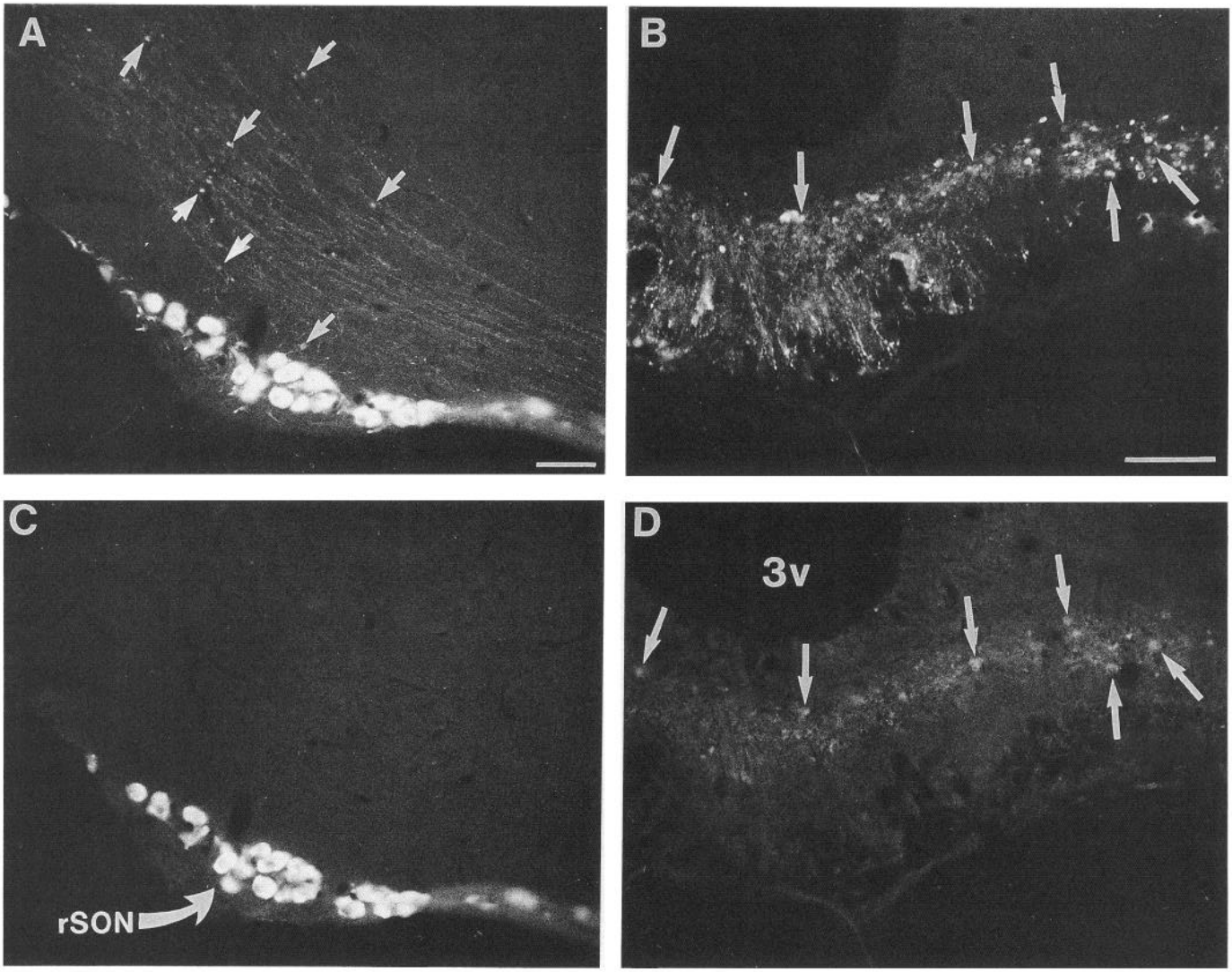

Figure 3. Colocalization of vasopressin peptide (*A, B,* fluorescein) and mRNA (*C, D,* fast red fluorescence) in a cryostat section through both the retrochiasmatic area of the SON (*rSON*; *A, C*) and the ME (*B, D*). Vasopressin peptide (*A, B*) was found in magnocellular neurons of the retrochiasmatic SON, in undilated parts of axons, and in many axonal swellings throughout the axonal trajectory (*A, arrows*), and in the ME (*B, arrows*). In contrast, vasopressin mRNA (*C, D*) was detected only in magnocellular perikarya (*C*) and in a subset of axonal swellings located in the ME (*D, arrows*). It was never detected in undilated portions of axons or in swellings of the axons passing through the lateral hypothalamic retrochiasmatic area (*C*). In the ME, most of the mRNA-containing swellings were immunoreactive (*B, D, arrows*), but many swellings immunoreactive for vasopressin peptide (*B*) were devoid of detectable amount of mRNA (*D*). *3v,* third ventricle. Scale bar, 50 μm.

Simultaneous detection of vasopressin peptide and mRNA

Double fluorescence labeling to detect vasopressin mRNA by *in situ* hybridization and vasopressin peptide by immunochemistry in the same sections was performed on salt-loaded animals only. Using the alkaline phosphatase–fast red detection system, vasopressin mRNA could be detected in magnocellular perikarya (Fig. 3*C*) as well as in swellings located in the ME (Fig. 3*D*) and PP (data not shown). Using the immunofluorescence technique, vasopressin peptide could be detected in magnocellular perikarya (Fig. 3*A*), in undilated axonal segments, and in many swellings located in the ME (Fig. 3*B*), the PP, and also everywhere along the magnocellular neuron axons (Fig. 3*A*). The combination of both techniques did not decrease significantly the sensitivity of either method. Thus, the comparative localization of vasopressin mRNA and peptide in the same series of structures could be readily ascertained (Fig. 3*A–D*). Although some swellings of the ME did contain both vasopressin mRNA and peptide, the majority of undilated axons as well as many other swellings in the ME or other areas of the hypothalamus (e.g., the retrochiasmatic area; Fig. 3*A*) contained vasopressin peptide without detectable vasopressin mRNA (Fig. 3*C*).

