

Clathrin-coated Vesicles in Nervous Tissue Are Involved Primarily in Synaptic Vesicle Recycling

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Abstract. The recycling of synaptic vesicles in nerve terminals is thought to involve clathrin-coated vesicles. However, the properties of nerve terminal coated vesicles have not been characterized. Starting from a preparation of purified nerve terminals obtained from rat brain, we isolated clathrin-coated vesicles by a series of differential and density gradient centrifugation steps. The enrichment of coated vesicles during fractionation was monitored by EM. The final fraction consisted of >90% of coated vesicles, with only negligible contamination by synaptic vesicles. Control experiments revealed that the contribution by coated vesicles derived from the axo-dendritic region or from nonneuronal cells is minimal.

The membrane composition of nerve terminal-derived coated vesicles was very similar to that of synaptic vesicles, containing the membrane proteins synaptophysin, synaptotagmin, p29, synaptobrevin and the 116-kD subunit of the vacuolar proton pump, in simi-

lar stoichiometric ratios. The small GTP-binding protein rab3A was absent, probably reflecting its dissociation from synaptic vesicles during endocytosis. Immunogold EM revealed that virtually all coated vesicles carried synaptic vesicle proteins, demonstrating that the contribution by coated vesicles derived from other membrane traffic pathways is negligible. Coated vesicles isolated from the whole brain exhibited a similar composition, most of them carrying synaptic vesicle proteins. This indicates that in nervous tissue, coated vesicles function predominantly in the synaptic vesicle pathway. Nerve terminal-derived coated vesicles contained AP-2 adaptor complexes, which is in agreement with their plasmalemmal origin. Furthermore, the neuron-specific coat proteins AP 180 and auxilin, as well as the α_2 - and α_1 -adaptins, were enriched in this fraction, suggesting a function for these coat proteins in synaptic vesicle recycling.

CHEMICAL neurotransmission occurs via the regulated exocytosis of synaptic vesicles in nerve terminals. Synaptic vesicles are highly specialized organelles containing a unique set of membrane proteins such as synapsin, synaptophysin, synaptobrevin, synaptotagmin, and also the small GTP-binding protein rab3A. Synaptic vesicles are thought to originate from the *trans*-Golgi network and to be transported by axonal transport to the nerve terminal where they undergo repeated cycles of exo-endocytosis (for review see Kelly, 1988; De Camilli and Jahn, 1990; Trimble et al., 1991; Südhof and Jahn, 1991).

The details of synaptic vesicle membrane trafficking in the nerve terminal are still poorly understood. In particular, the mechanism of vesicle membrane retrieval from the plasma membrane following exocytosis remains controversial. There is agreement that recycling via coated vesicles is involved at least under certain conditions (Heuser and Reese, 1973; Miller and Heuser, 1984). However, the extent to

which alternative endocytic pathways are involved is unclear. It has been proposed that the majority of the vesicle membrane is retrieved directly to reform synaptic vesicles, without the formation of intermediate coated stages. This is supported by the lack of correlation between the number of coated vesicles and the intensity of stimulation (Ceccarelli and Hurlbut, 1980; Torri-Tarelli et al., 1987). Synaptic vesicles may also re-form via large cisternal intermediates which pinch off directly from the plasma membrane. Such intermediates are frequently observed by EM after strong stimulation (Heuser and Reese, 1973; Miller and Heuser, 1984). Despite these alternatives, the recycling of synaptic vesicles via clathrin coated vesicles can be regarded as a prominent pathway (for a discussion of this topic see, e.g., Ceccarelli and Hurlbut, 1980; Heuser, 1989).

The coat protein composition of brain-derived clathrin-coated vesicles has been studied in detail and is known to contain several neuron-specific protein components. The clathrin heavy chain and two light chains (LC_a and LC_b), each a separate gene product, are present in all tissues (reviewed in Brodsky et al., 1991). In neurons, two addi-

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tional isoforms of LC_a and one additional form of LC_b exist. They are generated by alternative splicing events resulting in the insertion of short stretches of amino acids (Jackson et al., 1987). Similarly, neuron-specific variants of α - and β -adaplin chains were identified (Robinson, 1989; Ponnambalam et al., 1990). These proteins are components of the adaptor protein complexes which are thought to link the clathrin coat to membrane components (reviewed in Pearse and Robinson, 1990). Two such adaptor complexes have been identified, AP-1 and AP-2, that are present in all cells. Furthermore, two additional coat components have been identified that are specific for neurons: AP 180 (Ahle and Ungewickell, 1986) which is probably identical with NP 185 (Kohtz and Puszkin, 1988) and AP 3 (Murphy et al., 1991), and auxilin (Ahle and Ungewickell, 1990).

It is still largely unknown to what extent populations of coated vesicles operating in different steps of membrane trafficking differ from each other with regard to coat composition. It was demonstrated that the adaptor complex AP-1 is specific for coated vesicles functioning at the exit site of the *trans*-Golgi network, whereas AP-2 is specific for coated vesicles involved in endocytosis of plasma membrane components (Ahle et al., 1988; for review see Morris et al., 1989; Keen, 1990). However, the functional significance of the neuron-specific coat proteins and their variants is still an enigma. The recycling of synaptic vesicles in nerve terminals represents a highly specialized and greatly amplified membrane trafficking pathway and it is possible that coated vesicle function in this step is also specialized, requiring specific coat components. Therefore, it is important to evaluate which of the coat proteins expressed in neurons are components of the coated vesicles involved in this step.

In the present study, we have attempted the biochemical characterization of the coated vesicle subpopulation involved in synaptic vesicle recycling. For this purpose, we have developed a procedure to isolate coated vesicles from nerve terminals free of contamination by coated vesicles derived from glial cells or neuronal cell bodies. To identify the origin of the coated vesicle membrane, we have analyzed the membranes for the presence of synaptic vesicle proteins. Our results indicate that coated vesicles isolated from nerve terminals are exclusively derived from synaptic vesicles, with no evidence for other pathways, and that these coated vesicles contain AP-2 adaptor components as well as the neuron-specific coat proteins auxilin and AP 180. Furthermore, our data indicate that the synaptic vesicle membrane composition remains virtually unchanged during recycling, with the exception of the G-protein rab3A.

