Microvesicles of the Neurohypophysis Are Biochemically Related to Small Synaptic Vesicles of Presynaptic Nerve Terminals

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Abstract. Nerve endings of the posterior pituitary are densely populated by dense-core neurosecretory granules which are the storage sites for peptide neurohormones. In addition, they contain numerous clear microvesicles which are the same size as small synaptic vesicles of typical presynaptic nerve terminals. Several of the major proteins of small synaptic vesicles of presynaptic nerve terminals are present at high concentration in the posterior pituitary. We have now investigated the subcellular localization of such proteins. By immunogold electron microscopy carried out on bovine neurohypophysis we have found that three of these proteins, synapsin I, Protein III, and synaptophysin (protein p38) were concentrated on microvesicles but were not detectable in the membranes of neurosecretory granules. In addition, we have studied the distribution of the same proteins and of the synaptic vesicle protein p65 in subcellular fractions of bovine posterior pituitaries obtained by sucrose density centrifugation. We have found that the intrinsic membrane proteins synaptophysin and p65 had an identical distribution and were restricted to low density fractions of the gradient which contained numerous clear microvesicles with a size range the same as that of small synaptic vesicles. The peripheral membrane proteins synapsin I and Protein III exhibited a broader distribution extending into the denser part of the gradient. However, the amount of these proteins clearly declined in the fractions preceding the peak of neurosecretory granules.

Our results suggest that microvesicles of the neurohypophysis are biochemically related to small synaptic vesicles of all other nerve terminals and argue against the hypothesis that such vesicles represent an endocytic byproduct of exocytosis of neurosecretory granules.

THE neurohypophysis is primarily composed of nerve endings of hypothalamic neurons. The known function of these nerve endings is to secrete peptide neurohormones into the blood stream (Scharrer and Scharrer, 1954; Morris et al., 1978; Brownstein et al., 1980). Nerve endings are aligned along the basal membrane of capillary walls, and are densely populated by neurosecretory granules (NSGs),[1] ~100–300 nm in diameter, in which peptide neurohormones are stored. NSGs are assembled in the neuronal perikarya and are transported to the posterior lobe of the pituitary along the hypothalamo-hypophyseal tract (Morris et al., 1978; Brownstein et al., 1980; Russell, 1987).

In addition to NSGs, nerve endings of the neurohypophysis contain a high number of spherical microvesicles (MVs) which have an electron-translucent core and the same average diameter as small synaptic vesicles (SSVs) of typical presynaptic terminals (Klein et al., 1982; Tweedle, 1983). These MVs are in general clustered at the surface of the terminals facing the endothelial wall (Klein et al., 1982; Tweedle, 1983). The physiological function of MVs remains elusive. It had been proposed that MVs represent endocytic organelles involved in the retrieval of NSG membrane from the plasmalemma after granule exocytosis (Douglas et al., 1970). This idea was suggested by the finding that the core of MVs becomes labeled by the tracer when the terminals of the neurohypophysis are stimulated in the presence of extracellular tracers (Douglas et al., 1970; Nagasawa et al., 1971; Broadwell et al., 1984). It has been reported, however, that stimulation of peptide hormone release from isolated neural lobes does not lead to an increase in the number of microvesicles (Nordmann and Morris, 1976; Morris and Nordmann, 1980; Morris et al., 1981). In contrast, a significant numerical increase of a population of vacuoles with a diameter larger than 85 nm was observed (Nordmann et al., 1974; Nordmann and Morris, 1976). These vacuoles, as well, were labeled by extracellular tracer. These findings

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1. *Abbreviations used in this paper*: LDCVs, large dense-core vesicles; MVs, microvesicles; NSGs, neurosecretory granules; SSVs, small synaptic vesicles.

suggested that vacuoles, rather than MVs, participate in the retrieval of NSG membranes after exocytosis (Morris and Nordmann, 1980; Morris et al., 1981). Other possible functions of MVs have been proposed such as their involvement in the calcium homeostasis of nerve terminals (Nordmann and Chevallier, 1980; Morris et al., 1981). Moreover, the striking similarity between these MVs and SSVs of presynaptic nerve terminals has been noted and it has been suggested that MVs might have a secretory function of their own (Gerschenfeld et al., 1960; Alonso and Assenmacher, 1979; Morris and Nordmann, 1980; Nordmann and Chevallier, 1980; Morris et al., 1981; Broadwell et al., 1984).

Recent studies on SSVs have indicated that a number of major membrane polypeptides are shared by all SSVs irrespective of the specific classical neurotransmitter they contain (Matthew et al., 1981; De Camilli et al., 1983*a*,*b*; Jahn et al., 1985; Wiedenmann and Franke, 1985; Navone et al., 1986; Buckley and Kelly, 1985; Browning et al., 1987; Obata et al., 1987; Baumert et al., 1989). In addition, studies carried out in our laboratories by immunogold electron microscopy have indicated that at least some of these polypeptides, namely, synapsin I, Protein III, synaptophysin, and synaptobrevin, are not present at detectable concentrations in the membranes of large dense-core vesicles (LDCVs) (Navone et al., 1984; Navone et al., 1986; Navone et al., 1988; Baumert et al., 1989); i.e., the secretory vesicles of presynaptic nerve endings involved in the release of peptide neurotransmitters (Lundberg and Hokfelt, 1983; Coulter et al., 1988). These results suggest that SSVs and LDCVs have an independent biogenesis (De Camilli and Navone, 1987; Navone et al., 1988) and are consistent with evidence that SSVs and LDCVs have different life-cycles in nerve endings and are released under different conditions of stimulation and by different regulatory mechanisms (Lundberg and Hokfelt, 1983; Matteoli et al., 1988; De Camilli and Jahn, 1990).