Control experiments

For all techniques used in this study, some experiments were done in which the labeled oligonucleotide probes were omitted, while all other parameters were unchanged. In these cases, no significant labeling could be found either in hypothalamic cell bodies or in swelling of the ME or PP (not shown).

In some experiments performed in salt-loaded animals, the oxytocin oligonucleotide (Fig. 2*B,D*) or the POMC oligonucleotide (Fig. 1*D*) was used instead of the vasopressin probe. These controls showed that, although the oxytocin oligonucleotide gave a strong labeling in magnocellular neurons located in the dorsal

part of the SON (Fig. 2*B*), it did not label significantly any swelling in the ME (Fig. 2*D*). In contrast, the VP1 oligonucleotide labeled numerous swellings in another section from the same salt-loaded animal (Fig. 2*C*). Concerning the posterior lobe of the pituitary (Fig. 1*C,D*), the POMC oligonucleotide strongly labeled most of the cells located in the intermediate lobe and also some endocrine cells (presumably corticotropes) of the anterior lobe, but gave no significant labeling in the posterior lobe (Fig. 1*D*). Neurohypophyseal sections processed for vasopressin mRNA detection consistently showed a significant labeling, but only in some swellings of the posterior lobe (Fig. 1*C*).

Electron microscopy

In general, the morphology obtained with either technique appeared to be well preserved, especially in the magnocellular hypothalamic nuclei. Thus, regardless of fixation and permeabilization technique used, most of the perikaryal organelles including mitochondria, RER, smooth endoplasmic reticulum (SER), Golgi apparatus, neurosecretory granules, synaptic vesicles, and lysosomes could be identified in SON profiles. However, the permeabilization using the freeze–thaw method gave better morphology than Triton treatment. In the ME and the PP, the morphology of axonal profiles was less well preserved. In general, we noted a loss of small synaptic vesicles, most probably due to underfixation and permeabilization.

In the magnocellular perikarya, the labeling was very strong in those peripheral parts of the cytoplasm enriched with RER (Nissl bodies) (Fig. 4*B*). In fact, the labeling was intense in discrete areas along the RER membranes, on their external side (Figs. 4*B,C*; 5*A,B*). The RER lumen was never labeled. Some parts of the RER were devoid of label (Fig. 5*B*). An additional weaker labeling was sometimes observed in punctate areas of the cytoplasm, without any clear association with any organelle (Figs. 4*B*, 5*A*). Finally, in some cases, some labeling could be found in the vicinity of the nuclear envelope, on the cytoplasmic surface (Fig. 4*B*). Some labeled cross sections of proximal dendrites were observed (Fig. 4*C*). They always contained some RER, and the labeling within these dendrites was associated with the RER membranes, as in the perikarya. Entire areas of the cytoplasm, mainly located between the nucleus and the Nissl bodies, and which did not contain any RER, were devoid of labeling (Figs. 4*B*, 5*A*). In all sections from all animals examined (control, salt-loaded, and lactating animals), the mitochondria, the Golgi apparatus, and the neurosecretory granules remained consistently unlabeled (Figs. 4*B*, 5*A*). In magnocellular neurons of all animals studied, the Golgi apparatus was consistently located in a perinuclear location, while the RER was in the peripheral cytoplasm. In addition, neurosecretory granules were frequently found between the Golgi apparatus and the nucleus.

In the ME of salt-loaded (Fig. 7*A*) or lactating animals (Fig. 6*A,B*), the positive swellings that were seen in the vibratome sections could be identified at the electron microscopic level as large axonal varicosities. The ultrathin sections of ME displayed a low background, and allowed the identification of such labeled profiles (Figs. 6*A,B*; 7*A*). Glial cells and axon cross sections in the same fields were devoid of labeling (Figs. 6*A*, 7*A*). Most of the positive swellings were unmyelinated axons, although occasionally they were surrounded by a few layers of repeated membranes (Fig. 7*A*). Occasional swellings surrounded by a few membranes were also observed in the ME of control animals. In the positive swellings, despite the relatively poor morphology, the mitochondria and some neurosecretory granules could be identified (Fig. 6*B*). In some cases, longitudinal sections of axons entering a swelling were obtained. Serial sections of such structures, presented in Figure 6, *A* and *B*, show that the labeling is restricted to the swelling only; the preterminal undilated axon was never significantly labeled. No significant labeling was observed at the electron microscopic level in MEs from control animals.

Whatever the localization of the positive swellings (ME or PP), when using digoxigenin-labeled oligonucleotides detected with peroxidase-DAB, the labeling in these swellings was diffuse and the subcellular localization of the labeling could not be ascertained. The use of biotinylated-labeled probes detected with the immunogold technique allowed the visualization of the ultrastructural organization of the mRNA-containing swellings. Few neurosecretory granules and mitochondria were identified within these swellings. However, abundant fibrillar material was a consistent component of these structures. Most of the silver grains were dispersed throughout the entire swelling (Fig. 8). Although few silver grains seemed to be close to mitochondria or neurosecretory granules, quantitation of silver grains in contact with organelles did not show a preferential association with any of these organelles (data not shown).

