CELL STRUCTURE AND FUNCTION 19: 253–262 (1994)
© 1994 by Japan Society for Cell Biology

Redistribution of Synapsin I and Synaptophysin in Response to Electrical Stimulation in the Rat Neurohypophysial Nerve Endings

Toshihiro Hayashi, Frederic Soulie, Takao Nakata, and Nobutaka Hirokawa†

Department of Anatomy and Cell Biology, School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

Key words: synapsin I/synaptophysin/synapses/neurohypophysis/exocytosis

ABSTRACT. **To understand the dynamics of synaptic vesicles and synapsin I, we have studied the localization of synapsin I and synaptophysin in resting and stimulated nerve endings by ultracryomicrotomy and colloidal gold-immunocytochemistry. First, we characterized microvesicles in resting nerve endings of the rat neurohypophysis, which was chosen as the model of nerve ending in this study. Synaptophysin was localized in microvesicles that were clustered beneath the plasma membrane. Quick-freeze deep-etching electron microscopy showed that short strands cross-linked microvesicles to each other, which highly resembly the structures observed in our studies of the presynaptic nerve terminals of central and peripheral nervous system and *in vitro* reconstitution of synapsin I and synaptic vesicles. Immunocytochemistry showed that synapsin I was localized to the region of cluster of microvesicles. Second, using this system, we examined localization of synapsin I and synaptophysin in nerve endings after electrical stimulation. Besides release of neurosecretory granules, clusters of microvesicles disappeared and both microvesicles and synaptophysin were scattered over nerve endings. These changes were also confirmed by quick-freeze, freeze-substitution. Immunocytochemistry of the stimulated sample revealed that synapsin I was also scattered. The results show that microvesicles in neurohypophysis have similar characteristics of typical synaptic vesicles and synapsin I has a role as a scaffold to cross-link microvesicles to be clustered in resting nerve endings. This scaffold of synapsin I was disengaged after stimulation to redistribute microvesicles and synapsin I itself, which may be the mechanism of synapsin I to regulate the availability of synaptic vesicles for release.**

Nerve terminals contain clusters of synaptic vesicle near the active zones. Depolarization of nerve terminals induces Ca^{2+} entry through Ca^{2+} channels at the active zones followed by exocytosis of synaptic vesicles (11). Synapsin I, a major peripheral membrane protein of synaptic vesicles, is thought to regulate synaptic transmission (for review, see 7). In nerve terminals of central and peripheral nervous system, synapsin I cross-links synaptic vesicles to each other and/or to cytoskeletal elements (5, 6, 14, 15, 18, 29). Synapsin I binds to synaptic vesicles, F-actin and other cytoskeletal elements in phosphorylation dependent fashion *in vitro* (1, 2, 30, 32). Injection of dephospho-synapsin I inhibits and injection of calcium/calmodulin-dependent protein kinase II (CaM kinase II) facilitates synaptic transmission in the squid giant axon (19, 21, 22). Introduction of synapsin I or CaM kinase II into rat synaptosomes also shows similar effect on transmitter release (26). Thus it is proposed that synapsin I restrains synaptic vesicles to be clustered as a reserve pool in resting nerve ending and that phosphorylation of synapsin I by CaM kinase II after depolarization and accumulation of Ca^{2+} liberates synaptic vesicles and enable them to move to the active zones for release.

To capture the translocation of synapsin I and synaptic vesicles in response to stimulation may provide important information on the regulatory role of synapsin I in synaptic transmission. High potassium depolarization of synaptosomes translocates synapsin I to the cytosol fraction in phosphorylation dependent manner (34). However, little is known about the *in situ* dynamics of synapsin I and clustered vesicles during depolarization. Intensive stimulation by a-Latrotoxin with extracellular Ca^{2+} failed to show redistribution of synaptophysin, an intrinsic membrane protein of synaptic vesicles, and synapsin I in the frog neuromuscular junction (37). Fully packed clusters of synaptic vesicles, rapid recycling of synaptic vesicles and diffuse distribution of synapsin I in typical nerve terminals may hamper the capture of changes after stimulation.

The neurohypophysis, consisting mainly of nerve endings of hypothalamic magnocellular neurosecretory neu-

† To whom correspondence should be addressed.

rons, is suitable to deal with this problem. Focal localization of clusters of microvesicles among an abundance of neurosecretory granules in resting nerve endings make it easier to observe the translocation of synapsin I besides microvesicles after stimulation. These microvesicles, morphologically resembling typical synaptic vesicles, often cluster beneath the plasma membrane facing the basal lamina. Recent biochemical work showing the existence of synaptic vesicle-specific proteins in microvesicles suggests a similarity between microvesicles and synaptic vesicles, implying their secretory role (25).

In this work, we first characterized clusters of microvesicles in resting nerve endings in the neurohypophysis. Next we examined the dynamics of microvesicles and synapsin I after stimulation. We found the redistribution of microvesicles in the nerve endings after stimulation by quick-freeze, freeze-substitution, which enabled us to capture membrane events without any artifact of chemical fixation. We carried out immunogold labeling on cryo-ultrathin sections from unstimulated or electrically stimulated neurohypophyses for synaptophysin (16, 27, 38), as a marker of microvesicles, and synapsin I. We also observed resting nerve endings of neurohypophysis by quick-freeze deep-etch electron microscopy (QF-DE). This report shows the redistribution of synapsin I and microvesicles in the nerve endings of neurohypophyses after stimulation.

MATERIALS AND METHODS

Sample preparation and electrical stimulation. Neurohypophyses were isolated after decapitation of etherized male Wistar rats (5 weeks old). They were incubated in the physiological incubation buffer of the following composition (mM): NaCl, 155; KCl, 5.0; $CaCl_2$, 2.0; $MgCl_2$, 1.0; NaH_2PO_4, 0.25; glucose, 10; HEPES, 10 adjusted to pH 7.3 with NaOH. The medium was maintained at 37°C with oxygenated. A single gland was stimulated with monophasic pulse train at 50 Hz for 20 s through a bipolar electrode. Each stimulus pulse had an intensity of 1 mA and duration of 2 ms (33).

Conventional electron microscopy. Stimulated or unstimulated neurohypophyses were rapidly fixed by immersion in an ice-cold solution of 2.5% glutaraldehyde and 2% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer at pH 7.3 for 30 min. They were then left at room temperature for 2 h to allow further fixation. Post fixation with OsO_4 and block staining with uranyl acetate was carried out. The neurohypophyses were dehydrated with ethanol and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate.