Materials and Methods

Antibodies

Polyclonal antibodies against the 116-kD subunit of the vacuolar proton pump (Perin et al., 1991) were kindly provided by Dr. T. C. Südhof (University of Texas, Dallas, Texas). Mouse mAbs raised against bovine β - and β' -adaplin (mAb 100/1) and α -adaplin (mAb 100/2) (Ahle et al., 1988), auxilin (mAb 100/4; Ahle and Ungewickell, 1990) and AP 180 (mAb AP180-1; Ahle and Ungewickell, 1986) were kind gifts of Dr. E. Ungewickell (Max-Planck-Inst. für Biochemie, Martinsried, Germany). mAbs against the Golgi-specific protein Gimpt (Yuan et al., 1987) were made available by Drs. Suarez-Qiuan (National Institutes of Health, Bethesda, MD) and I. V. Sandoval (University of Madrid, Madrid, Spain). mAbs directed against clathrin heavy chain were obtained from Boehringer (Mannheim, Germany). The following monoclonal and polyclonal antibodies directed

against synaptic vesicle proteins were described previously: rab3A (clone Cl 42.2; Matteoli et al., 1991); synaptophysin (clone C 7.2; Jahn et al., 1985); synaptobrevin (rabbit serum; Baumert et al., 1989); synaptotagmin (clone Cl 41.1; Brose et al., 1992); p29 (rabbit serum; Baumert et al., 1990). Polyclonal antibodies directed against the neuron-specific insert of clathrin light chain (LC_a) were obtained by immunizing rabbits with a synthetic peptide (CVADEAFYKQPFAD) corresponding to the first part of the insert (Jackson et al., 1987) which was coupled to hemocyanin as a carrier (Schneider et al., 1983). For immunocytochemistry, the serum was affinity purified using an enriched light chain-containing fraction as adsorbent which was separated by SDS-PAGE and blotted onto nitrocellulose membrane filters (Olmsted, 1987). The resulting antibodies were specific for neuronal clathrin light chains and showed no reaction with nonneuronal clathrin light chains (tested with adrenal gland) nor labeling of coated structures by immunofluorescence in nonneuronal cell lines. Western blotting revealed labeling of both neuronal light chains a and of light chain b, indicating that the epitopes are residing on the shared sequence elements of the inserts (data not shown).

Subcellular Fractionation

All steps were carried out at 4°C. Coated vesicles were purified from rat brain and rat liver according to a modification of several published procedures (see, e.g., Pearse, 1983). 30 rat brains were homogenized in 300 ml of buffer A (0.1 M MES/NaOH, pH 6.5, 1 mM EGTA, 0.5 mM MgCl₂) using a glass-teflon homogenizer (10 strokes at 1,500 rpm) using a glass-teflon homogenizer (10 strokes at 1,500 rpm). The homogenate was centrifuged at 20,000 g for 20 min (SS 34 rotor; Sorvall Du Pont, Wilmington, DE). The supernatant was collected and centrifuged at 55,000 g for 1 h (Ti45 rotor; Beckman Instruments, Inc., Palo Alto, CA). The pellets were resuspended in a total volume of 20 ml buffer A (three strokes at 2,000 rpm in a glass-teflon homogenizer, followed by dispersion through a 27-gauge needle). The suspension was then mixed with 20 ml of buffer A containing 12.5% (wt/vol) Ficoll and 12.5% (wt/vol) sucrose and centrifuged for 40 min at 40,000 g (SS34 rotor). The supernatant was removed, diluted 1:5 in buffer A and centrifuged for 1 h at 100,000 g (Ti45 rotor) to pellet coated vesicles. The pellet was resuspended as above in 30 ml of buffer A and cleared by centrifugation at 20,000 g for 20 min. The supernatant was layered on top of buffer A prepared with D₂O containing 8% (wt/vol) sucrose and centrifuged for 2 h at 25,000 rpm in a Beckman SW28 rotor. The final pellet containing purified coated vesicles was resuspended in 0.6 ml of buffer A.

For the preparation of coated vesicles from nerve terminals, synaptosomes were isolated and washed to remove any contaminating coated vesicles derived from other cellular compartments. For this purpose, 60 rat brains were homogenized in 0.32 M sucrose in a glass-teflon homogenizer (600 rpm, 10 strokes). The homogenate was centrifuged for 2 min at 5,000 rpm (SS34 rotor). The supernatant was removed and centrifuged for 12 min at 11,000 rpm (SS34 rotor). The pellet was resuspended in 0.1 M K₂-tartrate (pH 7.3) and centrifuged at 6,500 rpm for 4 min (SS34 rotor). This was sufficient to resediment synaptosomes due to the lower density and viscosity of the tartrate buffer. The outer, white part of the pellet was resuspended in tartrate buffer (avoiding the mitochondria-containing reddish core of the pellet) and recentrifuged. The entire pellet was resuspended and recentrifuged as before. The pellets were then resuspended in 40 ml of 10 mM glucose, 5 mM KCl, 140 mM NaCl, 5 mM NaHCO₃, 1 mM MgCl₂, 1.2 mM Na₂HPO₄, and 20 mM Hepes, pH 7.4, and incubated at 37°C for 15 min with stirring. The resuspension was diluted 1:2 with 0.1 M K₂-tartrate (pH 7.3) and centrifuged at 6,500 rpm for 4 min (SS34 rotor). The pellets were resuspended in 40 ml tartrate buffer and diluted 1:10 in H₂O and immediately homogenized (three strokes at 2,000 rpm, glass-teflon homogenizer). The solution was adjusted to isoosmolarity by adding 1/10 vol of a 10 \times stock of buffer A. The subsequent steps were performed exactly as described above for the homogenate.

For removal of the clathrin coat, 40 μ l of coated vesicle suspension were diluted into 1 ml of 0.3 M Tris-Cl, pH 9.0, and rotated for 1 h at 37°C. The sample was centrifuged for 15 min at 120,000 g in a Beckman TLA 100.3 rotor and the pellet was resuspended in 40 μ l of buffer A. Synaptic vesicles were immunisolated as described previously (Burger et al., 1989).

Immunocytochemistry

Light microscopy immunofluorescence of frozen sections was carried out as described (De Camilli et al., 1983; Baumert et al., 1990). Sprague Dawley rats (175–250 g) were anesthetized and transcardially perfused with ice-cold 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer.