In the PP, the background was very low with all methods used. Pituicytes rarely showed more than trace labeling, and only occasional label was found over endothelial cells of pituitary capillaries. In salt-loaded animals, some labeled profiles resembled the labeled swellings in the ME (Figs. 7*B*, 8). Their distribution within the PP was heterogeneous. In some fields, they were quite abundant and many of them were grouped (see

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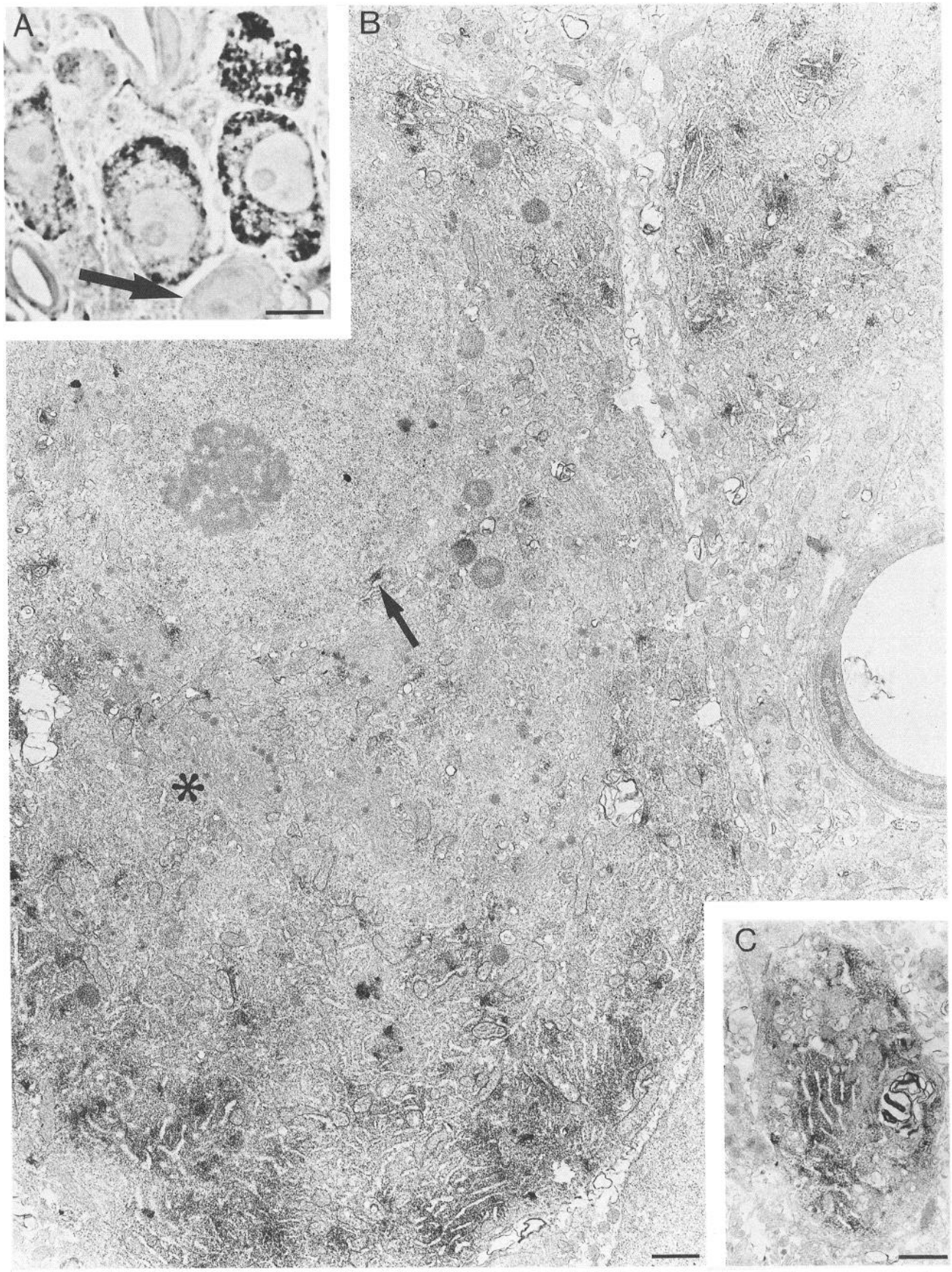
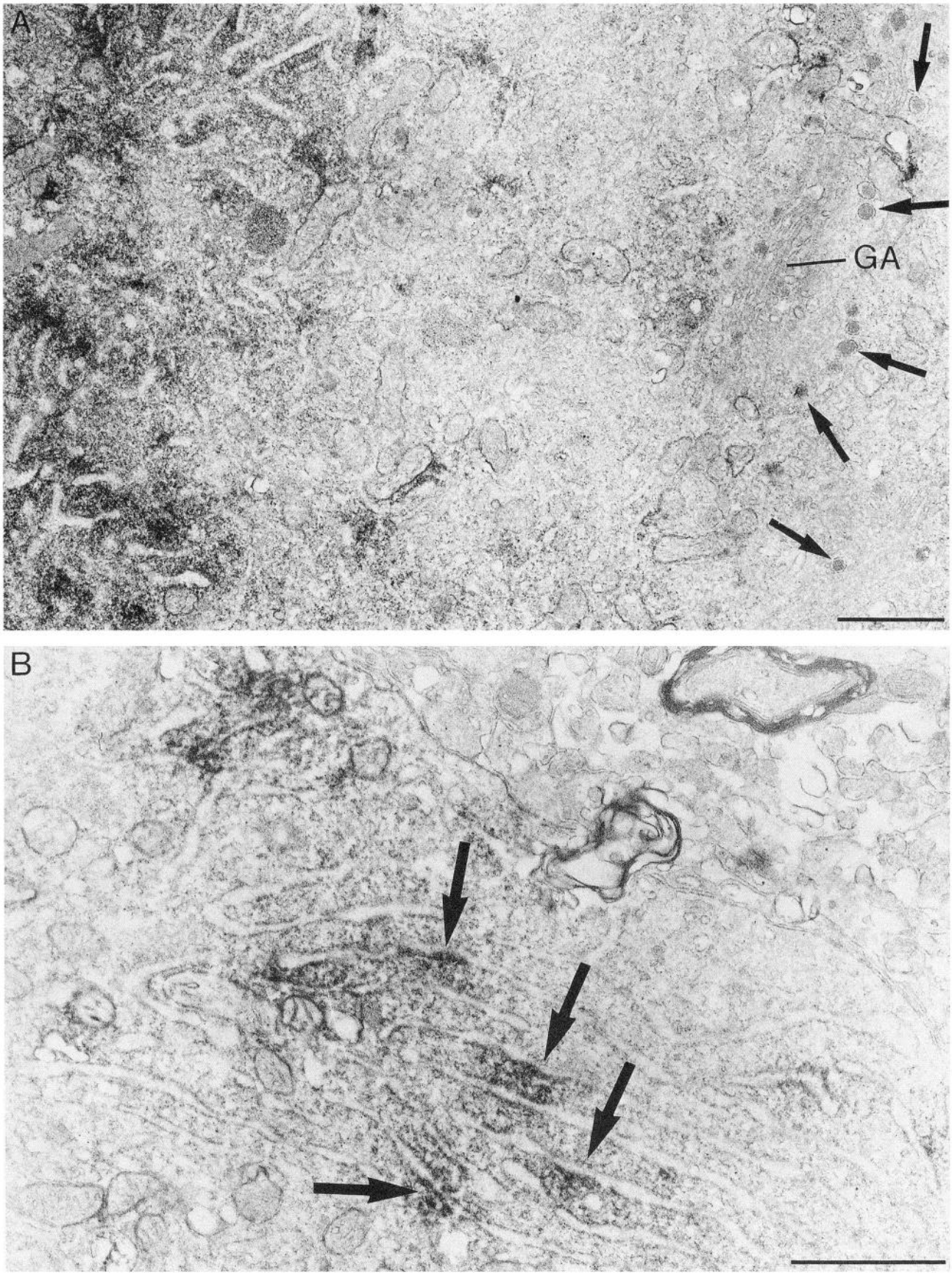
Figure 4. Subcellular localization of vasopressin mRNA in the SON of a control animal (*A, C*) and a 3 d lactating animal (*B*). Vasopressin mRNA detection was performed using either four digoxigenin-labeled oligonucleotides (VP1–VP4) detected using the ABC-DAB (*A, C*), or one digoxigenin-labeled oligonucleotide (VP1) detected with the ABC-DAB-Ni (*B*). The permeabilization was performed using either Triton (*A, C*) or the freeze–thaw method (*B*). *A,* Semithin plastic section (0.5 μm) shows labeling confined to the cytoplasm of several positive magnocellular neurons. An unlabeled magnocellular neuron is seen at lower right (*arrow*). In the positive neurons, the label was abundant in peripheral cytoplasmic areas of the perikarya. These areas are enriched in RER (see *B*). *B,* Labeled perikarya at the ultrastructural level. The cellular morphology is well preserved and the labeling is prominent on the RER. The nucleus is not labeled but a weak significant label is often observed close to the nuclear envelope, in the cytoplasmic compartment (*arrow*). The *asterisk* shows an area of the magnocellular neuron illustrated at a higher magnification in Figure 5*A*. *C,* Cross section of a medium-size dendrite in the SON. Part of the dendrite is occupied by labeled RER. Scale bars: 10 μm in *A*, 1 μm in *B* and *C*.