We report here that four major membrane proteins of SSVs—synapsin I (De Camilli et al., 1983*a*,*b*), Protein III (Browning et al., 1987), synaptophysin (Jahn et al., 1985; Wiedenmann et al., 1985), and p65 (Matthew et al., 1981) (Table I)—are present in the membranes of MVs of the neurohypophysis and that, as in presynaptic terminals, they are not present at significant concentrations in the membranes of peptide-containing secretory granules.

Materials and Methods

Antibodies

Rabbit polyclonal IgGs directed against synaptophysin or synapsin I were prepared, affinity purified, and characterized as previously described (Navone et al., 1986; De Camilli et al., 1983*a*) (see also Fig. 1, lanes *A* and *B*). Polyclonal IgGs directed against both Protein III and synapsin I were obtained by injecting rabbits with purified Protein III. These IgGs recognized both proteins even after affinity purification on either a synapsin I or a Protein III–Sepharose column (Fig. 1, lane *C*). This is probably explained by the high degree of sequence homology between synapsin I and Protein III (Südhof et al., 1989).

Mouse monoclonal antibodies (IgGs) directed against Protein III were prepared as previously described (Browning et al., 1987). These antibodies recognized Protein III specifically (both Proteins IIIa and IIIb) and not synapsin I (Fig. 1, lane *D*). Mouse monoclonal antibodies directed against neurophysin I were a gift from Dr. M. Treiman (Copenhagen, Denmark), and rabbit sera directed against secretogranin II and against p65 were gifts from Dr. A. Zanini (Milano, Italy) and from Drs. J. L. Bixby and L. F. Reichardt (San Francisco, CA), respectively.

Light Microscopy Immunocytochemistry

Sprague-Dawley albino rats were anesthetized and fixed by transcardial perfusion with 4% formaldehyde (freshly prepared from paraformaldehyde) in 120 mM sodium phosphate buffer as previously described (De Camilli et al., 1983*a*). Pituitary glands were subsequently dissected out and epon embedded. 1-μm-thick plastic sections were prepared and processed for light microscopy immunofluorescence by an indirect immunorhodamine procedure as previously described (De Camilli et al., 1983*a*, Navone et al., 1986). Rhodamine-conjugated secondary antibodies (goat anti-rabbit IgGs and goat anti-mouse IgGs) were from Organon Teknika-Cappel, Malvern, PA).

Electron Microscopy Immunocytochemistry

Electron microscopy immunocytochemistry of posterior pituitary homogenates was carried out as described for brain homogenates (Navone et al., 1984). Briefly, fresh bovine pituitary glands were obtained from a local slaughterhouse and within 1 h of the death of the animal the posterior lobe was dissected out, finely minced with the help of a razor blade, and homogenized in a glass-Teflon homogenizer by 10 up and down strokes at 900 rpm in 4 vol of ice cold buffer (0.25 M sucrose, 5 mM sodium phosphate buffer, pH 7). The homogenate was then fixed (3% formaldehyde [freshly prepared from paraformaldehyde]/0.25% glutaraldehyde) in either isotonic or hypotonic conditions (De Camilli et al., 1983*b*). This fixative did not preserve NSGs effectively (especially when the fixation was performed in hypotonic conditions), but was previously found to be compatible with good immunolabeling of vesicle antigens (De Camilli et al., 1983*b*). Qualitatively similar results were obtained using either isotonic or hypotonic fixation conditions. The fixed homogenate was then further processed for agarose embedding, immunogold labeling, epon embedding, and electron microscopy as previously described (Navone et al., 1986). Immunogold labeling was performed using protein A–gold conjugates (5–6 nm) prepared according to Slot and Geuze (1983), when the primary antibodies were rabbit IgGs, and with goat anti-mouse IgG–gold conjugates (5 nm; Janssen Biotech N.V., Belgium) when the primary antibodies were monoclonal antibodies.

Subcellular Fractionation

Bovine posterior pituitaries (6 for each experiment) were homogenized in 4 vol of 0.3 M sucrose, 4 mM Hepes, pH 7.0, 2 μg/ml pepstatin (Boehringer Mannheim, FRG) previously solubilized in DMSO, 0.1 mM PMSF (Sigma

Table I. Proteins of SSV Membranes Investigated in This Study

Protein	Topology in the membrane	M_r*	References
Synapsin I	Cytoplasmic surface	86,000 (a) 80,000 (b)	De Camilli et al., 1983*a*,*b*
Protein III	Cytoplasmic surface	74,000 (a) 55,000 (b)	Browning et al., 1987
Synaptophysin	Transmembrane	38,000	Jahn et al., 1985 Wiedenmann and Franke, 1985
Protein p65	Transmembrane	65,000	Matthew et al., 1981

* M_r given for the bovine proteins.

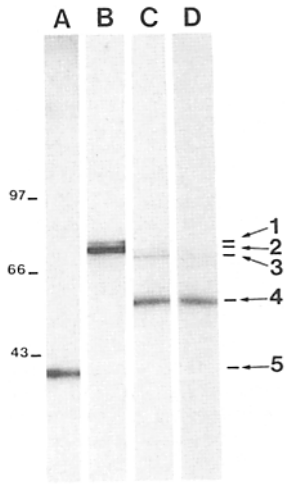

Figure 1. Western blots of total homogenates of rat brain showing the specificity of the antibodies directed against synaptophysin, synapsin I, and Protein III used in this study. Identical blots were radioimmunolabeled with the following antibodies: (*A*) affinity-purified antibodies directed against synaptophysin; (*B*), affinity-purified antibodies directed against synapsin I; (*C*) affinity-purified antibodies raised against Protein III which also recognize synapsin I, although with lower affinity; (*D*) monoclonal antibodies directed against Protein III. *1*, syn Ia; *2*, syn Ib; *3*, Protein IIIa; *4*, Protein IIIb; *5*, synaptophysin. Numbers at left show position of mol wt markers.