Negative staining and immunogold labeling of isolated subcellular frac-

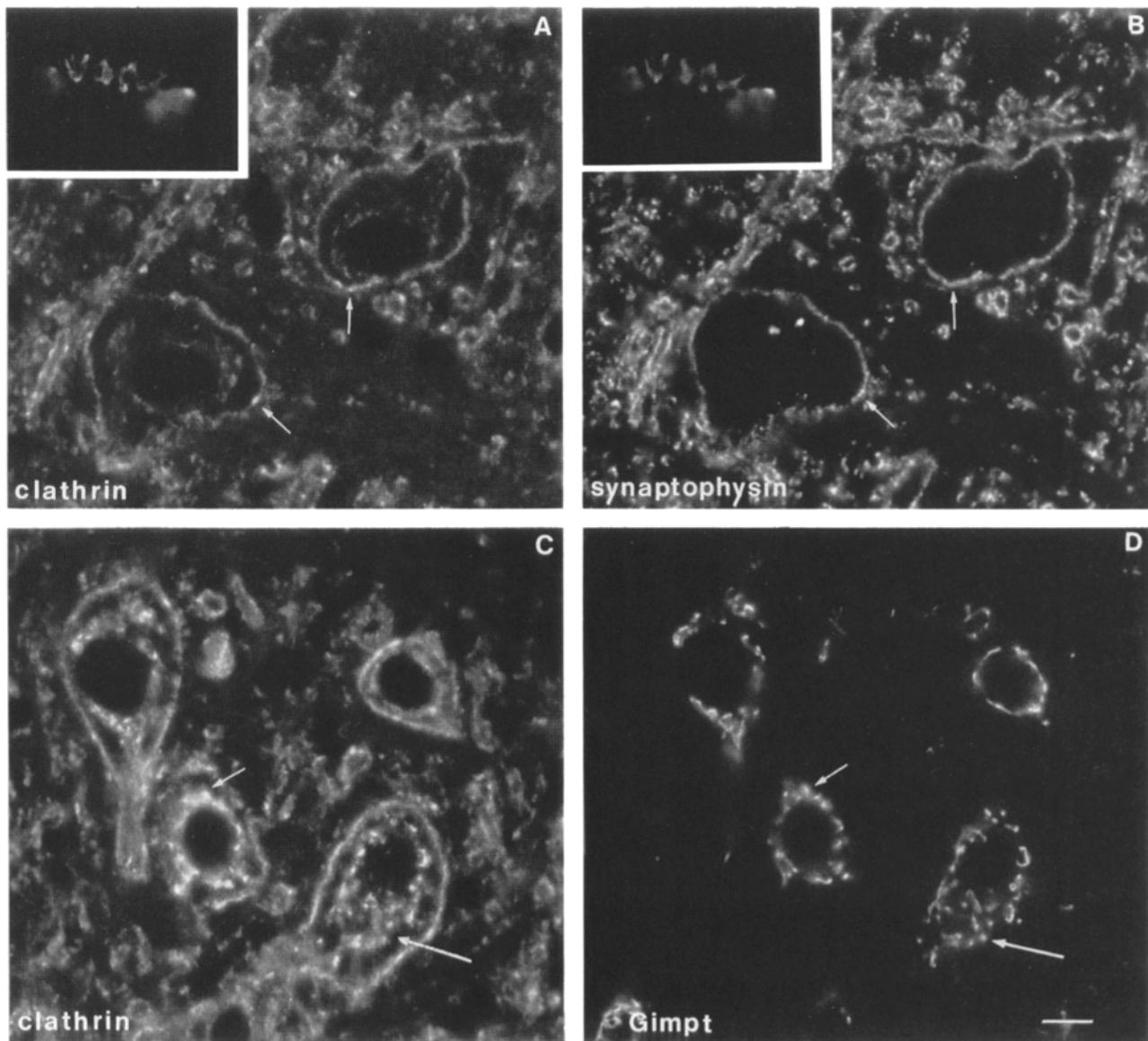


Figure 1. Double-immunofluorescence micrographs showing the localization of the neuronal forms of clathrin light chains (*A* and *C*) in comparison to the synaptic vesicle protein synaptophysin (*B*) and the *trans*-Golgi marker, Gimpt (*D*) in rat brain sections (*A–D*) and the neuromuscular junction (*A* and *B*, insets). Clathrin light chain and synaptophysin immunoreactivity is present in axon terminals which outline neuronal perikarya and dendrites (*A* and *B*, see arrows). Clathrin light chain immunoreactivity is also present in the nerve terminals of the neuromuscular junction, where it is colocalized with synaptophysin immunoreactivity (*A* and *B*, insets). Clathrin light chain immunoreactivity is also observed in the region of the Golgi complex, represented by a network of labeled perinuclear particles. The *trans*-Golgi marker, Gimpt, clearly defines the Golgi complex area (see arrows, *C* and *D*). Note that the mAb against synaptophysin does not recognize the perinuclear pool of the protein, in contrast to polyclonal sera used in earlier studies (see Navone et al., 1986). Bar, 10 μ m.

tions was performed as described (Jahn and Maycox, 1988). For immunolocalization of synaptophysin, mAb C 7.2 (Jahn et al., 1985) was used.

Other Methods

SDS-PAGE was performed according to Laemmli (1970). Immunoblotting was carried out according to Towbin et al. (1979), using radioiodinated protein A as a detection system (Jahn et al., 1985). For the analysis of clathrin coat proteins, SDS-PAGE was performed using gradient gels consisting of 10% acrylamide/0.3% bisacrylamide–18% acrylamide/0.6% bisacrylamide. This allowed all of the coat proteins and the individual adaptor subunits to be resolved by a single one-dimensional gel. Protein was determined by the method of Bradford (1976).

Results

Immunocytochemical Localization of the Neuronal Forms of Clathrin Light Chain

Using affinity-purified antibodies directed against neuron-specific clathrin light chains, immunostaining of frozen sections obtained from various areas of the central and peripheral nervous systems revealed a distinct staining pattern highly reminiscent of that described for synaptic vesicle membrane proteins (e.g., Navone et al., 1986), in particular that of p29 (Baumert et al., 1990). In all sections of the CNS,

a punctate pattern surrounding the cell bodies and the dendrites was visible (Fig. 1, *A* and *C*). Virtually identical patterns were observed when sections were double-stained with mAbs directed against the vesicle proteins synaptophysin (p38, Fig. 1 *B*) or rab3A (not shown). In addition, strong labeling of irregularly shaped structures surrounding the nucleus was observed. Double labeling with a mAb directed against the Golgi-specific protein Gmpt resulted in an identical staining pattern (Fig. 1, *C* and *D*). Similar double-labeling patterns were also observed in the peripheral nervous system, with highly specific labeling of nerve terminals (Fig. 1 *A*). No staining was observed in other regions of the neuron including the soluble compartment, or in any non-neuronal cell type (e.g., glial cells, etc.).