Immunostaining on Cryo-ultrathin Sections. Sample prefixation was performed as described above except for fixative; a solution containing 0.1% glutaraldehyde and 2.5% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer at pH 7.3 was used for immunocytochemistry. Cryo-ultrathin sections were prepared as described previously (35). The sections were washed with 50 mM glycine in PBS for 15 min and blocked with 5% normal goat serum in PBS for 20 min. The sections were incubated for 1 h at 37°C with either diluted anti-synapsin I antibody or anti-synaptophysin antibody, whose characterization was perfomed elsewhere (9, 14, 27–29). Antibodies were diluted in 1% bovine serum albumin in PBS. After washing with 0.2% bovine serum albumin in TBS, pH 8.2, the sections were incubated for 1 h at 37°C with 5 nm colloidal gold-labeled second antibody (goat anti-rabbit IgG for anti-synapsin I labeled sections, goat anti-mouse IgG for anti-synaptophysin; Amersham International, Amersham, UK). Second antibodies were diluted 20-fold with 0.2% bovine serum albumin in TBS, pH 8.2. Following thorough wash, fixation with 3% glutaraldehyde in PBS and wash with distilled water, the sections were embedded in a mixture of uranyl acetate and polyvinyl alcohol according to Tokuyasu (36). Nonspecific rabbit IgG and mouse IgG instead of anti-synapsin I antibody and anti-synaptophysin antibody, respectively, were used as control.

Quick-Freeze, Deep-Etch Electron Microscopy of the Neurohypophysis. Isolated neurohypophyses, rinsed briefly with the physiological incubation buffer and hemisected along the sagittal plane, were quick-frozen with a liquid helium-cooled freezing machine. Quick-frozen samples were freeze-fractured, deep-etched, and rotary-replicated with platinum and carbon as described previously (10, 13).

Freeze Substitution. Quick-frozen samples were substituted in 2% OsO_4 in acetone at −80°C for 2 days. Samples were then incubated at −20°C, 4°C, and room temperature for 2 hrs respectively. After block staining in 1% uranyl acetate in acetone overnight, the samples were embedded in Epon 812 as described previously (12).

Electron Microscopy. Samples were examined at 100 kV with a JEOL 1200EX electron microscope (JEOL, Tokyo, Japan).

RESULTS

Localization of synaptophysin in microvesicles in resting nerve endings of the rat neurohypophysis. Nerve endings of neurohypophysis contain clusters of microvesicles of 50 nm in diameter beneath the plasma membrane facing the basal lamina among neurosecretory granules (Fig. 1). To characterize microvesicles, we examined localization of synaptophysin in nerve endings of resting neurohypophysis. Synaptophysin was localized in the region of the clustered microvesicles (Fig. 2). Only a few gold particles were observed on other membrane organelles such as neurosecretory granules, the plasma membrane, and mitochondria. This observation confirms that microvesicles are distinct membrane organells from neurosecretory granules and have similar characteristics of synaptic vesicles, allowing the use of synaptophysin as a marker of microvesicles.

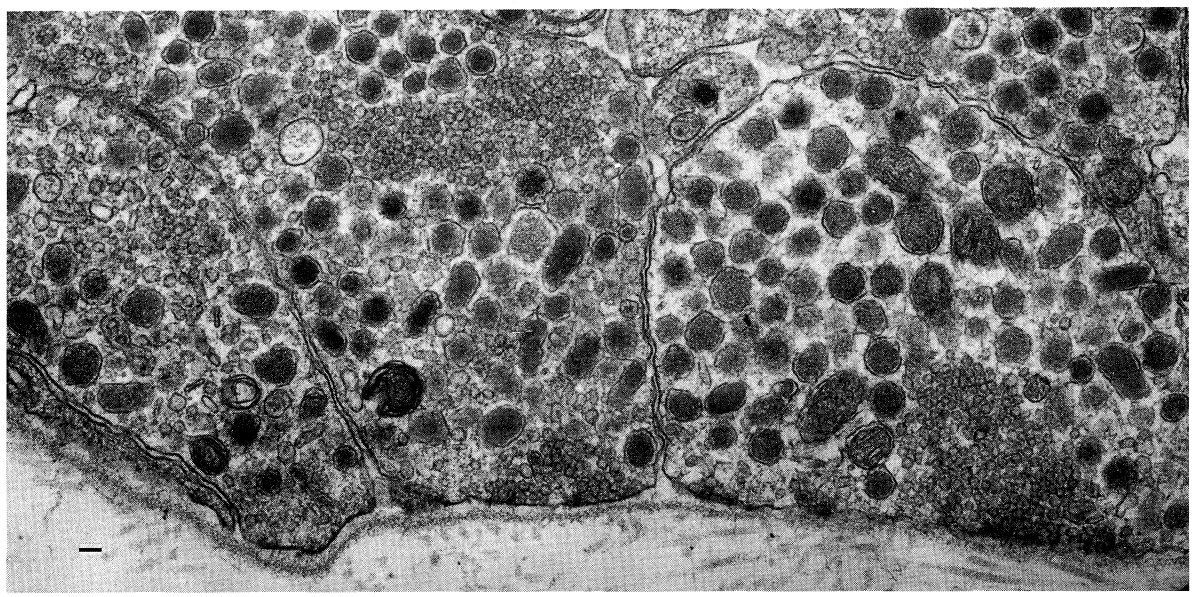


Fig. 1. Electron micrograph of resting neurohypophysial nerve endings in contact with the basal lamina. Among densely cored neurosecretory granules of 200 nm in diameter, nerve endings contain clusters of spherical microvesicles of 50 nm in diameter beneath the plasma membrane. Bar: 0.1 μm.