Chemical Co., St. Louis, MO). The homogenate was centrifuged at 4,000 *g* for 15 min in a rotor (SA600; Sorvall, Norwalk, CT) to remove cell debris, nuclei, and intact neurosecretosomes. The pellet was resuspended, rehomogenized, and centrifuged again under the same conditions. The two supernatants (total volume ~5.6 ml) were pooled, loaded on a 0.4–2-M sucrose gradient and spun for 5 h in a rotor (SW27; Beckman Instruments, Inc., Palo Alto, CA) at 65,000 *g*. The gradient was prepared in a 40-ml cellulose nitrate tube by overlaying above a 2-ml cushion of 2.5 M sucrose, 17 aliquots (1.8 ml each) of decreasing concentrations of sucrose (ranging from 2 to 0.4 M, with a difference of 0.1 M between adjacent fractions). All the sucrose solutions were prepared in 4 mM Hepes buffer, pH 7.0. The gradient was left at 4°C for 24 h before use. At the end of the centrifugation, 1.4-ml aliquots of the gradient were collected from the bottom of the tube by inserting a stainless steel cannula just above the densest sucrose cushion. A small sample of each fraction was immediately fixed and processed for electron microscopic analysis as described previously (Huttner et al., 1983). The remaining portions of the fractions were frozen until used for SDS-PAGE.

SDS Gel Electrophoresis and Immunoblotting

Proteins of total homogenates or of equal volumes of the sucrose gradient fractions were separated on SDS-polyacrylamide gels according to Laemmli (1970). After electrophoresis, the gels were either stained with Coomassie blue or electrophoretically blotted onto nitrocellulose paper (Towbin et al., 1979). Strips of paper blots were immunolabeled for the various antigens according to Borgese et al. (1982) either by an indirect immunoperoxidase procedure (Jahn et al., 1985) or by radioimmunolabeling (^{125}I-protein A in the case of rabbit antibodies, ^{125}I-goat anti-mouse IgGs in the case of mouse monoclonal antibodies).

Results

Localization of Synapsin I, Protein III, and Synaptophysin in the Rat Neurohypophysis by Immunofluorescence

The distribution of synapsin I, Protein III, and synaptophysin immunoreactivities in the intermediate and posterior lobes of the rat pituitary gland at the light microscopic level of resolution is illustrated in Fig. 2. The figure shows 1-μm-thick plastic sections of the intermediate and posterior pituitary stained by immunofluorescence for the three antigens. All of them are highly concentrated in the posterior pituitary, where they have a similar distribution. Immunoreactivity appears in the form of brightly fluorescent dots, many of which are aligned to outline the profile of large, irregularly shaped lacunae. These lacunae represent the lumena of blood capillaries. The distribution of immunoreactivity is therefore coincident with the localization of nerve terminals, which are tightly packed at the surface of the capillary walls (Tweedle, 1983).

Fig. 2 also shows a portion of the intermediate lobe of the pituitary. This lobe, which consists primarily of endocrine cells, is known to receive a sparse innervation (De Camilli et al., 1979). The scattered immunoreactive puncta visible on this lobe in the four fields of Fig. 2 represent these sparse nerve terminals.

Localization of Synapsin I, Protein III, and Synaptophysin in Nerve Terminals of the Bovine Neurohypophysis by Immunogold Electron Microscopy

To study the subcellular localization of synapsin I, Protein III, and synaptophysin in the neurohypophisis we have labeled subcellular particles of tissue homogenates embedded in an agarose matrix (De Camilli et al., 1983*b*; Navone et al., 1986). Because of the extremely small size of rat neurohypophyses, bovine pituitaries were used for these experiments. Homogenates of posterior pituitary tissue contain a large number of pinched-off nerve endings ("neurosecretosomes"). Depending upon our conditions of homogenization and fixation, a variable proportion of such neurosecretosomes had a partially disrupted plasmalemma although they still contained NSGs, MVs, and other organelles typical of intact nerve terminals. NSGs were not optimally preserved by our fixation conditions, which are primarily designed to maximize antigen accessibility. Their membranes were often discontinuous and their dense content appeared partially extracted. However, they were clearly recognizable. As in intact nerve endings, microvesicles were often grouped in clusters.

Fig. 3, *a–e*, and Fig. 4 show the localization of synapsin I, Protein III, and synaptophysin in such preparations as demonstrated by immunogold labeling. The pattern of labeling observed for the three antigens was very similar. Gold particles were concentrated in neurosecretosomes and within neurosecretosomes the great majority of gold particles was associated with MVs. No significant labeling of NSGs was observed. Many NSGs were completely devoid of gold particles even when their surface did not appear to be shielded from labeling by the presence of adjacent organelles. Some minor differences, however, were observed between preparations immunolabeled for synapsin I and Protein III (two peripheral membrane proteins [Huttner et al., 1983; Browning et al., 1987]) and preparations immunolabeled for synaptophysin (an integral membrane protein [Jahn et al., 1985; Wiedenmann and Franke, 1985]). Abundant synaptophysin immunoreactivity was detected on a few large vacuoles with a clear core. Similar structures had been found to be positive for synaptophysin in typical presynaptic nerve endings (Navone et al., 1986). Such structures were not labeled to any significant extent by synapsin I and Protein III antibodies which, on the other hand, produced a very low level of label-

