

Exo-endocytotic Recycling of Synaptic Vesicles in Developing Processes of Cultured Hippocampal Neurons

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Abstract. In mature neurons synaptic vesicles (SVs) undergo cycles of exo-endocytosis at synapses. It is currently unknown whether SV exocytosis and recycling occurs also in developing axons prior to synapse formation. To address this question, we have developed an immunocytochemical assay to reveal SV exo-endocytosis in hippocampal neurons developing in culture. In this assay antibodies directed against the luminal domain of synaptotagmin I (Syt I), an intrinsic membrane protein of SVs, are used to reveal exposure of SV membranes at the cell surface. Addition of antibodies to the culture medium of living neurons for 1 hr at 37°C resulted in their rapid and specific internalization by all neuronal processes and, particularly,

by axons. Double immunofluorescence and electron microscopy immunocytochemistry indicated that the antibodies were retained within SVs in cell processes and underwent cycles of exo-endocytosis in parallel with SV membranes. In contrast, another endocytotic marker, wheat germ agglutinin, was rapidly cleared from the processes and transported to the cell body. Antibody-labeled SVs were still present in axons several days after antibody loading and became clustered at presynaptic sites in parallel with synaptogenesis. These results demonstrate that SVs undergo multiple cycles of exo-endocytosis in developing neuronal processes irrespective of the presence of synaptic contacts.

SYNAPTIC vesicles (SVs)¹ are highly specialized secretory organelles by which neurons secrete nonpeptide neurotransmitters. They are highly homogeneous in size (average diameter, 50 nm) and are clustered at release sites in nerve endings. These clusters represent the key morphological features of synapses. SVs undergo regulated fusion with the plasmalemma, but differ in several important characteristics from secretory granules of the classical regulated secretory pathway which store and secrete peptide hormones and neurotransmitters and which in neurons are referred to as large dense-core vesicles (Matteoli and De Camilli, 1991).

Specific features of SVs include a unique membrane protein composition, an extremely short latency between the stimulus and the exocytotic response, the occurrence of their exocytosis at highly specialized plasmalemma sites, and their property to undergo exo-endocytotic recycling within the nerve terminal. At each exo-endocytotic cycle, SVs are refilled locally with neurotransmitters (Smith and Augustine, 1988; De Camilli and Jahn, 1990; Südhof and Jahn, 1991).

Most of these properties were established for SVs in ma-

ture presynaptic nerve endings. Much less is known about SVs or SV precursor organelles at stages of neuronal development which precede synaptogenesis. The presence of SV proteins has been described in processes of developing neurons before the formation of synaptic contacts, both in situ (De Camilli et al., 1983; Mason, 1986; Chun and Shatz, 1988; Bergmann et al., 1991) and in primary cultures (Fletcher et al., 1991). Fletcher et al. (1991) demonstrated that in cultured hippocampal neurons the compartmentalization of SV proteins occurs in two stages. Before synaptogenesis, vesicles containing SV proteins are preferentially concentrated in the axons, where they have a diffuse distribution. Upon contact with postsynaptic cells, SV proteins become clustered at the synaptic sites. It is unclear, however, whether at stages preceding synapse formation, SV proteins are already assembled in bona fide SVs and whether SVs undergo exo-endocytosis. An important question is whether the formation of a presynaptic specialization represents a prerequisite for SV biogenesis and fusion, or whether synapse formation simply correlates with a relocation of sites of SV fusion to specific regions of the axonal plasmamembrane.

Given the lack of a postsynaptic site, the classical assay to reveal SV exocytosis, i.e., postsynaptic recording (Del Castillo and Katz, 1956), cannot be used to study SV fusion in developing axons. To overcome these limitations, ingenious experimental systems based on in vitro systems and on

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1. *Abbreviation used in this paper:* SV, synaptic vesicle.

reporter cells or plasmamembrane patches attached to a recording pipette which is micromanipulated in close proximity to developing cell processes have been designed (Hume et al., 1983; Young and Poo, 1983; Sun and Poo, 1987; Xie and Poo, 1986). These studies have demonstrated neurotransmitter release from developing axons in culture and suggested a pulsatile release of the neurotransmitter (Young and Poo, 1983), but they did not establish whether release takes place by SV exocytosis.

To address this question, SV exocytosis assays which detect vesicle fusion and which are independent of the release of content molecules are required. Recently, assays based on changes in plasmalemma capacitance have been employed to monitor exocytosis of secretory organelles in a variety of cell systems (Penner and Neher, 1989; Fidler Lim et al., 1990). These assays, however, are of limited use to monitor exocytosis of SVs because of the small size of these vesicles and of the rapid reinternalization of SV membranes which, in general, closely follows exocytosis. In experiments carried out at the frog neuromuscular junction, it was shown that, even during sustained rates of SV exocytosis, the amount of SV membranes that are incorporated at any given time in the presynaptic plasmalemma may be insignificant (Valtorta et al., 1988; Torri Tarelli et al., 1990).