Figure 5. Ultrastructural localization of vasopressin mRNA within perikarya of 3 d lactating animals. Vasopressin mRNA was detected using the digoxigenin-labeled VP1 probe detected with Elite-ABC peroxidase-DAB-Ni method. The permeabilization of the tissue was performed using the freeze–thaw method. *A,* Higher magnification of Figure 4*B* showing the lack of label on the Golgi apparatus (*GA*) and neurosecretory granules (*arrows*). *B,* Within the RER, the labeling is located on discrete areas (*arrows*). The same pattern of labeling was observed in all groups of animals studied at the ultrastructural level (control, salt-loaded, and lactating animals). Scale bars, 1 μm.


A
B
C
*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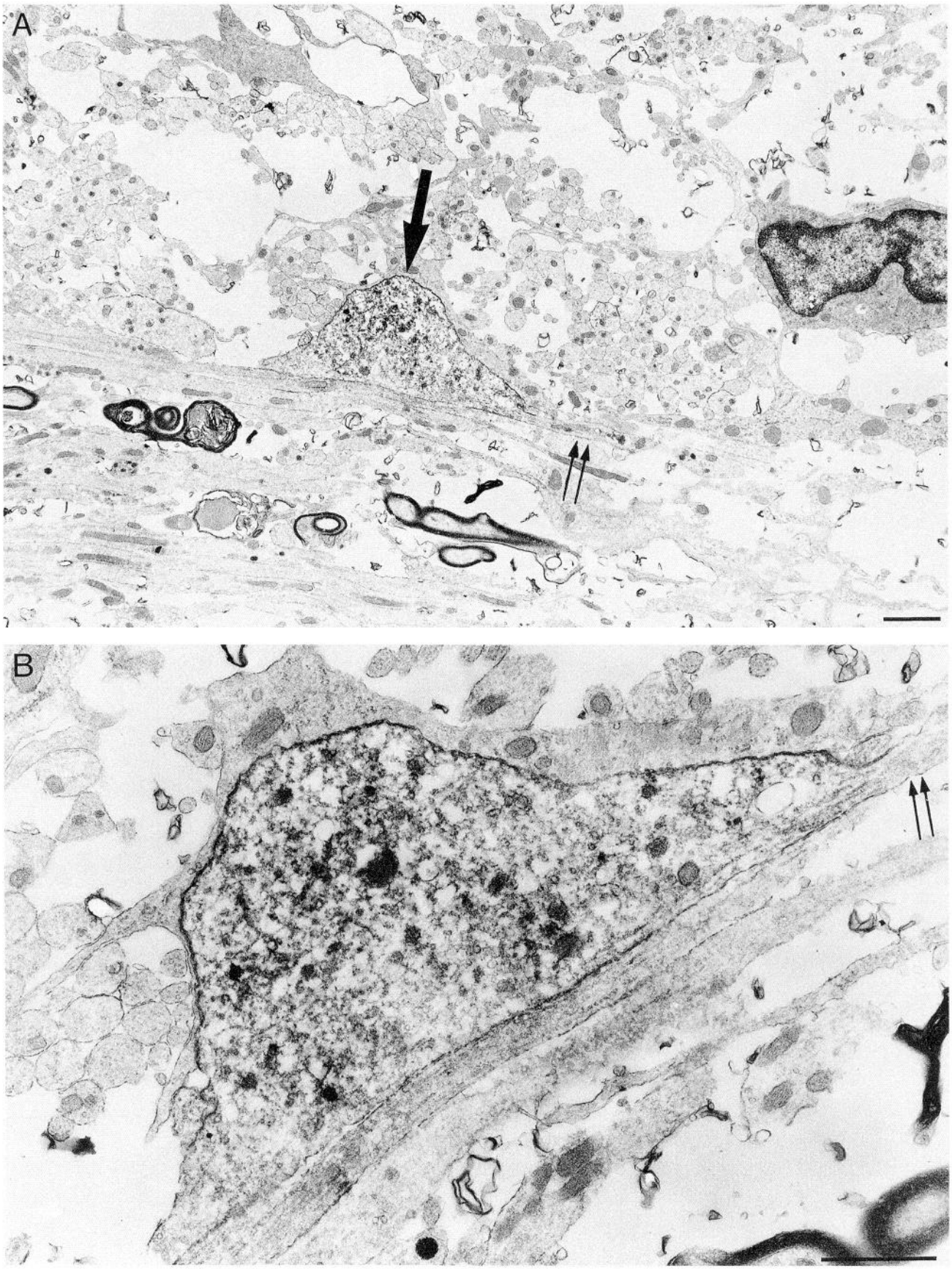