These observations demonstrate that those coated vesicle populations that carry neuron-specific light chains are colocalized with the major pools of synaptic vesicle membranes, namely in the *trans*-Golgi area and the nerve terminals. The localization of neuron-specific forms of the light chains in synapses and cell bodies has also been reported recently using a mAb (Wong et al., 1990). In addition, the staining pattern obtained with our light chain antibodies is similar to that obtained with antibodies generated against clathrin heavy chain in earlier studies (Cheng et al., 1980; Bloom and Puszkin, 1983).

Characterization of Coated Vesicles Isolated from Nerve Terminals

The immunocytochemical evidence suggests that the major pools of neuronal-coated vesicles are functionally associated with the synaptic vesicle pathway in the nerve terminal and possibly also in the Golgi complex area. For a full characterization of the coated vesicle membrane and coat composition, we have isolated coated vesicles from nerve terminals and whole brain and analyzed these preparations for the presence of coat proteins and synaptic vesicle membrane proteins both by immunoblotting and immunogold EM.

For the isolation of coated vesicle subpopulations derived from nerve terminals, we purified synaptosomes and isolated coated vesicles from their interior after osmotic rupture of the synaptosomal membrane (see Materials and Methods). To ensure that our synaptosome preparation was not contaminated by coated vesicles released from other parts of the cell or from other cell types, synaptosomes were washed several times with isotonic tartrate buffer before lysis. As shown in Fig. 2, hypotonic treatment of synaptosomes released large quantities of coated vesicle and synaptic vesicle protein constituents into the supernatant which were pelleted by a subsequent centrifugation at high speed (Fig. 2, lanes *B*). In contrast, no material was released when the incubation was performed in isotonic buffer (Fig. 2, lanes *C*). This experiment shows that our synaptosome preparation was free from adherent coated vesicles. This is supported by the lack of electron microscopic evidence for adherent coated vesicles on the synaptosomal surface (data not shown). In some experiments, synaptosomes were further purified by Ficoll-step gradients (Fischer von Mollard et al., 1991) before lysis. No significant differences were observed.

A major concern during the purification of clathrin-coated vesicles from nerve terminals is contamination by synaptic vesicles. These organelles are similar in size and are far more abundant than coated vesicles. For these reasons, we

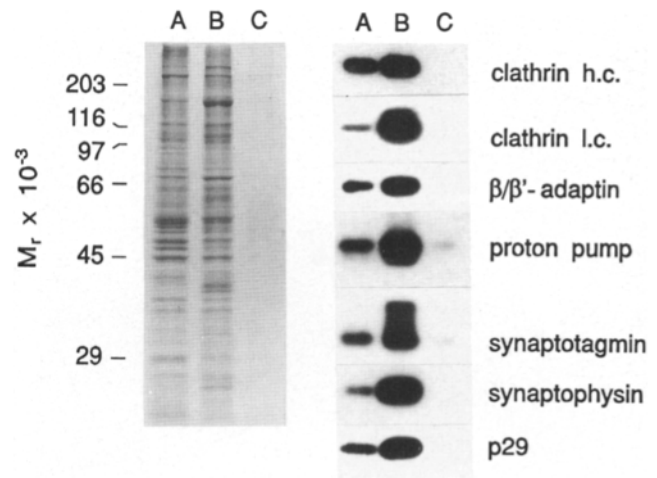


Figure 2. Coated vesicles are only released from synaptosomes when osmotic rupture of the plasma membrane is performed. Isolated synaptosomes were lysed by resuspension and homogenization in hypoosmotic buffer or resuspended with isotonic K_2 -tartrate buffer (control, see Materials and Methods). After removal of synaptosomal membranes by centrifugation, membranous particles released into the supernatant were pelleted at 100,000 g for 20 min in a Beckman TLA 100.3 rotor. The resulting pellets were resuspended and analyzed by SDS-PAGE and immunoblotting (equal proportional amounts in both cases). (Lane *A*) Synaptosomes before lysis ($5 \mu\text{g}$ of protein/lane); (lane *B*) high-speed membrane pellet derived from synaptosomes treated with hypoosmotic buffer ($5 \mu\text{g}$ of protein/lane); (lane *C*) high-speed membrane pellet derived from synaptosomes treated with isoosmotic buffer (equal proportion of the resuspended pellet as in lane *B* was loaded). (Left) Coomassie blue staining; (right) immunoblots.

monitored the enrichment of coated vesicles both by EM and by immunoblotting.

Fig. 3 shows the last three stages of coated vesicle purification from synaptosomes as observed by negative staining. The first enrichment step after synaptosomal lysis involved differential centrifugation. Although in comparison to earlier fractions (not shown) coated vesicles are significantly enriched, the fraction still contains numerous small profiles reminiscent of synaptic vesicles (Fig. 3 *a*) which were all positive for synaptic vesicle proteins in immunogold EM (not shown). Ficoll density gradient centrifugation resulted in a significant reduction of synaptic vesicle contamination (Fig. 3 *b*). The final purification step (D_2O /sucrose cushion) resulted in a clathrin-coated vesicle fraction of high purity, with only a minor proportion of uncoated vesicular profiles (Fig. 3 *c*).

The enrichment of coated vesicles as observed by EM was paralleled by a concomitant enrichment of coat proteins. Fig. 4 shows the electrophoretic profile of subcellular fractions obtained during coated vesicle purification. After immunoblotting, staining for the coat components clathrin heavy chain and the β/β' -adaptins (not differentiated in this experiment; see below), revealed a parallel enrichment of these proteins with the coated vesicles.