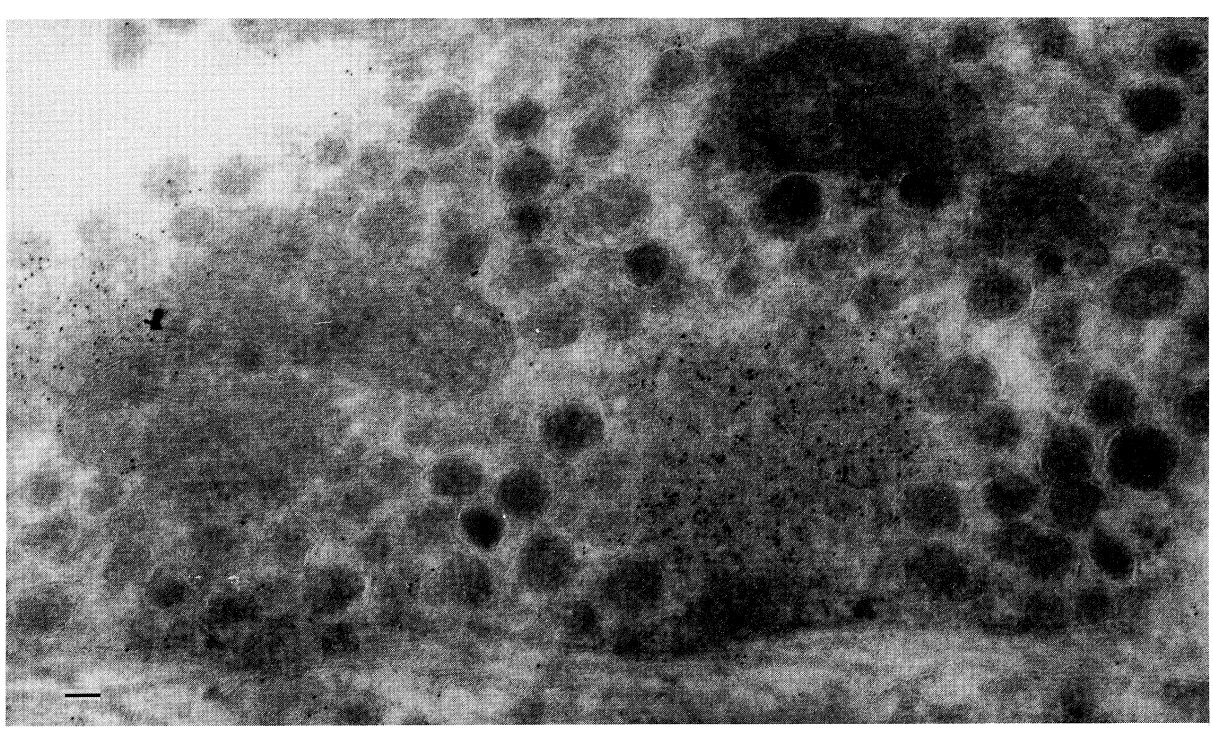


Fig. 2. Ultrathin cryosections of resting neurohypophysial nerve endings stained with anti-synaptophysin and colloidal gold-labeled second antibody. Gold particles are specifically observed in the region of the clusters of microvesicles. Few gold particles were observed on other membrane organelles such as neurosecretory granules, the plasma membrane, and mitochondria. Bar: 0.1 μm.

Cross-bridges of Synapsin I between clustered microvesicles. To examine clusters of microvesicles, we observed the molecular architecture of nerve endings of neurohypophysis by QF-DE (Fig. 3). The nerve endings were filled with neurosecretory granules. Among them, microvesicles were clustered beneath the plasma membrane. These micrographs clearly showed that short strands (30–60 nm) cross-link microvesicles (arrowheads) to make microvesicles clustered. These strands seemed to be specific to microvesicles, since neurosecretory granules were devoid of them. These findings highly resemble our previous observations by QF-DE of the presynaptic terminals and *in vitro* reconstitution of synapsin I and synaptic vesicles, which show that synapsin I are a component of cross-linking short strands (30–60 nm) between synaptic vesicles in presynaptic terminals (14).

To confirm that synapsin I is a component of short strands between microvesicles, we examined the localization of synapsin I in unstimulated nerve ending. Labeling patterns were similar to those of synaptophysin in unstimulated nerve ending. Gold particles were restrictively localized in the region of the clusters of microvesicles, whereas neurosecretory granules, the plasma membrane, and mitochondria were almost free of gold particles (Fig. 4B). Synapsin I are associated with microvesicles in high magnification view (Fig. 4A).

These observations show that synapsin I is indeed a component of short cross-bridges between microvesicles and that synapsin I constructs a scaffold (a cytoskeletal barrier) in which microvesicles are embedded. This fact and the localization of synaptophysin in microvesicles suggest the similarity in molecular architecture between clustered microvesicles in neurohypophyses and synaptic vesicles in typical presynaptic terminals.

Redistribution of synaptophysin after stimulation. To know the fate of clustered microvesicles after stimulation, we examined the localization of synaptophysin in the electrically stimulated nerve endings. Microvesicles were observed over the nerve endings after stimulation (Fig. 5). This change was confirmed with quick-freeze, freeze-substitution (Fig. 6). The number of neurosecretory vesicles was reduced as a result of exocytosis, and large vacuoles and pleomorphic vesicles, some of them thought to derive from membrane of neurosecretory granules, were observed instead. Synaptophysin was also scattered, showing that synaptophysin positive-clustered microvesicles are redistributed after stimulation (Fig. 7). In fact, clustered microvesicles or concentrated labeling of synaptophysin was hardly observed. Some labels were found near the plasma membrane.

Redistribution of synapsin I after stimulation. To determine whether synapsin I, now shown to be a part