Figure 6. Ultrastructural localization of vasopressin mRNA within a swelling (*single arrow*) of the ME from a 17 d lactating animal. Vasopressin mRNA was detected with one digoxigenin-labeled oligonucleotide (VP1) and using the Elite-ABC peroxidase-DAB-Ni method. Permeabilization: freeze–thaw method. *A* and *B* represent serial sections of the labeled swelling; *A* is a lower magnification of *B*. The longitudinally cut undilated axon emerging from the swelling is devoid of labeling (*double arrows*). The diffuse peroxidase reaction product precludes precise localization of the mRNA. Scale bars, 1 μm.

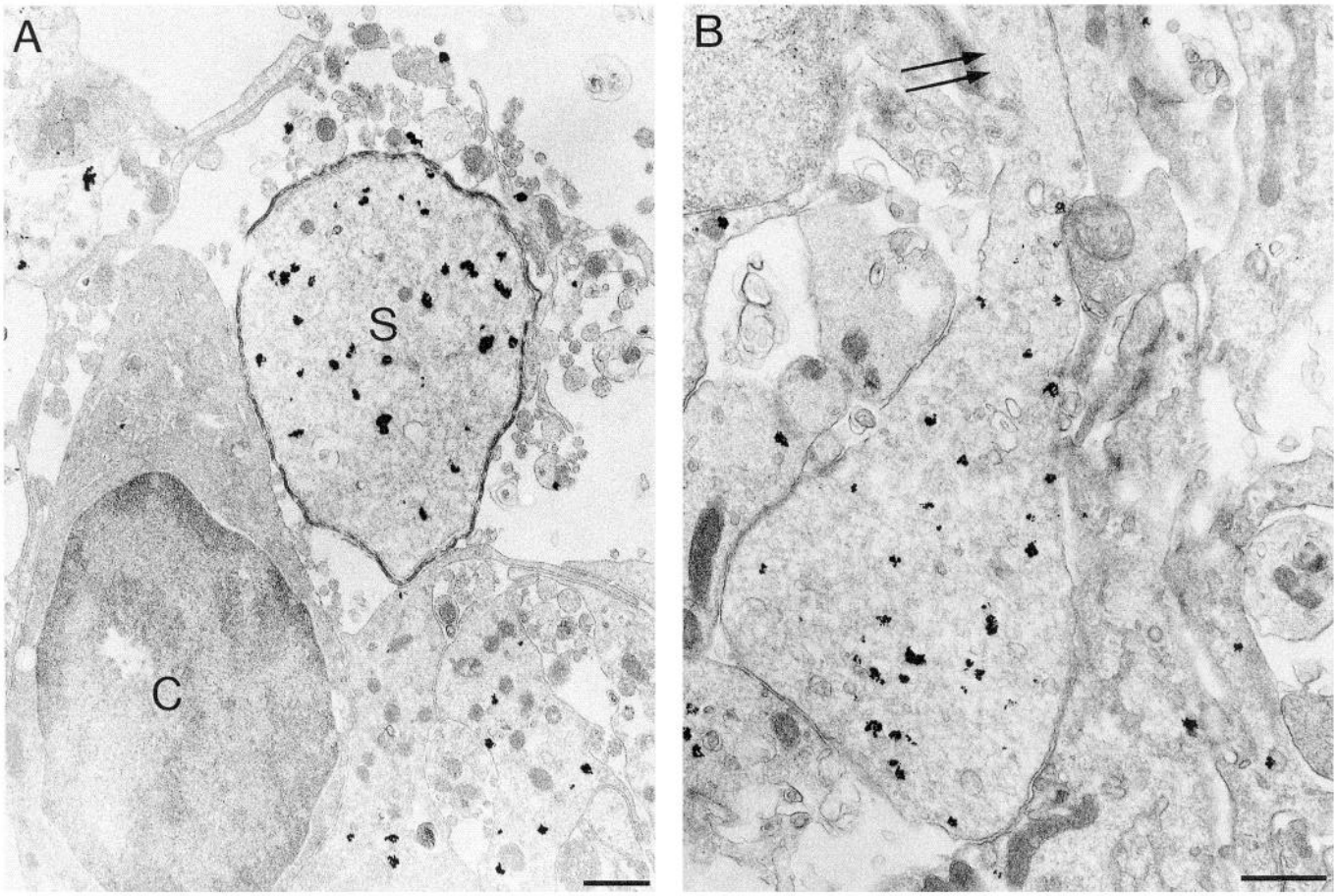

Figure 7. Ultrastructural visualization of vasopressin mRNA in swellings of the ME (*A*) and the PP (*B*) of a 7 d salt-loaded animal, after Triton permeabilization of vibratome sections. Vasopressin mRNA hybridization was performed using four biotin-labeled oligonucleotides (VP1–VP4) detected using the immunogold-silver enhancement-gold toning method. *A,* Labeled swelling (*S*) adjacent to unlabeled cell (*C*). *B,* An unlabeled axon (*double arrows*) terminates in a labeled swelling. Scale bars, 1 μm.