Comparison of Nerve Terminal Coated Vesicles with Synaptic Vesicles and Coated Vesicles Isolated from Whole Brain

Once this pure preparation of nerve terminal-coated vesicles

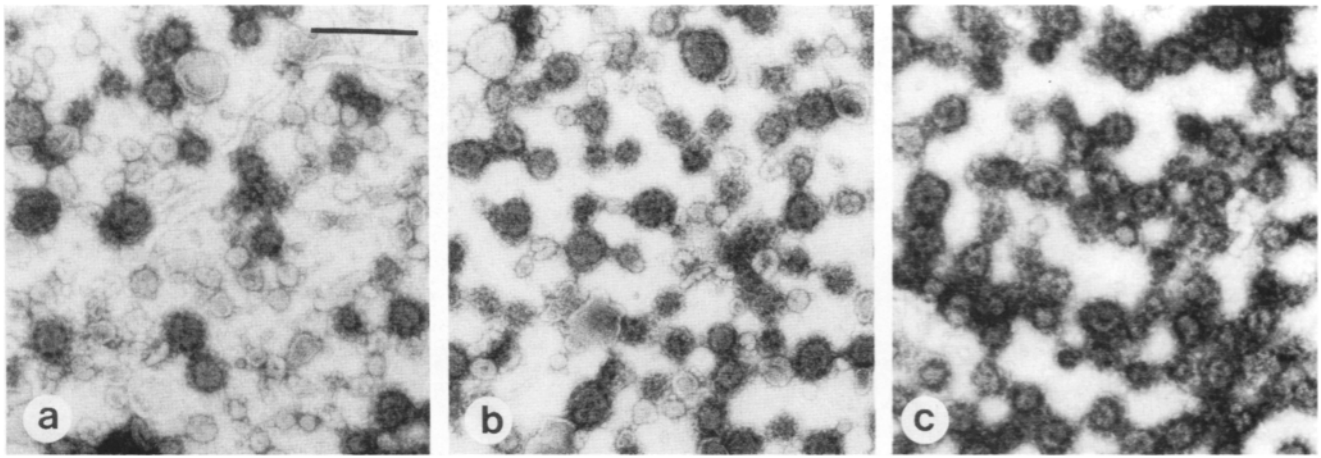
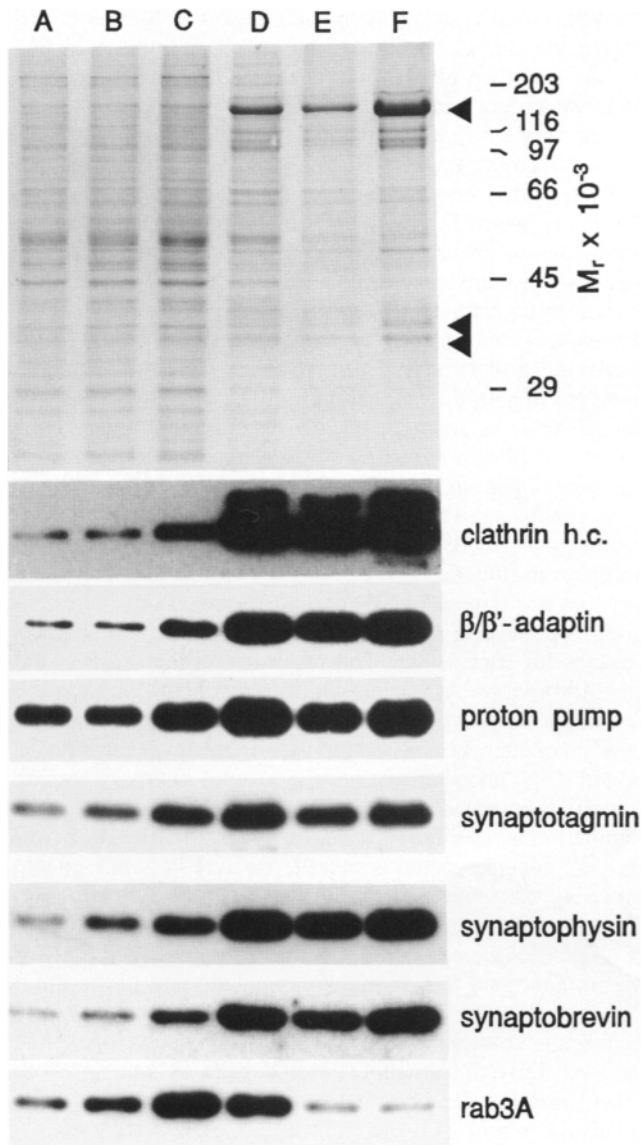


Figure 3. Enrichment of coated vesicles released from nerve terminals during subsequent Ficoll- and D₂O-sucrose-density gradient centrifugations, monitored by EM (negative staining). (a) 55,000-g pellet, obtained after lysis of synaptosomes and removal of heavy particles (see Materials and Methods). (b) Supernatant obtained after Ficoll-density gradient centrifugation (analyzed after dilution and centrifugation at 100,000 g, see Materials and Methods). (c) Pellet obtained after D₂O-sucrose density gradient centrifugation. Bar, 200 nm.



was available, we investigated the degree of similarity between the membrane protein components of coated vesicles and synaptic vesicles, i.e., to determine whether these coated vesicles contained membrane proteins previously shown to be specific for synaptic vesicles. Therefore, the distributions of the vesicle proteins synaptotagmin, synaptophysin, synaptobrevin, and rab3A were monitored in parallel. In addition, the distribution of the 116-kD subunit of the vacuolar proton pump was analyzed. This pump was first identified in coated vesicles (Stone et al., 1983), but was later also shown to be present in synaptic vesicles (Stadler and Tsukita, 1984; Hell et al., 1988; Cidon and Sihra, 1989). With the exception of rab3A, all of these proteins enriched together with the coat components although not to the same extent (Fig. 4). Rab3A did not copurify but rather enriched in the final purification steps. Similarly, none of the synapsin isoforms enriched with coated vesicles (data not shown).

Together these data suggest that a major proportion of the coated vesicles in nerve terminals carries synaptic vesicle-derived membranes and is involved in the recycling of synaptic vesicles. For comparison, we isolated coated vesicles and synaptic vesicles from total brain homogenate and analyzed their membrane composition. As shown by both

Figure 4. Enrichment of coated vesicle coat proteins and synaptic vesicle membrane proteins during the purification of coated vesicles from nerve terminals, monitored by SDS-PAGE and immunoblotting (8 μ g of protein/lane). (Top) Coomassie blue stain (arrowheads indicate the positions of clathrin heavy and light chains, respectively); (bottom) immunoblot. (A) Homogenate; (B) crude synaptosomes; (C) washed synaptosomes; (D) 55,000-g pellet, obtained after lysis of synaptosomes and removal of heavy particles (see Materials and Methods). (E) Supernatant obtained after Ficoll density gradient centrifugation (analyzed after dilution and centrifugation at 100,000 g; see Materials and Methods). (F) Pellet obtained after D₂O-sucrose density gradient centrifugation.