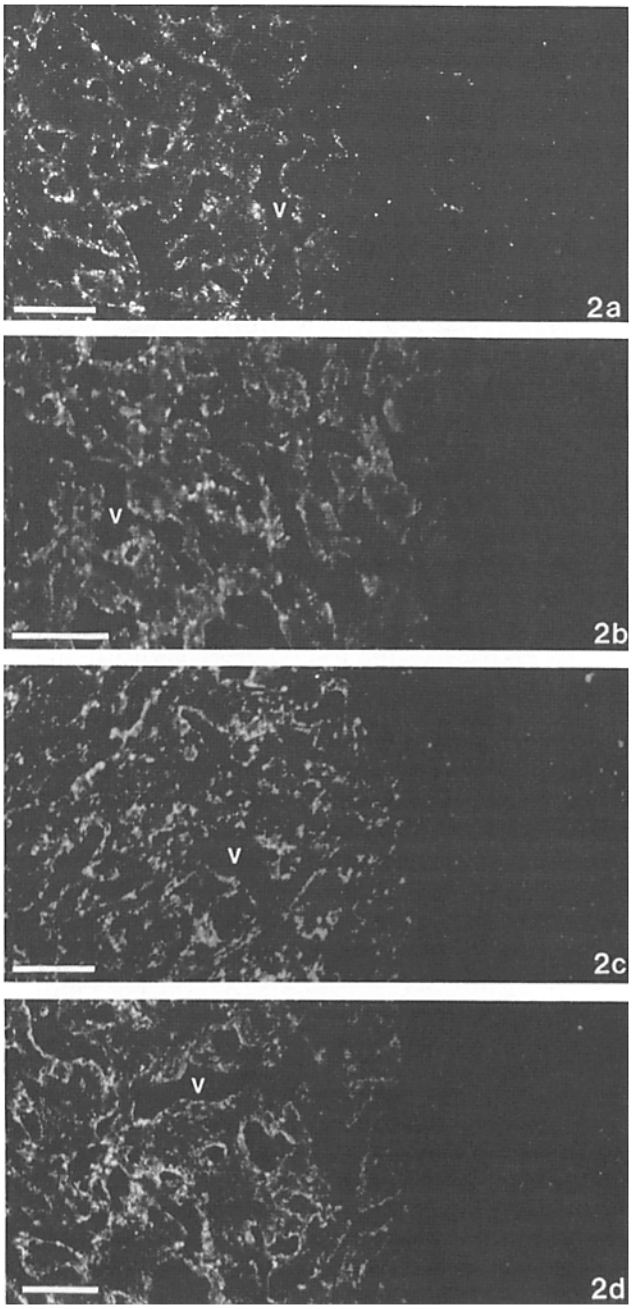

Figure 2. Localization of synapsin I, Protein III, and synaptophysin in rat pituitary tissue demonstrated by indirect immunofluorescence. 1-μm-thick plastic sections including a portion of the posterior lobe (*left*) and intermediate lobe (*right*). Primary antibodies used for the immunostain are as follows: polyclonal rabbit IgGs which recognize only synapsin I (*a*), monoclonal mouse IgGs specific for Protein III (*b*), polyclonal rabbit IgGs which recognize both synapsin I and Protein III (*c*), and polyclonal rabbit IgGs directed against synaptophysin (*d*). In all cases immunoreactivity is primarily concentrated in the posterior lobe and has a similar pattern of distribution represented by immunoreactive dots which outline black anastomosed spaces. These represent blood capillaries (*v*). The apparent size of the dots, which represent individual nerve terminals, is different in the four fields. This variability is to some extent a photographic artifact related to the different level of fluorescence obtained with the four antibodies. Sparse dots of immunoreactivity are visible in the intermediate lobe. These dots represent the few nerve terminals scattered in this lobe. In sections stained for synaptophysin (but not in those stained for synapsin I or Protein III), a moderate level of immunoreactivity was also visible in cells of the intermediate pituitary, in agreement with the previous demonstration that synaptophysin is also present in endocrine cells. Such staining is not visible in *d*, because this micrograph has been overexposed during printing to make staining intensity in the neurohypophysis similar to that of the other fields. Bars, 10 μm.

ing of most organelles, including fragments of the RER (see Discussion). Virtually no gold particles were observed in preparations labeled with nonimmune IgGs (Fig. 3 *f*).

Partition of Synapsin I, Protein III, Synaptophysin, and p65 in Sucrose Density Gradients

The distribution of SSV proteins and of neurosecretory granule marker proteins was analyzed by sucrose density centrifugation of a crude extract of the neurohypophysis. Because of their high protein content, neurosecretory granules exhibit a high buoyant density which allows their separation from most intracellular membranes, including MVs (Dean and Hope, 1967; Russell and Thorn, 1975). Therefore, organelles of a homogenate cleared of nuclei and unbroken neurosecretosomes by low speed centrifugation were separated on a linear sucrose density gradient and the various fractions analyzed for SSV and NSG marker protein content by Western blot. To obtain a quantitative estimate of the distribution of the different proteins, equal volumes of each fraction were assayed. SSV proteins analyzed were synapsin I, Protein III, synaptophysin, and, in addition, p65, which, like synaptophysin, is an intrinsic membrane protein (Matthew et al., 1981). As marker proteins for NSGs, neurophysin I and secretogranin II were used. Neurophysin I is a specific secretory peptide of posterior pituitary nerve endings (Nordmann et al., 1984). Secretogranin II is a peptide of still unknown function which is stored together with well-established peptide hormones in secretory granules of a large number of neurons and endocrine cells (Rosa et al., 1985).