Another possible approach to study SV exocytosis independently of the release of their content may rely on the detection of luminal epitopes of SVs which become exposed to the extracellular medium as a result of fusion with the plasmalemma. If antibodies directed against these epitopes are added to the extracellular medium, the amount of antibodies which become cell-bound should reflect the number of exocytotic events irrespective of whether SV membranes are reinternalized.

To set up an exocytotic assay based on the latter approach, we have used a rabbit polyclonal antibody (Syt_{lum}-Abs) directed against a peptide corresponding to the luminal NH₂ terminus of synaptotagmin I (Syt I), a major SV protein (Matthew et al., 1981; Perin et al., 1990, 1991a,b). Syt I is a member of the synaptotagmin family which is represented by at least three highly similar isoforms which are differentially expressed in the nervous system (Geppert et al., 1991; Wendland et al., 1991). All three isoforms have a short luminal domain which contains the NH₂-terminus, a single transmembrane region, and a large cytoplasmic domain with two copies of an internal repeat homologous to the C2 regulatory domain of protein kinase C and which may play a role in the Ca⁺⁺-dependent fusion of SVs with the plasmalemma (Perin et al., 1990, 1991; Petrenko et al., 1991).

We have shown here that antibodies to the NH₂ terminus of Syt I recognize the native form of the molecule and we have used these antibodies to monitor SV exocytosis from hippocampal neurons in primary cultures. Our findings demonstrate an active rate of SV exocytosis and recycling not only at synaptic contacts of mature neurons, but also in the growing processes of isolated neurons. These observations suggest an important role of exocytotic neurotransmitter secretion from developing neuronal processes. They also demonstrate that Syt_{lum}-Abs may be a useful marker to study the traffic of SVs and the onset of synaptogenesis in living neurons.

Materials and Methods

Antibodies

Rabbit polyclonal antibodies directed against the luminal domain of rat Syt I (Syt_{lum}-Abs) were generated by using a peptide corresponding to residues 1-11 (NH₂ terminus) of the protein with an additional cysteine at the NH₂ terminus (CMVSASHPEALA) (Perin et al., 1990, 1991a). The peptide was conjugated to keyhole limpet hemocyanin via an NH₂-terminal cysteine as described (Johnston et al., 1989b). Polyclonal rabbit antibodies directed against the cytoplasmic domain of rat synaptophysin were generated using a 15-mer peptide corresponding to the COOH terminus of synaptophysin as described (Johnston et al., 1989b; Johnston and Südhof, 1990). Antibodies were affinity purified using the corresponding peptides, as described (Südhof et al., 1989). Rabbit polyclonal antibodies directed against the cytoplasmic domain of rat Syt I were raised using a recombinant Syt I protein expressed in *E. coli* that lacks the first 78 amino acids of the protein, including the transmembrane region (Perin et al., 1991a). Polyclonal and monoclonal antibodies directed against rat synaptophysin were the kind gift of Dr. R. Jahn (Yale University, New Haven, CT) and were previously described (Navone et al., 1986). mAbs directed against SV2 (Buckley and Kelly, 1985) and MAP2 (De Camilli et al., 1984) were kind gifts of Dr. K. Buckley (Harvard University, Boston, MA) and Dr. R. Vallee (Worcester Foundation for Experimental Biology, Worcester, MA) respectively. mAbs directed against β -tubulin were purchased from Amersham Corp. (Arlington Heights, IL).

Brain Homogenates and Cell Extracts

Total homogenates from rat brains and hippocampi were prepared as described (Huttner et al., 1983). Cultured hippocampal neurons and astrocytes were solubilized in 3% SDS, 5% 2-mercaptoethanol, 65 mM Tris-HCl (pH 6.8), and 10% sucrose as described (Fletcher et al., 1991).

Hippocampal Cell Cultures

Primary neuronal cultures were prepared from the hippocampi of 18-d-old fetal rats as described by Banker and Cowan (1977) and Bartlett and Banker (1984a). Briefly, hippocampi were dissociated by treatment with trypsin (0.25% for 15 min at 37°C), followed by trituration with a fire-polished Pasteur pipette. Dissociated cells were plated on poly-L-lysine-treated glass coverslips in MEM with 10% horse serum at densities ranging from 10,000 cells/cm² to 20,000 cells/cm². After a few hours, coverslips were transferred to dishes containing a monolayer of cortical glial cells (Booher and Sensenbrenner, 1972), so that they were suspended over the glial cells but not in contact with them (Bartlett and Banker, 1984a). Cells were maintained in MEM (Gibco Laboratories, Grand Island, NY) without sera, supplemented with 1% HL1 (Ventrex, Portland, ME), 2 mM glutamine and 1 mg/ml BSA (neuronal medium).