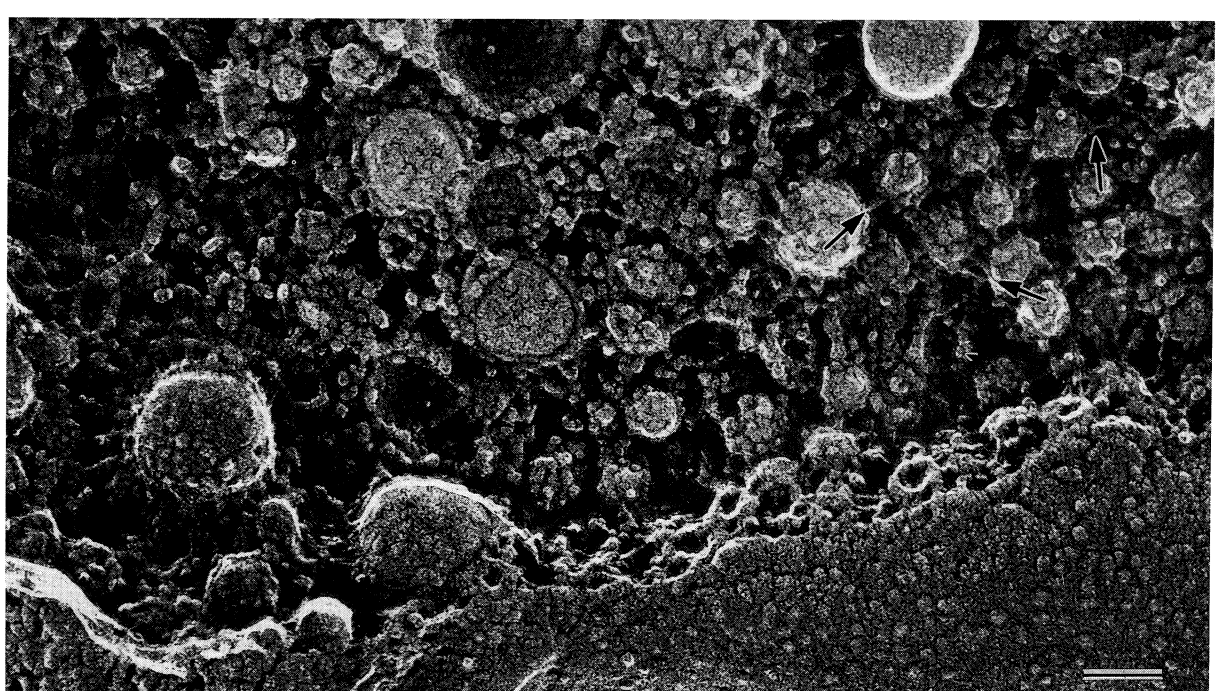


Fig. 3. Quick-freeze, deep-etch electron micrograph of unstimulated neurohypophysial nerve endings. Short linking strands of 30–60 nm are found between microvesicles (arrows). These strands seemed to be specific to microvesicles. Clustered microvesicles beneath the plasma membrane are cross-linked to each other by these strands. Bar: 0.1 μm.

of the scaffold of clustered microvesicles, remains localized in the restricted area or sparsely distributed when microvesicles are scattered after stimulation, we investigated the localization of synapsin I under the same conditions of stimulation used for the study of synaptophysin (Fig. 8). Our observation supports the latter to be

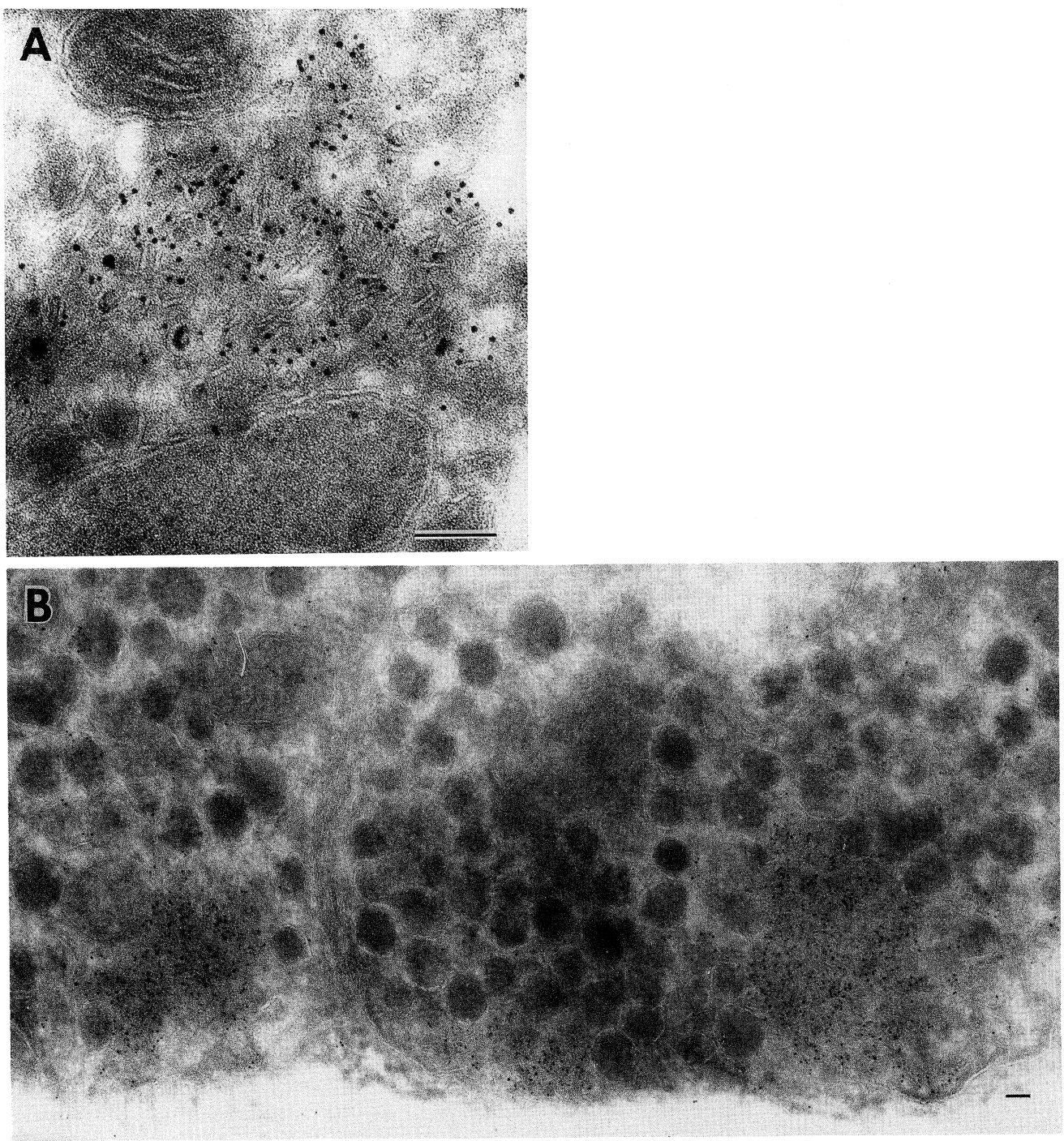


Fig. 4. Ultrathin cryosections of resting neurohypophysial nerve endings stained with anti-synapsin I and colloidal gold-labeled second antibody. The staining pattern of synapsin I is similar to that of synaptophysin (A). Gold particles are specifically observed in the region of the cluster of microvesicles and associated with microvesicles in high magnification view (B). Few gold particles were observed on other membrane organelles such as neurosecretory granules, the plasma membrane, and mitochondria. Bar: 0.1 μm.