Fig. 5 shows the immunoblots of the various fractions collected from the sucrose gradient. As expected from the known high buoyant density of NSGs, neurophysin I and secretogranin II were enriched in the denser (right in the figure) fractions of the gradient (peak at 1.64 M sucrose) (Fig. 5). As assessed by electron microscopy these fractions contained almost exclusively NSGs (see Fig. 6 *b* which shows the morphology of the fraction indicated by the double arrow in Fig. 5). In addition, neurophysin I and secretogranin II were also enriched in the first fractions of the gradient (left in Fig. 5). These corresponded to the "load" and contained the soluble proteins. Neurophysin I and secretogranin II in these fractions probably represented the content of granules which had been lysed during the homogenization.

The integral membrane proteins synaptophysin and p65 formed a single peak in the low density regions (maximal enrichment at 0.85 M sucrose) and were well segregated both from the neurosecretory granules and from the soluble proteins. The morphology of one of the fractions corresponding to the peak of synaptophysin and protein p65 is shown in Fig. 6 *a* (fraction indicated by a single arrow in Fig. 5). The fraction is heterogeneous but has a high content of 40–60-nm microvesicles, i.e., vesicles of the same diameter as MVs. The inset of the figure shows that the size of these small vesicles is identical to that of MVs decorated by immunogold for

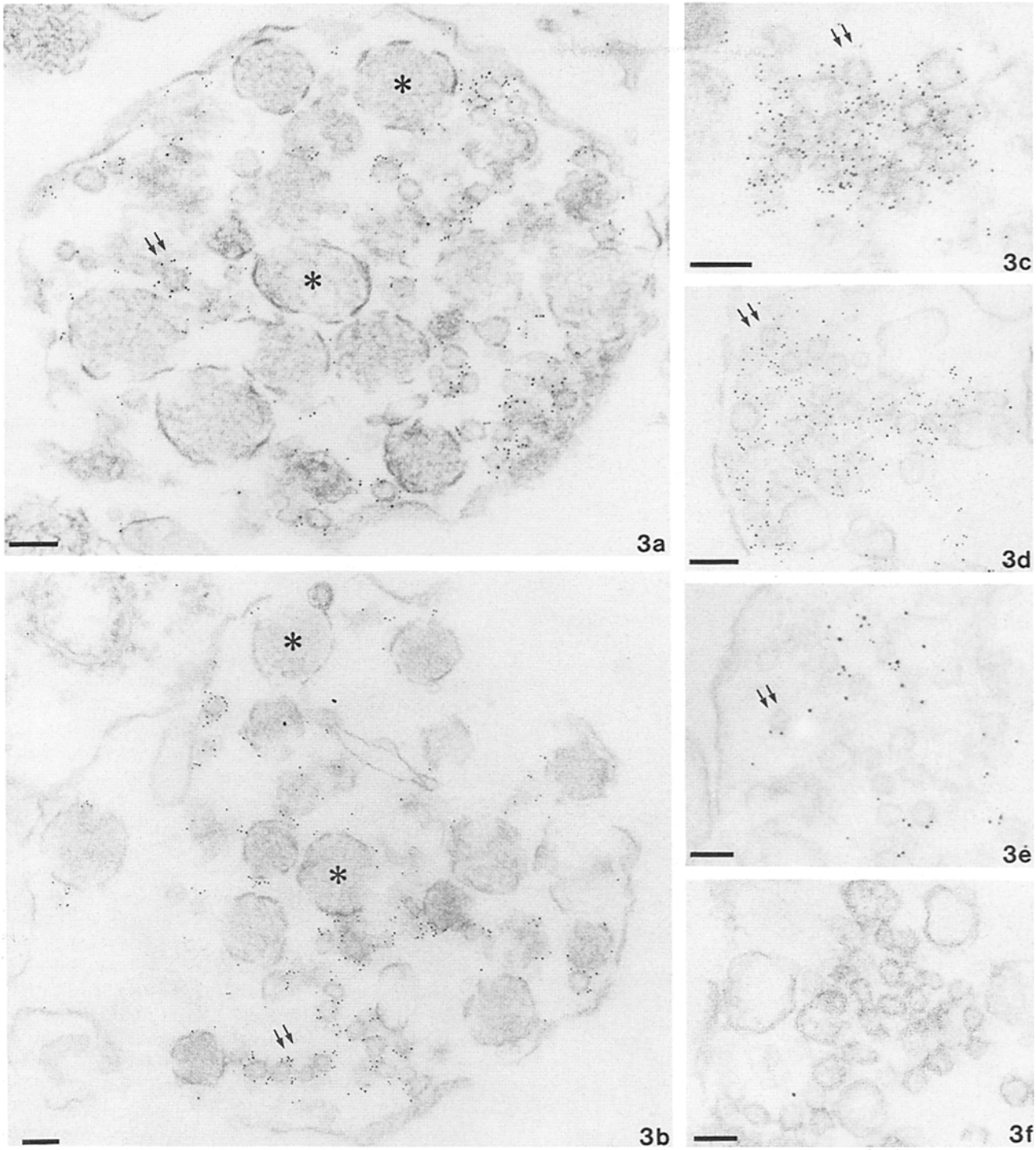

Figure 3. Neurosecretosomes of bovine neurohypophysis labeled by immunogold for synapsin I and for Protein III. Primary antibodies used for the immunolabeling were as follows: polyclonal rabbit IgGs which recognize only synapsin I (*a* and *c*), polyclonal rabbit IgGs which recognize both synapsin I and Protein III (*b* and *d*), monoclonal mouse IgGs specific for Protein III (*e*), and nonimmune control rabbit IgGs (*f*). In all fields, except in the control field, gold particles are concentrated on MVs (*small arrows*), which often form large clusters (*c* and *d*). Labeling intensity on MVs is much more prominent with polyclonal antibodies than with monoclonal antibodies. NSGs (*asterisks*) are partially broken and extracted because of the fixation conditions used. Bars, 100 nm.

synaptophysin in immunocytochemical experiments. In addition, the same fraction contains larger vacuolar profiles, some of which are in the same size range as the synaptophysin-positive vacuoles shown in Fig. 4.