Fig. 8). In other fields, they were rare. In control animals, rare but weakly labeled swellings were observed.

Discussion

In this work, the subcellular compartmentalization of vasopressin mRNA was studied in the magnocellular hypothalamo-neurohypophyseal system using two new *in situ* hybridization cytochemical techniques compatible with electron microscopic observations. We first discuss the specificity of these two new techniques, and then consider their application to the subcellular localization and potential function of vasopressin mRNA in perikaryal and axonal compartments.

Specific labeling of mRNA by nonisotopic in situ *hybridization*

The specificity of the labeling was demonstrated by several observations. Concerning the cell bodies, regardless of the vasopressin oligonucleotide used, alone or in combination, the pattern of perikaryal labeling was consistent and in accordance with the literature describing the hypothalamic distribution of vasopressin peptide (Sofroniew, 1985; Hou Yu et al., 1986) or mRNA (Uhl et al., 1985). Concerning the labeling of axonal swellings, specificity was demonstrated by several experiments. The absence of any reaction in swellings when the probes were omitted or when using heterologous probes (e.g., POMC probe) showed that the detection systems and the labeled tail of the probes produced no false positive reactions. In addition, multiple other data indirectly support the specific mRNA labeling of axonal swellings: (1) these labeled swellings were found in the internal layer of the ME, which is known to be the layer of passage of axons coming from the hypothalamic magnocellular nuclei (Sofroniew, 1985); (2) the range of sizes of these swellings was comparable in the ME and the PP; (3) at the electron microscopic level, these positive swellings could clearly be identified as large axonal varicosities; (4) the positively labeled swellings were clearly more abundant in the ME and PP of salt-loaded rats compared to control animals; and (5) no oxytocin mRNA–positive swellings were observed in salt-loaded animals. This last observation is in accordance with Northern blot and radioactive *in situ* hybridization results showing that vasopressin mRNA concentration is dramatically increased, 17-fold in the PP after 7 d salt loading, while oxytocin mRNA increases only about threefold (Mohr et al., 1991). Finally, our double labeling experiments in which both vasopressin mRNA and peptide were detected together in single sections showed that vasopressin mRNA was detected in vasopressin peptide–containing perikarya and swellings.

Subcellular compartmentalization of vasopressin mRNA in magnocellular neurons and its functional significance

Perikarya. Within the perikarya, vasopressin mRNA was mainly associated with the Nissl bodies, which are very prominent in magnocellular neurons. Such a localization has been previ-