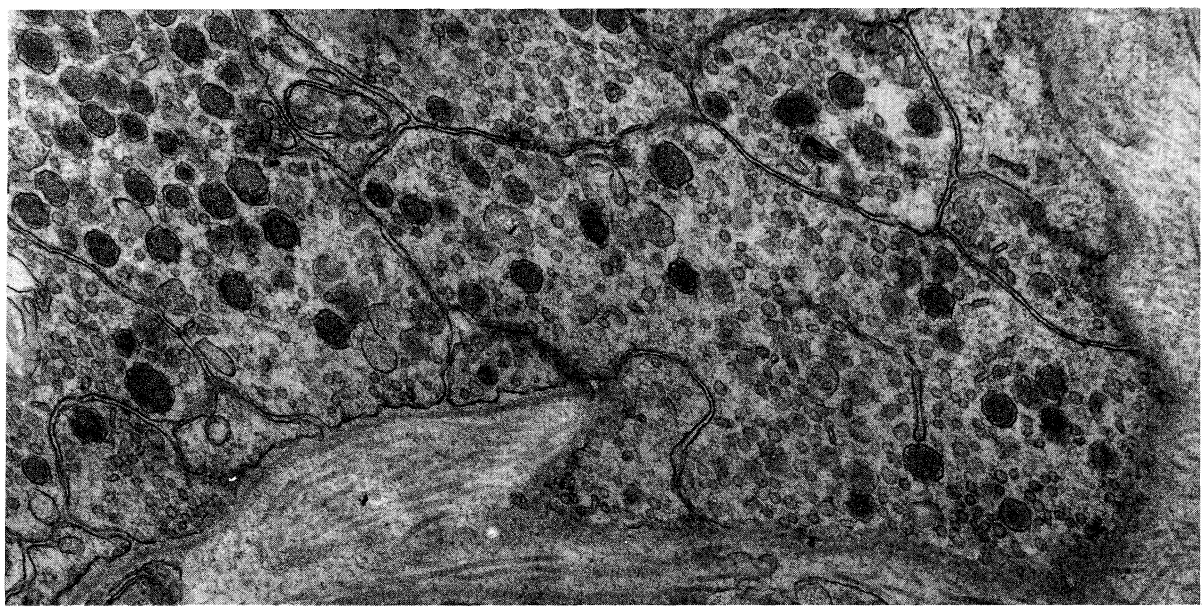


Fig. 5. Electron micrograph of neurohypophysial nerve endings electrically stimulated at 50 Hz for 20 s. The number of neurosecretory vesicles was reduced as a result of exocytosis after stimulation. Clusters of microvesicles disappeared and microvesicles were scattered over the nerve endings. Endosome-like pleomorphic vesicles are observed. Bar: 0.1 μm.

likely. Synapsin I was scattered among the nerve endings after stimulation; selectively concentrated localization as found in unstimulated nerve endings was not observed. These observations show that after stimulation, synapsin I is redistributed sparsely in the nerve endings in addition to the redistribution of microvesicles, rahter than remaining localized in the restricted area.

DISCUSSION

This study shows that microvesicles in neurohypophysis have similar characteristics of typical synaptic vesicles, and synapsin I has a role as a scaffold to cross-link microvesicles to be clustered in resting nerve endings. This scaffold of synapsin I is disengaged after stimulation to redistribute microvesicles and synapsin I itself. In unstimulated nerve endings, we observed by QF-DE that short strands cross-linked microvesicles to cluster (Fig. 3) and by immunocytochemistry that synapsin I was localized in the area with clusters of microvesicles (Fig. 4). In stimulated nerve endings, synapsin I was scattered (Fig. 8) and microvesicles were also sparsely distributed (Fig. 7).

Observations in unstimulated nerve endings are highly consistent with our earlier studies that synapsin I is a component of short cross-bridges between synaptic vesicles. QF-DE showed that short strands cross-link synaptic vesicles in the presynaptic terminal. Quite similar cross-bridges were observed by *in vitro* reconstitution of synaptic vesicles and synapsin I. Immunocytochemistry on ultracryosection showed the localization of synapsin I in synaptic vesicle-rich region (14). Furthermore, QF-DE after freeze-thaw immunocytochemistry showed that cross-bridges between synaptic vesicles are immuno-labeled with antisynapsin I antibody and immunogold, confirming that synapsin I is the constituent of cross-bridges (9). This consistency moreover lends credence to our present conclusion of a prevalent role of synapsin I in the scaffold to restrain synaptic vesicles to aggregate in resting nerve terminals.

Experiments in stimulated nerve endings were carried out to test the following two possible behaviors of synapsin I after stimulation that was found to cause redistribution of microvesicles. One possibility is that the scaffold, in which synapsin I may be a main component, remains; only microvesicles are released from the scaffold and redistributed in the nerve endings. The second possibility is that the scaffold is disengaged, and concurrently microvesicles are scattered. Our observation that synapsin I also scattered (Fig. 8) supports the second view. Since phosphorylation of synapsin I decreases its binding affinity with synaptic vesicles (32) and reduces its interaction with actin filaments (1, 30), phosphorylation of synapsin I by CaM kinase II after stimulation may detach synapsin I from microvesicles and/or F-actin, causing liberation of microvesicles and synapsin I from clusters and redistribution of microvesicles and synapsin I. In accordance with this, high potassium depolarization of synaptosomes results in translocation of synapsin I to the cytosol fraction in phosphor-



Fig. 6. Electron micrographs of quick-freeze, freeze-substitution of stimulated neurohypophysial nerve endings. The membrane organization was similar to those observed in conventional electron microscopy (Fig. 1 and 5.), while each membrane is smoother. Bar: 0.1 μm.

ylation dependent manner (34).

In contrast to our results in stimulated nerve endings, an earlier study using intensive stimulation by α-Latrotoxin with extracellular Ca^{2+} failed to show redistribution of synaptophysin and synapsin I in the frog neuromuscular junction (37). We suspect that their results by venom stimulation might not reflect the physiologically stimulated state of nerve terminals. Rapid recycling of synaptic vesicles may also hamper the capture of any change because they used 1 hour of stimulation. Our results by electrical stimulation (20 s) and rapid fixation are more likely to reflect physiological change in nerve endings under stimulation. Turnover of microvesicles in the neurohypophysis might not be so rapid as that in the neuromuscular junction. In addition, both high potassium depolarization and chronic dehydration also cause redistribution of synapsin I in the neurohypophysial nerve ending (unpublished data).