The peripheral membrane proteins synapsin I and Protein III exhibited a sedimentation pattern somewhat different from that of synaptophysin and p65 (Fig. 5). They had a broader distribution extending into the denser part of the gradient. However, the amount of these proteins clearly declined in the fractions preceding the peak of NSG markers.

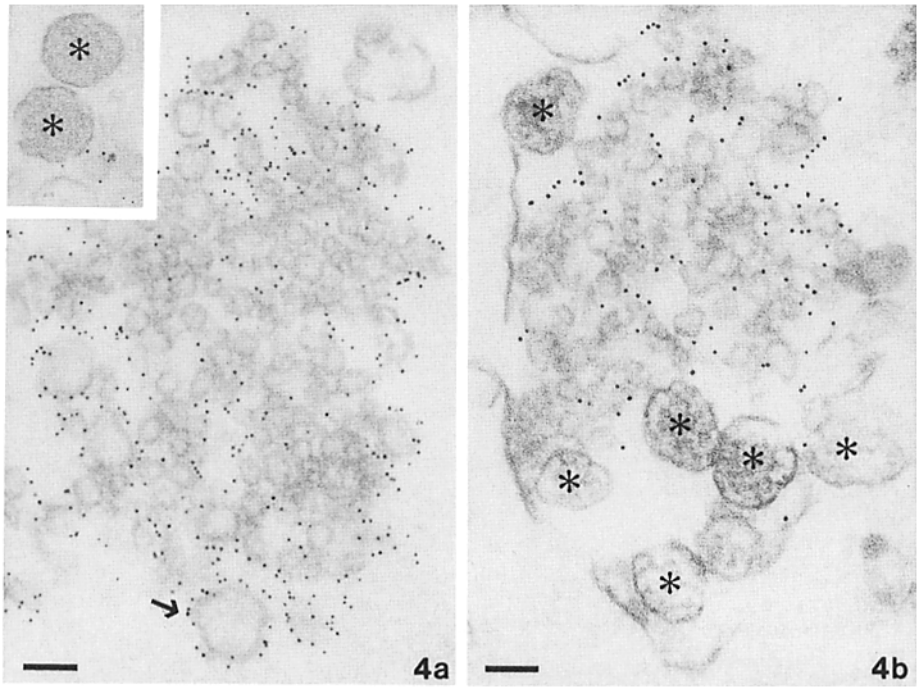

Figure 4. Lysed neurosecretosomes of bovine neurohypophysis labeled by immunogold for synaptophysin. Immunogold labeling is specifically associated with MVs and with larger vacuoles with a clear core (*arrow*). NSGs (*asterisks*) are unlabeled. Bar, 100 nm.

Low amounts of Protein III were also present in the fractions containing the soluble proteins (left in Fig. 5) suggesting that a small pool of Protein III may become soluble under our conditions of homogenization.

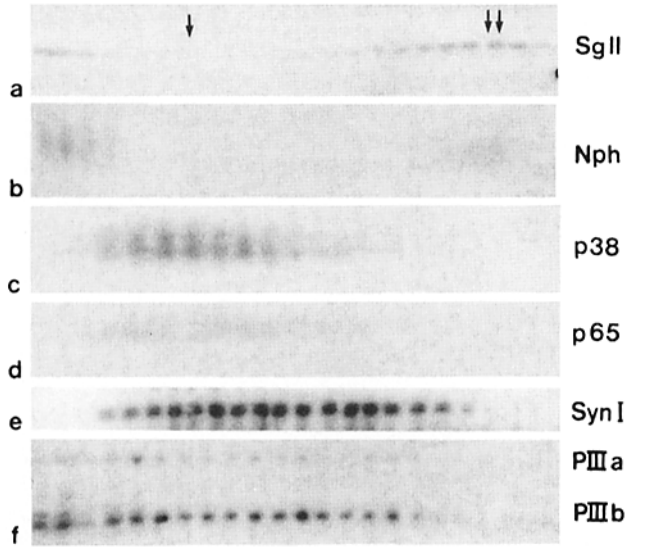

Figure 5. Western blot showing separation of SSV and NSG marker proteins in a sucrose gradient. A 4,000 *g* supernatant of a total homogenate of the bovine neurohypophysis was loaded onto a sucrose gradient ranging from 0.4 to 2 M. The first five fractions on the left correspond to the "load" of the gradient. Equal volumes of the various fractions were separated by SDS-PAGE, transferred electrophoretically to nitrocellulose paper, and paper strips corresponding to the molecular weight of interest were immunolabeled for the following proteins by immunoperoxidase (*a–d*) or radioimmunolabeling (*e* and *f*): secretogranin II (*a*), neurophysin (*b*), synaptophysin I p38 (*c*), protein p65 (*d*), synapsin I (*e*), and Protein III (*f*). A single arrow and a double arrow point to the two fractions whose morphology is shown in Fig. 6, *a* and *b*, respectively.