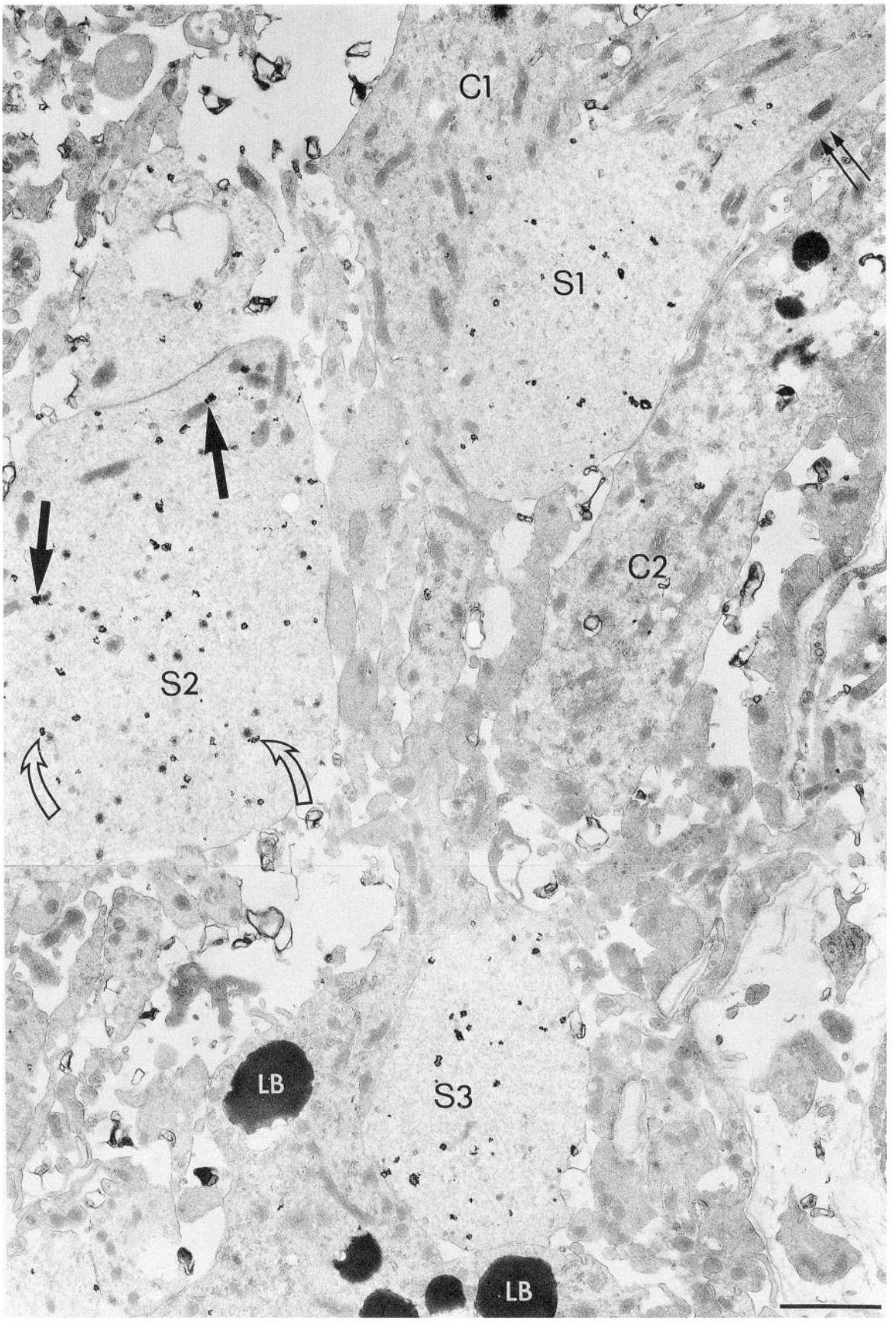

Figure 8. Ultrastructural detection of vasopressin mRNA in swellings of the PP of a 7 d salt-loaded animal. The mRNA detection was performed using four biotin-labeled oligonucleotides (VP1–VP4), revealed by the immunogold-silver enhancement-gold toning technique, after Triton permeabilization of the vibratome sections. In this field, three swellings (*S1, S2, S3*) are heavily labeled. In contrast, surrounding cell processes (*C1, C2*)

ously reported for this mRNA using a radioactive (Guitteny and Bloch, 1989) or a biotinylated probe (Trembleau et al., 1991). However, the former study was of very poor spatial resolution, since the marker used was ^{35}S, and the use of paraformaldehyde fixation in the latter study limited structural preservation. Thus, in both of these studies, the precise pattern of localization of the mRNA within the Nissl bodies could not be rigorously ascertained. Conversely, in the present study, the well-preserved morphology combined with the good resolution of the peroxidase reaction product allowed us to obtain new information concerning vasopressin mRNA distribution within the Nissl bodies. The labeling was widely distributed through the Nissl bodies: many of the RER cisternae, regardless of their location within the neuron, were at least partially labeled. We did not observe any large areas of Nissl bodies devoid of labeling. Instead, every part of the cell containing some RER appeared to have some label. Some labeled RER was found even in medium-size cross sections of dendrites. The labeling was always found on the external side of the RER membranes, and the lumen of the cisternae was never labeled.

A significant discrete vasopressin mRNA labeling was also found close to the nuclear envelope, always in the cytoplasmic compartment, as well as in cytoplasmic areas between the nucleus and the RER, without a clear relationship with any cellular organelle. A nuclear envelope–associated label has been previously reported in a study describing the isotopic detection of vasopressin mRNA (Guitteny and Bloch, 1989), and it has been shown later using a peroxidase detection technique that this label was never intranuclear, but consistently cytoplasmic (Trembleau et al., 1991). Such labeling could represent mRNA in transit from the nucleus to the RER, although a possible translation of vasopressin mRNA at the level of the external membrane of the nuclear envelope cannot be excluded, since neurophysin immunoreactivity has already been reported in the cisternae of the nuclear envelope (Broadwell et al., 1979).

The wide distribution of vasopressin mRNA within the RER demonstrates that it is transported from the nucleus in every direction, to almost all cytoplasmic areas containing some RER, including proximal parts of dendrites. This suggests quite a long intraperikaryal mRNA transport since, in magnocellular neurons, the RER is typically located in the periphery of the perikaryon, where it constitutes the Nissl bodies.

The presence of vasopressin mRNA within the Nissl bodies, and the absence of labeling inside the lumen of the RER cisternae, both fit well with the current knowledge about the mechanisms of translation for mRNAs encoding secreted peptides.

At the level of individual RER cisternae, the labeling was not ubiquitous. Instead, RER labeling was restricted to discrete areas along the RER membranes, constituting "patches." This patchy pattern of labeling of the RER may be due to a differential accessibility of the mRNA to the oligonucleotide probes along the RER. Alternatively, vasopressin mRNA may be restricted to specific sites of the RER. Interestingly, a functional compartmentalization of the RER of vasopressinergic magnocellular neurons has recently been proposed by van Leeuwen (for review, see van Leeuwen, 1992), based on the pattern of immunoreactivity for coexisting peptides in the vasopressin deficient Brattleboro rat. van Leeuwen has proposed that vasopressin mRNA, together with some coexisting neuropeptide mRNAs, such as angiotensin, would be translated on different patches of the RER than other peptide mRNAs, such as dynorphin and galanin. Our results, showing patches of vasopressin mRNA labeling within the RER, are in accord with his hypothesis. However, further morphological evidence, such as the simultaneous detection of two mRNAs (i.e., vasopressin mRNA and dynorphin mRNA) with putative distinct locations within the RER, is needed to determine whether this hypothesis is valid.

In control animals, as well as in salt-loaded or in lactating animals, the Golgi apparatus as well as the perikaryal neurosecretory granules were never labeled by *in situ* hybridization for vasopressin mRNA. In lactating animals, an axonal transport of oxytocin mRNA has also been recently documented by nonradioactive *in situ* hybridization employing HRP reactivity as the reporter (Jirikowski et al., 1990). Since this work suggested an association between oxytocin mRNA and neurosecretory granules in the PP, the possibility existed that axonal transport of mRNA occurred via the classical neurosecretory pathway, through the Golgi apparatus and the neurosecretory granules. However, the present results, showing a lack of labeling both of the Golgi apparatus and of the neurosecretory granules within the perikarya of salt-loaded animals, strongly suggest that vasopressin mRNA and peptide may use different macromolecular mechanisms for their axonal transport.