In this study, we also show that microvesicles in nerve endings of neurohypophysis have a similar characteristic of typical synaptic vesicles. First, synaptophysin and synapsin I were shown to be specifically localized in microvesicles by immunogold-ultracryotomy, which confirms the results of biochemical study (25). Second, mo-

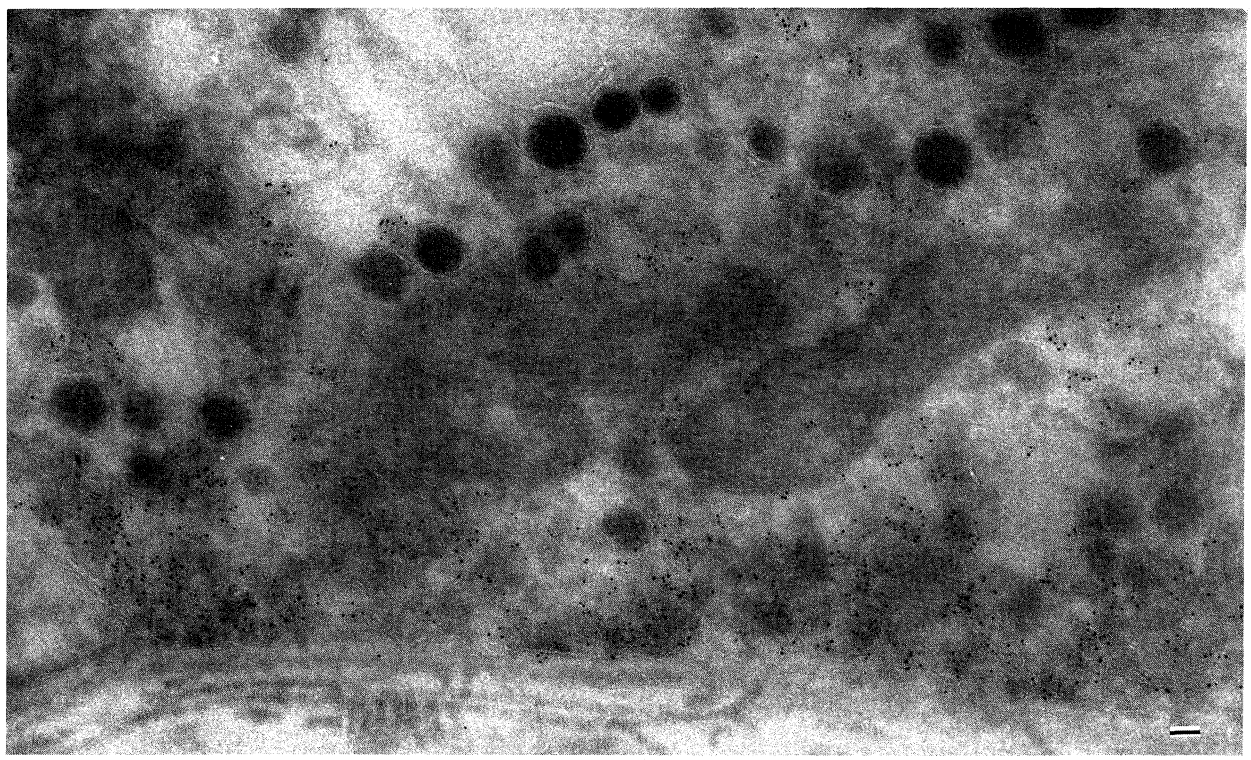


Fig. 7. Ultrathin cryosections of neurohypophysial nerve endings electrically stimulated at 50 Hz for 20 s stained with anti-synaptophysin and colloidal gold-labeled second antibody. Gold particles are diffusely distributed among the nerve endings. The view of clustered microvesicles or concentrated labeling of synaptophysin is hardly found. Some labels were found near the plasma membrane. Bar: 0.1 μm.

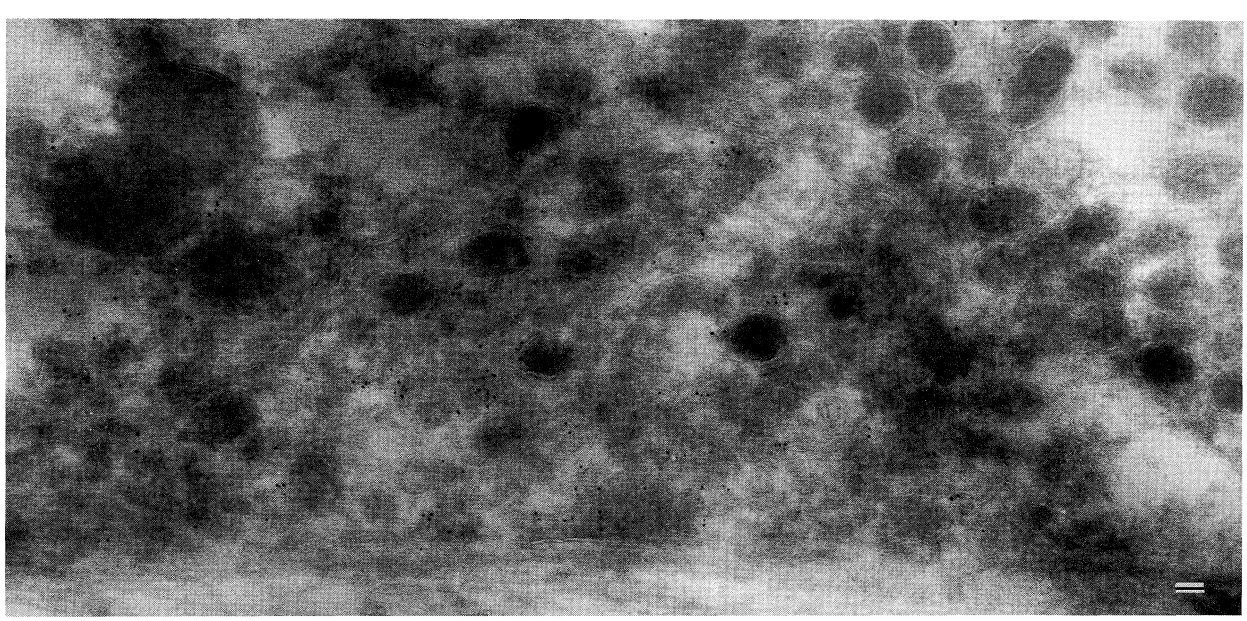


Fig. 8. Ultrathin cryosections of neurohypophysial nerve endings electrically stimulated at 50 Hz for 20 s stained with anti-synapsin I and colloidal gold-labeled second antibody. Gold particles were scattered in the nerve endings after stimulation; selectively concentrated localization of synapsin I as observed in unstimulated nerve endings was not found. Bar: 0.1 μm.