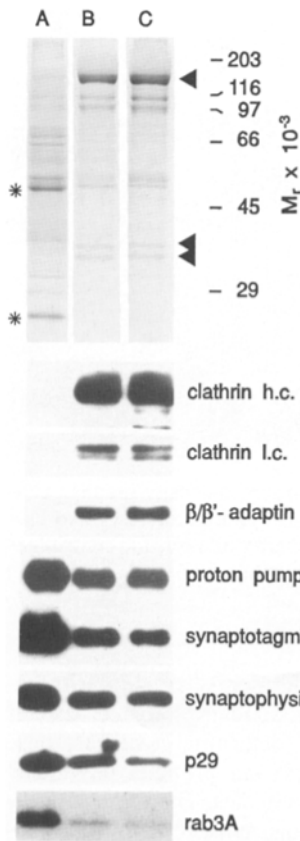


Figure 5. Comparison of the protein composition of synaptic vesicles (lane *A*) with that of coated vesicles isolated from whole brain (lane *B*) and nerve terminals (lane *C*). (Top) Coomassie blue stain of a SDS-polyacrylamide gel. Asterisks indicate the position of the heavy and light chains of mAb C 7.2 used for immunoprecipitation of synaptic vesicles. Arrowheads indicate the positions of clathrin heavy and light chains, respectively. (Bottom) Immunoblots. (Lane *A*) 10 μ g of protein (including IgG); (lanes *B* and *C*) 4 μ g of protein.

Coomassie blue staining and immunoblot analysis (Fig. 5), the protein composition of the two coated vesicle fractions is very similar. Furthermore, the ratio of the vesicle membrane proteins (again with the exception of rab3A, see above) is very similar in both coated and the synaptic vesicle fraction suggesting that these proteins are transported together during vesicular membrane traffic. P29 appears to be an exception to this pattern, being somewhat more abundant in the coated vesicle fraction isolated from whole brain homogenate. This is in agreement with our earlier observation that p29 immunoreactivity in the Golgi complex area is more intense than that of the other synaptic vesicle proteins (Baumert et al., 1990).

In the following experiments, we investigated which of the coat proteins are present on nerve terminal coated vesicles and how the coat composition of nerve terminal coated vesicles differs from that of whole brain coated vesicles. As shown in Fig. 6, nerve terminal-derived coated vesicles contain less of the β '-adaptin than total brain-derived coated vesicles. In contrast, all α -adaptins are present in the nerve terminal-derived coated vesicle fraction, with a slight enrichment of the α_{a1} - and α_{c1} -adaptins. This enrichment profile suggests that coated vesicles in nerve terminals contain predominantly, or even exclusively, AP-2 adaptor protein complexes. In addition, the distribution of the brain-specific coat components AP 180 and auxilin was studied. Both proteins were abundant in the nerve terminal-derived coated vesicle fraction, with a slight enrichment in comparison to whole brain-derived coated vesicles, demonstrating that both proteins are components of nerve terminal coated vesicles.

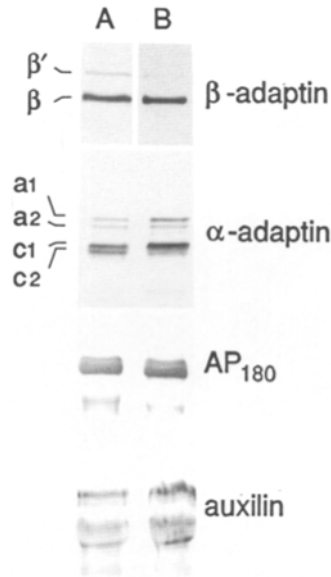


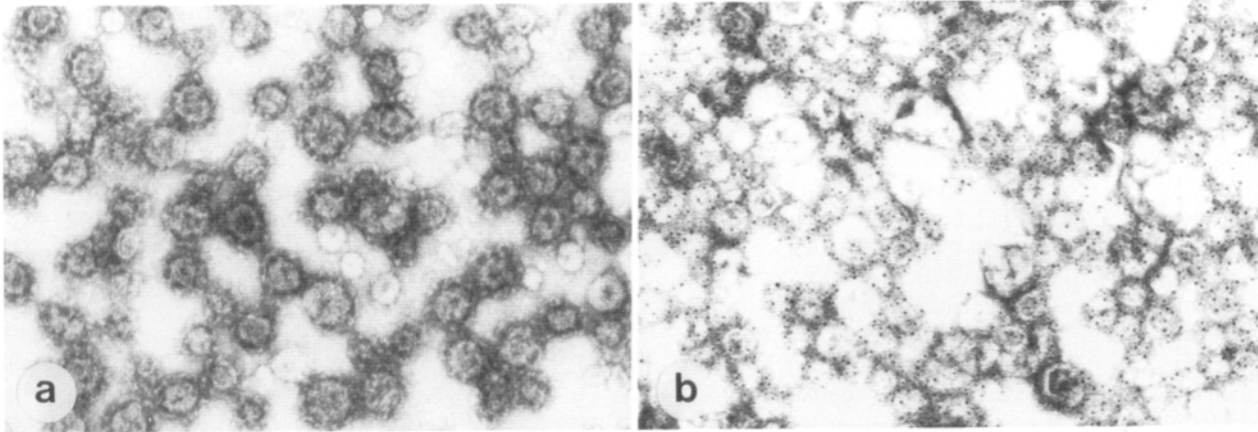
Figure 6. Analysis of the coat-associated proteins of clathrin coated vesicles from whole brain coated vesicles (*A*) and coated vesicles purified from nerve terminals (*B*), showing the presence of AP-2 adaptor components and the neuron-specific coat proteins auxilin and AP 180 in nerve terminal coated vesicles. The proteins were separated on special gradient gels (see Materials and Methods), transferred to nitrocellulose membrane filters and immunolabeled for β/β '-adaptin, α -adaptin, AP 180, and auxilin with mAbs 100/1, 100/2, AP180-1, and 100/4, respectively.

In the last series of experiments, both the nerve terminal-derived coated vesicle fraction and the whole brain-derived coated vesicle fraction were analyzed by immunogold EM for the presence of synaptophysin (Fig. 7). These experiments were performed to determine which proportion of coated vesicles in each fraction contains synaptic vesicle-derived membranes.