Axonal compartment. In the present study, vasopressin mRNA was found in the axons of hypothalamic magnocellular neurons. Thus, our study provides the first direct structural evidence for an axonal transport of this mRNA. However, in contrast to the peptide, vasopressin mRNA was found only in a subset of axonal dilatations (swellings) located in the internal layer of the ME and in the PP. This finding suggests that within magnocellular neurons, the accumulation of mRNA is restricted to a specific compartment of the axons. These labeled swellings were abundant in salt-loaded animals, detectable but less abundant in lactating animals, and very rare or absent in control animals. As discussed above, such labeling is likely to be specific, as strongly suggested by the control experiments. Moreover, it has previously been shown using radioactive *in situ* hybridization as well as Northern blot analysis that vasopressin mRNA concentration was dramatically increased in the ME and in the PP following chronic salt loading. For example, in the PP, there is on the seventh day of salt loading a 17-fold increase for vasopressin mRNA concentration (Mohr et al., 1991). Our morphological analyses, in which vasopressin mRNA was not clearly detectable in control animals but readily detectable in salt-loaded animals, are in agreement with these previous data. Sections from lactating animals displayed weaker labeling in swellings, at both 3 d and 17 d lactation, the only stages studied in this work. Since our nonradioactive methods are, at present, hardly compatible with quantitation, we did not determine whether the number of these labeled swellings varied during lactation. Further extensive studies at various stages of lactation may be necessary to answer this question. However, it was

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contain background levels of labeling. Within the swelling, most of the label is scattered. Occasional label is associated with either mitochondria (*solid arrows*) and secretory granules (*open arrows*) (see swelling *S2*). An undilated axon terminating in a labeled swelling is devoid of label (*double arrow, upper right*). *LB*, lipid bodies. Scale bar, 1 μm.

obvious that the number of these positive swellings observed in these lactating animals was lower than that in salt-loaded animals.

The significance of such an increase of vasopressin mRNA in the axonal compartment during lactation is not clear. However, it has been shown that vasopressin mRNA concentration was significantly increased in the SON during lactation (Lightman and Young, 1987). Moreover, Mezey and Kiss (1991) recently showed that urine osmolarity was also significantly increased during lactation. They concluded that the increase of vasopressin expression may be a response to the loss of water due to lactation. Thus, like the oxytocinergic system, the vasopressinergic system seems to be stimulated during lactation. Such a stimulation may lead to an increase of axonal transport of vasopressin mRNA.

The anatomical features of the axons of vasopressin magnocellular neurons have been previously well documented, using immunohistochemical approaches (see Sofroniew, 1985). It has been shown that these axons were subject to plasticity during salt loading. For example, Dellmann et al. (1988) reported that a striking increase in swelling number within hypothalamic axons otherwise depleted of immunoreactive material occurred within 7 d in salt-loaded animals. However, from these studies in which the axons were labeled using neurophysin immunohistochemistry, no differences were noted between swellings located along the intrahypothalamic course of the axons and the swellings observed in the ME and the PP.

The most striking result from the present study is the demonstration of differential and segregated mRNA content within the axonal compartment of the magnocellular neurons: in salt-loaded animals with the highest axonal labeling, some swellings located in the ME and the PP were heavily labeled, while undilated axons as well as other intrahypothalamic swellings in more proximal parts of the axons were never labeled. Thus, our observations strongly suggest that besides the positive swellings located in the ME and the PP, which seem to contain a high concentration of vasopressin mRNA, most other parts of these axons contain a very low concentration of mRNA. In fact, our results of colocalization of vasopressin peptide and mRNA demonstrate that within the axons, the distribution of vasopressin mRNA does not overlap that of vasopressin peptide. Therefore, all the swellings along the axons may not have the same biochemical and functional features. This finding is of interest because no biochemical differences between the proximal swellings and the distal swellings in the ME or PP have been previously reported. In fact, these swellings along the axons in osmotically stimulated animals have been considered the result of local variations in the transport velocity, the nondilated axon segments being regions of fast transport while swellings conceived as areas of slow transport, temporary storage, or disposal of vesicles (Castel and Hochman, 1976; Morris et al., 1978; Pena et al., 1988).

In this study, we attempted to define the ultrastructural features of the labeled swellings using electron microscopy. Such an approach confirmed that the labeled structures observed using light microscopy in the ME and the PP were truly large axonal varicosities. These positive structures were sometimes cut in a way that permitted visualization of physical continuity with preterminal undilated axons. In these cases, the labeling was always restricted to the swelling, but it was never present in the connected undilated axon. Within the labeled swellings, some mitochondria, as well as a few secretory granules, could be identified. In the PP, most of these labeled swellings contained a few mitochondria and secretory granules only. In fact, they looked like the "empty" swellings described by Morris et al. (1978) in dehydrated animals. However, most probably due to the low glutaraldehyde concentration in the fixative, and also to the numerous steps required for these techniques, the internal morphology of the swelling of the ME and the pituitary axons was suboptimal in our study and was not compatible with the preservation of some cellular organelles such as the SER. According to previous studies, the SER is prominent in some of the swellings containing few secretory granules (Castel et al., 1984). Thus, we cannot yet determine whether the swellings aggregating the mRNA correspond to SER-enriched structures.

In conclusion, vasopressin mRNA is specifically aggregated within a subset of distal swellings along the axons of magnocellular neurons. The functional significance of this intra-axonal mRNA remains unclear. The possibility that translation of the mRNA could occur in hypothalamo-neurohypophyseal axons has been considered very unlikely, since extensive ultrastructural studies have failed to provide any evidence for ribosomes in these axons.

Thus, axonal vasopressin mRNA may have another function. The dramatic increase of the axonal transport of vasopressin mRNA during dehydration, a circumstance in which dramatic neuronal plasticity occurs (Castel et al., 1984; Hatton, 1990), together with the observation that this mRNA is detectable in the developing PP at the time of the arrival of magnocellular neuron axons in this structure (Mohr et al., 1991), indicates a possible function of axonal mRNAs in relation to neuronal development and plasticity.

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