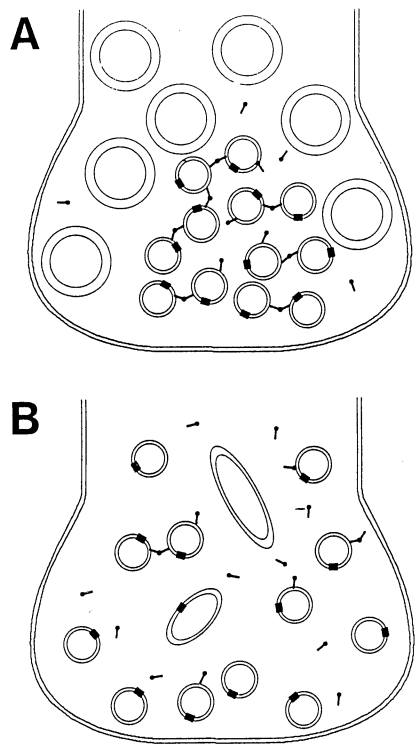


Fig. 9. Distribution of microvesicles, synapsin I and synaptophysin. (a) Resting nerve ending. Microvesicles in which synaptophysin is incorporated are clustered. Some synapsin I molecules are microvesicles crosslinking each other. (b) Stimulated nerve endings. Microvesicles are scattered among nerve ending. Pleomorphic vesicles are also seen. Some synapsin I are still binding to microvesicles and others are detached from microvesicles. Most crosslinks of synapsin I between microvesicles are disengaged.

lecular architecture of nerve endings of neurohypophysis revealed by QFDE, especially clustered microvesicles, were highly similar to those of the presynaptic terminals of central and peripheral nervous system (14). These observations support the hypothesis that microvesicles have characteristics similar to typical synaptic vesicles and that microvesicles and neurosecretory granules are distinct membrane organelles (25), rather than the hypothesis that microvesicles are the retrieved membrane of exocytosed neurosecretory granules (8, 24).

There was another finding that microvesicles and synaptophysin were scattered over nerve endings after stimulation (Figs. 5 and 6). We also found a few labels of synaptophysin near the plasma membrane. Morris and Nordmann (23) showed by morphometric study on the neurohypophysis that microvesicles are redistributed towards the plasma membrane facing the basal lamina under high potassium stimulation. These observations may imply regulated exocytosis of microvesicles. Indeed, one recent line of evidence suggests that synaptophysin-positive microvesicles perform exocytotic-endocytotic recycling. Synaptophysin is targeted into a pathway of recycling microvesicles in both neuroendocrine cell line PC12 and synaptophysin-transfected fibroblasts (17), and in PC12 cells synaptophysin is further sorted into specialized vesicle population similar to authentic synaptic vesicles (3, 4, 20). Synaptophysin-positive microvesicles in pancreatic β-cell are associated with GABA synthesizing enzyme and are suggested to store GABA (31). If microvesicles in neurohypophyses actually perform exocytosis, redistribution of microvesicles after stimulation may be meaningful because it agrees with the hypothesis that phosphorylation of synapsin I frees clustered vesicles and supplies releasable vesicles.

In summary, this study shows that synapsin I makes a scaffold in which microvesicles are incorporated by cross-linking in unstimulated nerve endings and that this scaffold is disengaged and microvesicles are also concurrently scattered in stimulated nerve endings (Fig. 9). Our conclusion provides *in situ* dynamics of synapsin I, which is thought to have a crucial role in regulation of availability of synaptic vesicles for release. We also confirm the similarity between microvesicles in the neurohypophysis and typical clear synaptic vesicles.

Acknowledgments. We thank Drs. Kenji Sobue and Kunihiko Obata for the supply of antibodies against synapsin I and synaptophysin and Dr. Katsuei Shibuki for his valuable advice. This work was supported by a Special Grant-in-Aid for Scientific Research from Ministry of Education, Science and Culture of Japan and grant from RIKEN to N. Hirokawa.

REFERENCES

1. Bähler, M. and Greengard, P. 1987. Synapsin I bundles F-actin in a phosphorylation-dependent manner. *Nature,* **326**: 704–707.
2. Benfenati, F., Bähler, M., Jahn, R., and Greengard, P. 1989. Interactions of synapsin I with small synaptic vesicles: distinct sites in synapsin I bind to vesicle phospholipids and vesicle proteins. *J. Cell Biol.,* **108**: 1863–1872.
3. Cameron, P.L., Südhof, T.C., Jahn, R., and De Camilli, P. 1991. Colocalization of synaptophysin with transferrin receptors: implications for synaptic vesicle biogenesis. *J. Cell Biol.,* **115**: 151–164.
4. Clift-O'Grady, L., Linstedt, A.D., Lowe, A.W., Grote, E., and Kelly, R.B. 1990. Biogenesis of synaptic vesicle-like structures in a pheochromocytoma cell line PC-12. *J. Cell Biol.,* **110**: 1693–1703.
5. De Camilli, P., Cameron, R., and Greengard, P. 1983. Synapsin I (protein I), a nerve terminal-specific phosphoprotein. I. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. *J. Cell Biol.,* **96**: 1337–1354.
6. De Camilli, P., Harris, S.J., Huttner, W.B., and Greengard, P. 1983. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. *J. Cell Biol.,* **96**: 1355–1373.
7. De Camilli, P., Benfenati, F., Valtorta, F., and

GREENGARD, P. 1990. The synapsins. *Annu. Rev. Cell Biol.,* **6**: 433–460.