Discussion

In the present study we report morphological and biochemical evidence indicating that MVs of the neurohypophysis have at least a set of integral and peripheral membrane proteins in common with SSVs. In addition, we report that these proteins are not present at significant concentration in the membranes of NSGs. Highly selective labeling for MVs was observed when antibodies directed against synapsin I, Protein III, or synaptophysin were used for immunogold electron microscopy. The absence of these proteins from NSGs was confirmed by experiments employing sucrose-density gradient centrifugation. In addition, in the latter experiments, synaptophysin and p65 showed very similar sedimentation characteristics and were restricted to fractions containing MVs. This is in contrast with the recent report that a significant amount of p65 is present in the membrane of NSGs (Fournier and Trifarò, 1988). The broader distribution observed for synapsin I and Protein III in the sucrose gradient may be due to a pool of these proteins not associated with microvesicles in vivo, or to a partial dissociation of these two peripheral membrane proteins from MVs after homogenization. In the morphological experiments this pool of nonvesicular synapsin I and Protein III is probably reflected by the very low concentration of gold particles observed on most organelles. The finding of some labeling for these two proteins on the RER supports the idea of an artifactual redistribution. In homogenates of the neurohypophysis the RER is contributed only by nonneuronal supportive cells which do not express synapsin I and Protein III.

Our present observations parallel our previous findings that synapsin I, Protein III, and synaptophysin are not detectable by immunocytochemistry in LDCVs of other neurons (Navone et al., 1984, 1986, 1988) and complement those results with a subcellular fractionation approach. In addition, they suggest that p65 may also be restricted to SSVs in presynaptic nerve terminals. This study strongly supports

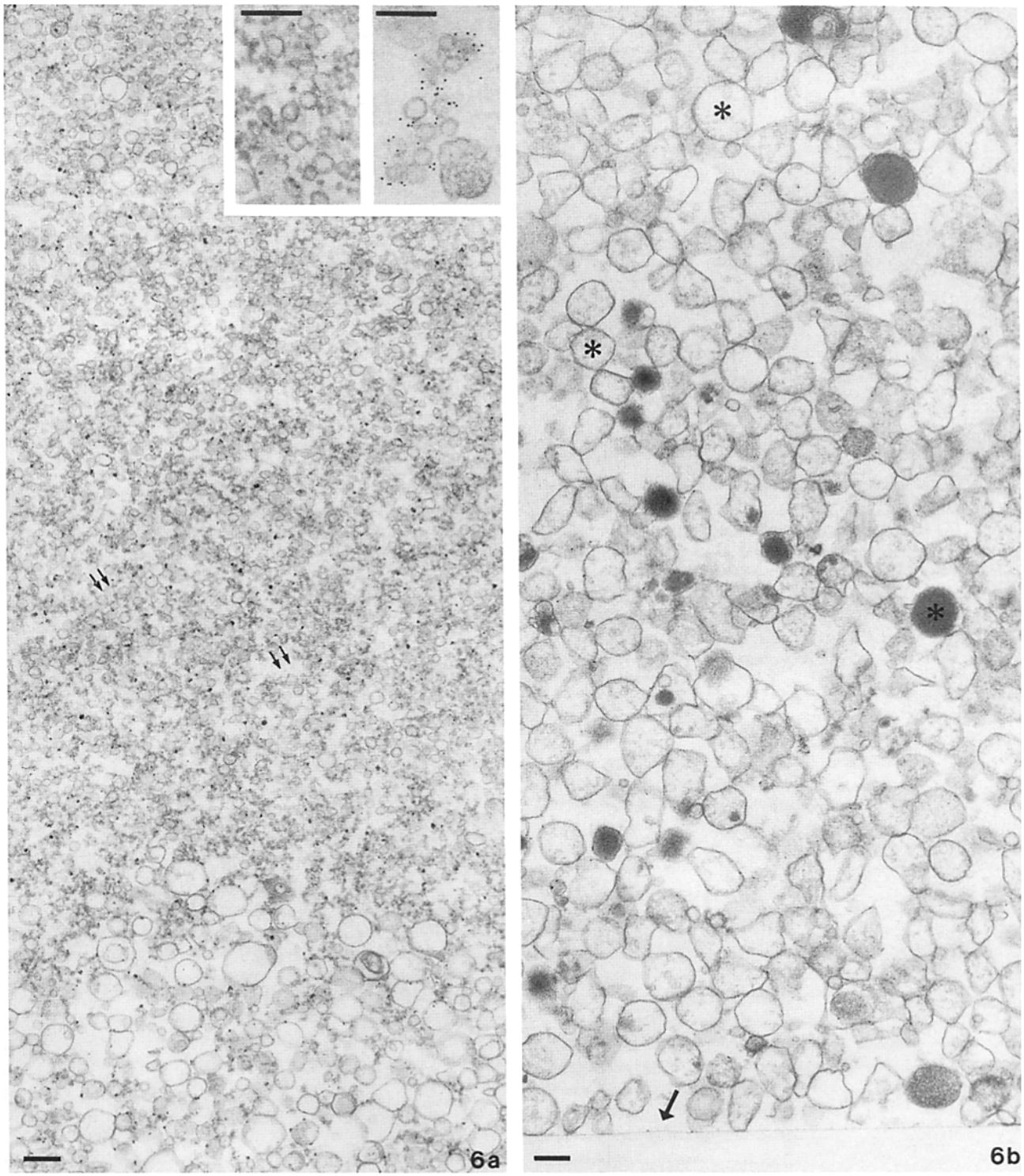

Figure 6. Electron microscopic appearance of organelles contained in the fractions indicated by an arrow (*a*) and by two arrows (*b*) in Fig. 5. The fraction shown in *a* has a heterogeneous composition but contains numerous small vesicles with the same average diameter as MVs (40–60 nm). The inset of *a* shows an enlargement of these vesicles (*left*) and a small cluster of MVs from a preparation of neurosecretosomes immunolabeled for synaptophysin (*right*). The two micrographs of the inset have been printed at the same final magnification (bars, 200 nm) and show that small vesicles of the sucrose gradient fraction have the same diameter as synaptophysin-positive MVs. The fraction shown in *b* is homogeneously composed of NSGs (*asterisks*). The variable density of the core of the granules is the result of the fixation (see also Figs. 3 and 4). Such variability is related to a partial lysis of the granules when fixation is performed at physiological pH. An arrow points to the bottom of the pellet. Bars, 200 nm.