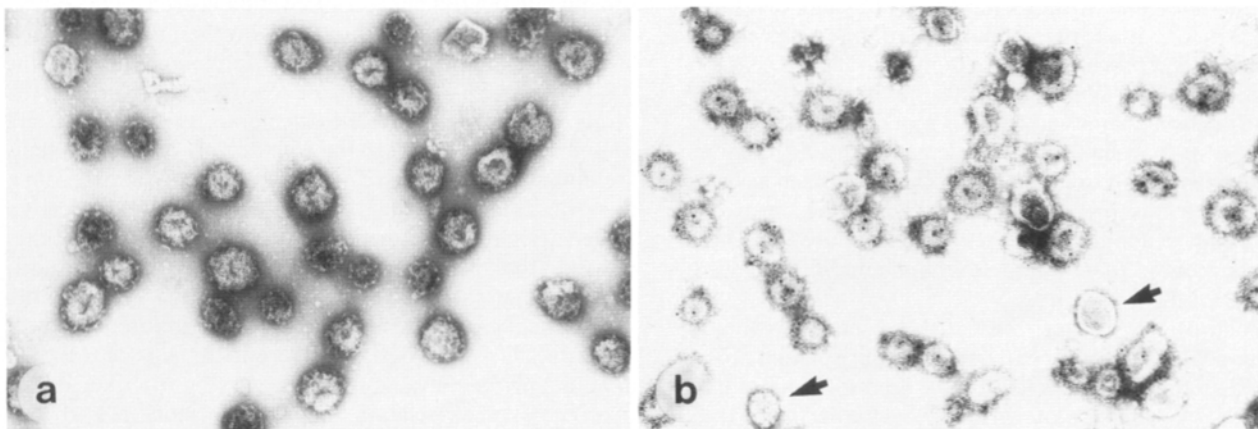
First, coated vesicles purified from nerve terminals (Fig. 7, upper) were decoated and the remaining membranes immunogold-labeled for synaptophysin. As shown in field b, virtually all membrane profiles were labeled (>95%). This demonstrates that essentially the entire population of nerve terminal coated vesicles is composed of recycling membranes derived from synaptic vesicles. This indicates that nerve terminals do not contain significant proportions of coated vesicles involved in nonvesicular pathways, e.g., plasma membrane recycling which is expected to occur during synaptogenesis and synapse turnover.

Surprisingly, an analysis of whole brain coated vesicles revealed that again the majority of vesicles was labeled for synaptophysin after decoating (Fig. 7, middle). The degree of labeling was determined by counting labeled and unlabeled profiles in several randomly selected fields, totaling >200 vesicles for each count. The result shows that 83% of the small profiles were labeled with immunogold, whereas the few larger profiles present were mostly unlabeled. Since the purified coated vesicle fraction used in this experiment contained <5% uncoated membrane profiles before decoating (most of them considerably larger than coated vesicles), contamination by free synaptic vesicles is negligible. As a control, we purified coated vesicles from liver in parallel using precisely the same purification and labeling protocol (Fig. 7, lower). Virtually no gold was found after decoating (<5% of all profiles were labeled), demonstrating the specificity of the immunogold procedure. We conclude that in the brain the synaptic vesicle pathway is dominant in coated vesicle membrane trafficking and that the contribution of coated vesicles derived from nonneuronal cells as well as from other, nonvesicular neuronal membrane traffic pathways is relatively minor.

coated vesicles isolated from nerve terminals



coated vesicles isolated from whole brain



coated vesicles isolated from liver

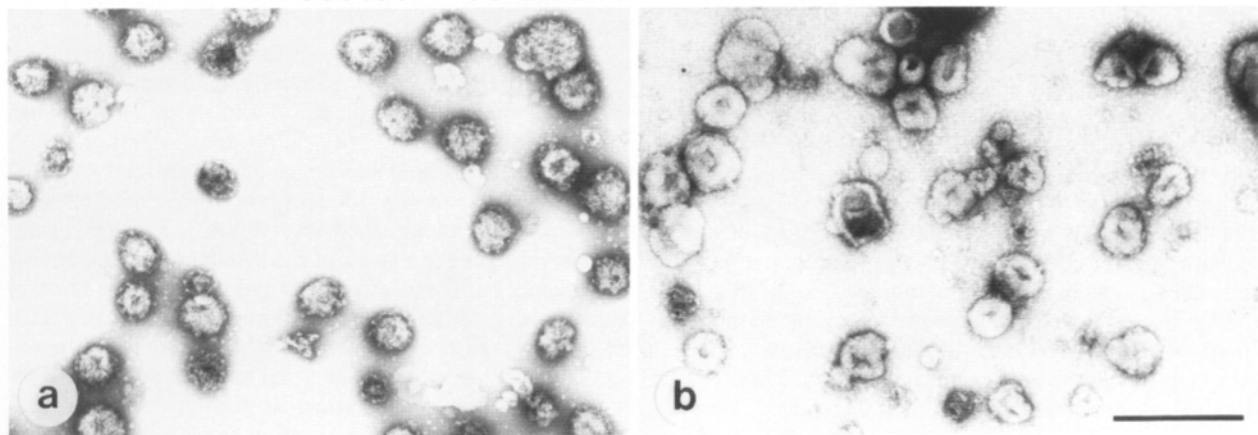


Figure 7. Immunogold labeling for synaptophysin on negatively stained coated vesicle membranes. The coat was stripped from the vesicles before labeling. (a) Coated vesicles before decoating. (b) Decoated membranes, immunogold labeled for synaptophysin. In the nerve terminal and in whole brain-derived coated vesicles, the majority of profiles are labeled for synaptophysin (arrows point to few unlabeled profiles in the whole-brain preparation). Virtually no labeling is observed in the liver sample which was used as a control. Bar, 200 nm.

Discussion

In the present study, we have utilized synaptosomes as starting material for the purification of clathrin-coated vesicles from nerve terminals in order to study their coat composition and their relationship with synaptic vesicles. Our data are consistent with a model in which coated vesicles in the nerve terminal function predominantly in vesicle recycling,

retrieving all synaptic vesicle proteins with the exception of rab3A and possibly the synapsins. Furthermore, their coat is enriched with components of AP-2 adaptor complex as well as with the neuron-specific coat proteins AP 180 and auxilin, suggesting a role for these proteins in synaptic vesicle recycling.