8. DOUGLAS, W.W., NAGASAWA, J., and SCHULZ, R. 1970. Electron microscopic studies on the mechanism of secretion of posterior pituitary hormones and significance of microvesicles ("synaptic vesicles"): evidence of secretion by exocytosis and formation of microvesicles and a by-product of this process. *Mem. Soc. Endocrinol.,* **19**: 353–377.
9. HARADA, A., SOBUE, K., and HIROKAWA, N. 1990. Developmental changes of synapsin I subcellular localization in rat cerebellar neurons. *Cell Struct. Funct.,* **15**: 329–342.
10. HEUSER, J.E. and SALPETER, S.R. 1979. Organization of acetylcholine receptors in quick-frozen, deep-etched and rotary replicated *Torpedo* postsynaptic membrane. *J. Cell Biol.,* **82**: 150–173.
11. HEUSER, J.E., REESE, T.S., DENNIS, M.J., JAN, Y., JAN, L., and EVANS, L. 1979. Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. *J. Cell Biol.,* **81**: 275–300.
12. HIROKAWA, N. and KIRINO, T. 1980. An ultrastructural study of nerve and glia cells by freeze-substitution *J. Neurocytol.,* **9**: 243–254.
13. HIROKAWA, N. and HEUSER, J.E. 1981. Quick-freeze, deep-etch visualization of the cytoskeleton beneath surface differentiation of epithelial cells. *J. Cell Biol.,* **91**: 339–409.
14. HIROKAWA, N., SOBUE, K., KANDA, K., HARADA, A., and YORIFUJI, H. 1989. The cytoskeletal architecture of the presynaptic terminal and molecular structure of synapsin I. *J. Cell Biol.,* **108**: 111–126.
15. HUTTNER, W.B., SCHIEBLER, W., GREENGARD, P., and DE CAMILLI, P. 1983. Synapsin I (protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. *J. Cell Biol.,* **96**: 1374–1388.
16. JAHN, R., SCHIEBLER, W., OUIMET, C., and GREENGARD, P. 1985. A 38,000-dalton membrane protein (p38) present in synaptic vesicles. *Proc. Natl. Acad. Sci. USA,* **82**: 4137–4141.
17. JOHNSTON, P.A., CAMERON, P.L., STUKENBROK, H., JAHN, R., DE CAMILLI, P., and SÜDHOF, T.C. 1989. Synaptophysin is targeted to similar microvesicles in CHO and PC12 cells. *Embo J.,* **8**: 2863–2872.
18. LANDIS, D.M., HALL, A.K., WEINSTEIN, L.A., and REESE, T.S. 1988. The organization of cytoplasm at the presynaptic active zone of a central nervous system synapse. *Neuron,* **1**: 201–209.
19. LIN, J.W., SUGIMORI, M., LLINÁS, R.R., MCGUINNESS, T.L., and GREENGARD, P. 1990. Effects of synapsin I and calcium/calmodulin-dependent protein kinase II on spontaneous neurotransmitter release in the squid giant synapse. *Proc. Natl. Acad. Sci. USA,* **87**: 8257–8261.
20. LINSTEDT, A.D. and KELLY, R.B. 1991. Synaptophysin is sorted from endocytotic markers in neuroendocrine PC12 cells but not transfected fibroblasts. *Neuron,* **7**: 309–317.
21. LLINÁS, R., MCGUINNESS, T.L., LEONARD, C.S., SUGIMORI, M., and GREENGARD, P. 1985. Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase 2 alters neurotransmitter release at the squid giant synapse. *Proc. Natl. Acad. Sci. USA,* **82**: 3035–3039.
22. LLINÁS, R., GRUNER, J.A., SUGIMORI, M., MCGUINNESS, T.L., and GREENGARD, P. 1991. Regulation by synapsin I and Ca^{2+}-calmodulin-dependent protein kinase II of the transmitter release in squid giant synapse. *J. Physiol. (Lond),* **436**: 257–282.
23. MORRIS, J.F. and NORDMAMM, J.J. 1980. Membrane recapture after hormone release from nerve endings in the neural lobe of the rat pituitary gland. *Neurosci.,* **5**: 639–649.
24. NAGASAWA, J., DOUGLAS, W.W., and SCHULZ, R.A. 1971. Micropinocytotic origin of coated and smooth microvesicles ("synaptic vesicles") in neurosecretory terminals of posterior pituitary glands demonstrated by incorporation of horseradish peroxidase. *Nature,* **232**: 341–342.
25. NAVONE, F., DI GIOIA, G., JAHN, R., BROWNING, M., GREENGARD, P., and DE CAMILLI, P. 1989. Microvesicles of the neurohypophysis are biochemically related to small synaptic vesicles of presynaptic nerve terminals. *J. Cell Biol.,* **109**: 3425–3432.
26. NICHOLS, R.A., CHILCOTE, T.J., CZERNIK, A.J., and GREENGARD, P. 1992. Synapsin I regulates glutamate release from rat brain synaptosomes. *J. Neurochem.,* **58**: 783–785.
27. OBATA, K., NISHIYE, H., FUJITA, S.C., SHIRAO, T., INOUE, H., and UCHIZONO, K. 1986. Identification of a synaptic vesicle-specific 38,000-dalton protein by monoclonal antibodies. *Brain Res.,* **375**: 37–48.
28. OBATA, K., KOJIMA, N., NISHIYE, H., INOUE, H., SHIRAO, T., FUJITA, S.C., and UCHIZONO, K. 1987. Four synaptic vesicle-specific proteins: identification by monoclonal antibodies and distribution in the nervous tissue and the adrenal medulla. *Brain Res,* **404**: 169–179.
29. OKABE, T. and SOBUE, K. 1987. Identification of a new 84/82 kDa calmodulin-binding protein, which also interacts with actin filaments, tubulin and spectrin, as synapsin I. *Febs Lett,* **213**: 184–188.
30. PETRUCCI, T.C. and MORROW, J.S. 1987. Synapsin I: an actin-bundling protein under phosphorylation control. *J. Cell Biol.,* **105**: 1355–1363.
31. REETZ, A., SOLIMENA, M., MATTEOLI, M., FOLLI, F., TAKEI, K., and DE CAMILLI, P. 1991. GABA and pancreatic β-cell: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. *EMBO J.,* **10**: 1275–1284.
32. SCHIEBLER, W., JAHN, R., DOUCET, J.P., ROTHLEIN, J., and GREENGARD, P. 1986. Characterization of synapsin I binding to small synaptic vesicles. *J. Biol. Chem.,* **261**: 8383–8390.
33. SHIBUKI, K. 1990. Activation of neurohypophysial vasopressin release by Ca^{2+} influx and intracellular Ca^{2+} accumulation in the rat. *J. Physiol. (Lond),* **422**: 321–331.
34. SIRA, T.S., WANG, J.K.T., GORELICK, F.S., and GREENGARD, P. 1989. Translocation of synapsin I in response to depolarization of isolated nerve terminals. *Proc. Natl. Acad. Sci. USA,* **86**: 8108–8112.
35. TOKUYASU, K.T. 1980. Immunochemistry on ultrathin frozen sections. *Histochem. J.,* **12**: 381–403.
36. TOKUYASU, K.T. 1989. Use of poly(vinylpyrrolidone) and poly(vinyl alcohol) for cryoultramicrotomy. *Histochem. J.,* **21**: 163–171.
37. TORRI-TARELLI, F., VILLA, A., VALTORTA, F., DE CAMILLI, P., GREENGARD, P., and CECCARELLI, B. 1990. Redistribution of synaptophysin and synapsin I during alphalatrotoxin-induced release of neurotransmitter at the neuromuscular junction. *J. Cell Biol.,* **110**: 449–459.
38. WIEDENMANN, B. and FRANKE, W.W. 1985. Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of presynaptic vesicles. *Cell,* **41**: 1017–1028.

(Received for publication, April 26, 1994
and in revised form, May 27, 1994)