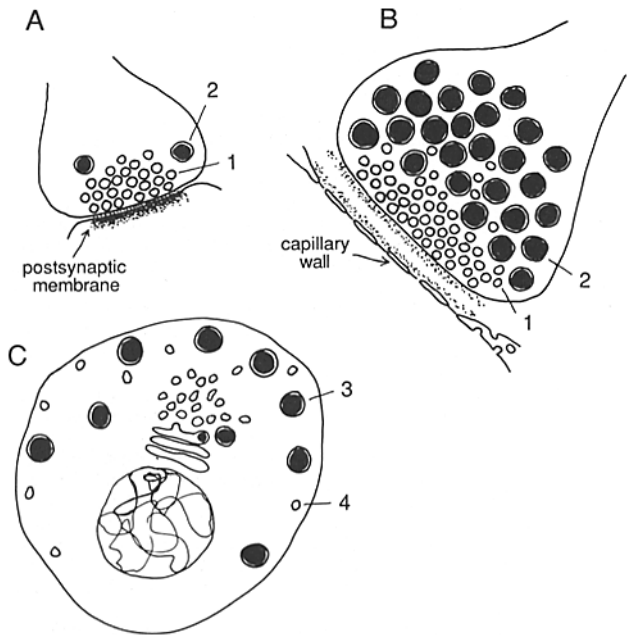

Figure 7. Schematic drawing illustrating the similarity between the organization of presynaptic nerve terminals (*A*) and terminals of the neurohypophysis (*B*), and the relation between vesicles of these terminals and vesicles of endocrine cells (*C*). Two vesicle populations, small clear vesicles (*1*) and larger granules with a dense core (*2*), coexist in the two types of nerve terminals. In both cases, small clear vesicles are clustered at specialized subplasmalemma sites (most likely exocytotic sites) and have in common some major membrane proteins which are not found on the membranes of the dense-core granules. We hypothesize that small clear vesicles have a related function in both types of terminals and have a biogenesis independent of dense-core secretory granules. The latter organelles are the equivalent of secretory granules of endocrine cells (*3*). The former are biochemically related to a subpopulation of microvesicles (*4*) of still unknown function present in endocrine cells (Navone et al., 1986; De Camilli and Navone, 1987). These endocrine microvesicles are slightly larger and more pleomorphic than SSVs and are concentrated at the *trans*-side of the Golgi complex.

the idea that MVs of the neurohypophysis are not an endocytic byproduct of exocytosis of NSGs. Were MVs derived from the membranes of NSGs, at least the two intrinsic membrane proteins synaptophysin and p65 would be found in the membranes of such granules. We cannot completely rule out the possibility that these proteins are present at a very low concentration in NSG membranes and that after NSG exocytosis they are selectively sorted and enriched into MVs. A similar model has been proposed by other authors to explain the generation of SSVs in presynaptic nerve terminals (Lowe et al., 1988; Kelly, 1988). However, we favor the hypothesis that MV membrane proteins travel to nerve terminals separately from NSGs, either as MVs or in some form of precursor membranes (De Camilli and Jahn, 1990).

The hypothesis that MVs of the neurohypophysis originate from NSG membranes had been generated by the finding that stimulation of posterior pituitary nerve terminals in the presence of an extracellular tracer leads to an increased uptake of the tracer into MVs (Douglas et al., 1970; Nagasawa et al., 1971). Our results indicate that MVs represent a distinct organelle which is structurally and possibly functionally related to SSVs. We hypothesize that stimulation of nerve terminals of the neurohypophysis triggers exocytosis of both NSGs and MVs and that the increased uptake of extracellular tracer into MVs after stimulation is likely to represent MV recycling. The same phenomenon is observed after stimulation of presynaptic nerve terminals (Holtzman et al., 1971; Heuser and Reese, 1973; Ceccarelli et al., 1973). Thus, our results are consistent with the possibility that retrieval of NSG membranes after exocytosis is achieved via large electrolucent vacuoles as suggested by some studies (Nordmann and Morris, 1976; Morris and Nordmann, 1980; Morris et al., 1981).

From this work, and from previous work of our laboratories, it appears that a common characteristic of all axon terminals, including presynaptic terminals, sensory terminals (De Camilli et al., 1988; Scarfone et al., 1988), and terminals of the neurohypophysis, is the presence of two types of secretory organelles with distinct content and membrane characteristics (Fig. 7). One is represented by dense-core vesicles (LDCVs of typical presynaptic terminals, NSGs of the neurohypophysis), which are assembled in the perikaryal region of the neurons and are primarily involved in the secretion of peptide molecules. These vesicles share many properties with, and are therefore highly related to, secretory granules of endocrine cells (Winkler, 1977; De Camilli and Navone, 1987; Russell, 1987; Johnson, 1987). The other population is represented by small clear vesicles (SSVs, MVs of the neurohypophysis) which undergo a local exo-endocytotic recycling in nerve terminals. It has been shown that these vesicles are biochemically related to a previously unknown population of microvesicles of endocrine cells (Navone et al., 1986; Wiedenmann et al., 1988). In presynaptic nerve endings small clear vesicles are involved in the secretion of classical neurotransmitters. The function of these organelles in sensory nerve endings and in terminals of the neurohypophysis, as well as that of the related microvesicles in endocrine cells, remains to be elucidated.

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