The ability to isolate synaptosomes represents a unique opportunity to obtain clathrin-coated vesicles in a pure form

from a distinct subcellular compartment which is specialized for regulated secretion and to use them as a starting point for functional studies. The isolation procedure we adopted overcame one of the main problems associated with the purification of these organelles from nerve terminals, namely contamination with synaptic vesicles. Under resting conditions, synaptic vesicles appear to be far more abundant than coated vesicles (see, e.g., Heuser and Reese, 1973; Torri-Tarelli et al., 1987), which is reflected by a high ratio of synaptic to coated vesicles in the first fraction obtained after synaptosomal lysis (see Fig. 3 *a*). A simplified version of this protocol was used for the isolation of clathrin-coated vesicles from whole brain and rat liver. The pure fractions (Figs. 3 *c* and 7) contained coated vesicles of heterogeneous size which indicates that the purification method does not select for clathrin-coated vesicle subpopulations of a certain size class.

It is unlikely that artificial re-coating of synaptic vesicles (i.e., coat formation after homogenization) occurs during our purification procedure. Our attempts to induce coat formation on purified synaptic vesicles *in vitro* by addition of soluble clathrin chains and adaptor protein complexes were unsuccessful (our unpublished observations). Furthermore, recent evidence from several laboratories suggests that coated pit formation must precede clathrin-coated vesicle formation *in vitro* and, in addition, requires specific incubation conditions not met by our procedure (Schmid and Smythe, 1991; Lin et al., 1991).

In contrast, we cannot rule out that some decoating of clathrin-coated vesicles occurred during our isolation procedure. To minimize loss of clathrin, we used solutions of high ionic strength which are known to prevent the decoating (see, e.g., Pearse, 1983 for review) observed in low ionic strength-sucrose buffers usually applied for organelle isolation. For example, synaptic vesicles isolated by conventional procedures (e.g., according to Huttner et al., 1983; Hell et al., 1988) are associated with significant amounts of adaptor proteins but not with clathrin (our unpublished observations), suggesting that they are contaminated with partially decoated vesicles. This was avoided under the more rapid and mild conditions of immunoisolation (Fig. 5).

One of the most striking observations from this study is the fact that coated vesicles from nerve terminals essentially contain synaptic vesicle-derived membranes, with no evidence for a significant contribution from any other membrane pool. With the exception of the membrane-associated proteins synapsin and rab3A (see below), the ratios between the individual integral membrane proteins were identical between coated and synaptic vesicles, suggesting that after exocytosis vesicle proteins recycle in bulk with almost fixed stoichiometry. These findings strongly support a central role of clathrin-coated vesicles in synaptic vesicle retrieval as suggested by Heuser and co-workers (see, e.g., Heuser, 1989). However, the existence of additional retrieval pathways cannot be excluded.

In contrast to the integral vesicle membrane proteins synaptotagmin, synaptophysin, p29 and synaptobrevin, the membrane-associated proteins synapsin (data not shown) and rab3A (Figs. 4 and 5) were barely detectable on clathrin-coated vesicles. Whereas the synapsins may have been lost during the purification due to high salt concentration (Huttner et al., 1983), the absence of rab3A probably reflects the

dissociation of this protein from the endocytotic limb of the membrane cycle. It was previously reported that rab3A dissociates from synaptic vesicles after stimulation of exocytosis (Fischer von Mollard et al., 1991), but it could not be determined at which step of the vesicle cycle this occurs. Irreversible stimulation of exocytosis by α -latrotoxin revealed that rab3A is transferred to the plasma membrane in a membrane-bound form (Matteoli et al., 1991). The absence of rab3A from clathrin-coated vesicles suggests that this protein is selectively segregated from the rest of the vesicle proteins during the clathrin-dependent membrane retrieval from the plasma membrane.

Our analysis of the coat proteins clearly suggests that several of the neuron-specific variants function in synaptic vesicle recycling. Thus, auxilin and AP 180 are major components of nerve terminal coated vesicles. A similar conclusion was reached recently by others for the neuron-specific coat protein NP 185 (Su et al., 1991) which is probably identical with AP 180 (Murphy et al., 1991). Furthermore, our analysis of the adaptins assigned the adaptor complexes to the AP-2 type which agrees with the plasma membrane-derived origin of the coated vesicles in nerve terminals. We are presently unable to determine whether any of these proteins are also present in coated vesicles derived from the somatodendritic area. This is due to the fact that cell body-derived coated vesicles cannot be isolated free from coated vesicles derived from nerve terminals. In our whole brain coated vesicle preparation, the proportion of coated vesicles from the perinuclear and dendritic region is therefore unknown. However, the slight but selective enrichments of AP 180, auxilin, and the α ₂- and α ₁-adaptins (Fig. 6) is suggestive of an exclusive association with nerve terminal coated vesicles. The resolution of this issue is, however, dependent on the availability of specific antibodies which are suitable for immunocytochemistry.

Surprisingly, coated vesicles isolated from total brain displayed an almost identical membrane composition to those isolated from nerve terminals, with >80% of the small profiles carrying synaptic vesicle proteins. Identical results were obtained when coated vesicles were purified from bovine brain by an entirely different procedure (free-flow electrophoresis; our unpublished observations). This again demonstrates the predominance of the synaptic vesicle pathway in the adult brain and shows that the contribution of other pathways, e.g., Golgi-derived pathways as well as of coated vesicles derived from glial cells, is minor. Due to the unknown percentage of nerve terminal-derived coated vesicles in this preparation (see above), we cannot determine with certainty whether the colocalization of clathrin light chain and synaptic vesicle proteins observed by immunocytochemistry in the perinuclear region reflects association with the same organelle. Thus, it remains to be determined whether coated vesicles are involved in synaptic vesicle formation and/or degradation in the neuronal cell body.

It should be mentioned that our findings differ from two earlier studies reporting that only a small proportion of coated vesicles (10–25%) was immunogold labeled with antibodies directed against the vesicle protein synaptophysin (Pfeffer and Kelly, 1985; Wiedenmann et al., 1985). It is possible that in these studies the preparation was contaminated by empty cages or that antibody access to the coated vesicle membrane was impaired which would explain the

discrepancies. The latter problem was circumvented in our study by labeling after removal of the clathrin coat to allow unrestricted access of the antibody. Taken together, our findings demonstrate that membrane components such as the vacuolar proton pump and a chloride channel that have been previously purified from brain coated vesicles, can be regarded largely as synaptic vesicle-derived (for review see Forgac, 1989; Xie et al., 1988).

In summary, our data show that the major function of neuronal coated vesicles is to participate directly in the synaptic vesicle pathway. Furthermore, the association of specific coat components with nerve terminal-derived coated vesicles suggests a functional specialization of the coat in nerve terminals to adapt to the specific requirements of synaptic vesicle recycling.

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