Labeling Protocols: Light Microscopy

Cultures were incubated with Syt_{lum}-Abs (serum and affinity-purified antibodies yielded similar results) or other rabbit antibodies for 1 h at 37 or 0°C. The carbonate buffer was substituted by a HEPES-based buffer when the incubation was performed at 0°C. In some cases, Syt_{lum}-Abs were applied to neurons together with FITC-conjugated wheat germ agglutinin (WGA) (Vector Laboratories, Burlingame, CA). Antibodies and WGA were diluted in the neuronal medium. At various times after this incubation (15 min to 6 days) cells were fixed by 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer containing 0.12 M sucrose. Fixed cells were detergent permeabilized and labeled with rhodamine-conjugated anti-rabbit antibodies (Boehringer Mannheim Biochemicals, Indianapolis, IN) as previously described (Cameron et al., 1991). Neurons were then counterstained for SV2, MAP2, or β -tubulin using mouse mAbs followed by FITC-conjugated secondary antibodies (Boehringer Mannheim Biochemicals) (Cameron et al., 1991). In some experiments 30 min or 24 h after the first antibody incubation, living neurons were incubated with rhodamine-conjugated anti-rabbit antibodies. Cells were then briefly washed and fixed. For microscopic observation coverslips were mounted in 70% glycerol in PBS containing 1 mg/ml phenylendiamine. Preparations were examined with a Zeiss axiophot microscope equipped with epifluorescence microscopy and photographed with T-MAX 100 (Kodak).

Labeling Protocols: Electron Microscopy

Living neurons were incubated with rabbit antibodies for 1 h at 37°C. 15 min from the end of this incubation neurons were exposed to a second incubation in the presence of colloidal gold-protein A conjugates (6 nm prepared as described by Slot and Geuze, 1983). Neurons were then washed, fixed with 2% glutaraldehyde prepared in culture medium and processed for electron microscopy essentially as described by Bartlett and Banker (1984a,b).

Other Procedures

SDS-PAGE electrophoresis and Western blotting were carried out as described (Laemmli, 1970; Towbin et al., 1979). Preparation and immunostaining of frozen sections from rat brain were performed as described (De Camilli et al., 1983).

Results

Antibodies That Recognize the Luminal Domain of Syt I in Living Neurons

Polyclonal antibodies directed against the intravesicular domain of Syt I were obtained by immunizing rabbits with a 12-mer peptide corresponding to the luminal NH₂-terminus of Syt I as previously described (Perin et al., 1991a). This domain was shown to be localized within the vesicle lumen (Perin et al., 1990, 1991a). The antibodies recognized a band with the expected mobility of Syt I (65 kD) when tested by Western blot on a total homogenate of rat brain (not shown), hippocampus (Fig. 1, A and B, lane 1) and hippocampal neurons in culture (Fig. 1 B, lane 2). This band was not recognized when the antibodies were tested on a total homogenate of cultured rat astrocytes (Fig. 1 B, lane 3).

Syt_{lum}-Abs produced a typical nerve ending staining pattern when tested by light microscopy immunocytochemistry on frozen sections of rat brain (Fig. 2 a). Double labeling of the same sections for synaptophysin immunoreactivity (Fig. 2 b) demonstrated that only a subpopulation of synapses were stained by Syt_{lum}-Abs, in agreement with the

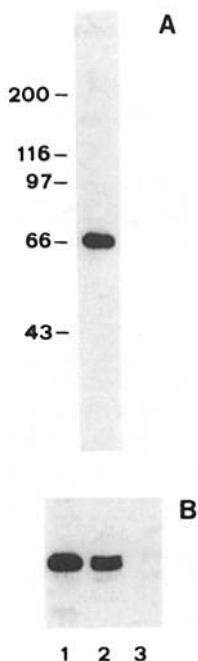


Figure 1. Western blots of total tissue and cell homogenates showing specificity of Syt_{lum}-Abs. Syt_{lum}-Abs recognize a 65-kD band in total homogenates of rat hippocampal neurons, but not in total homogenates of nonneuronal cells. (A) Western blot of a total homogenate of rat hippocampus. (B) Western blot of total homogenates of rat hippocampus (lane 1), of primary cultures of rat hippocampal neurons (10-d-old neurons, lane 2) and astrocytes (lane 3).

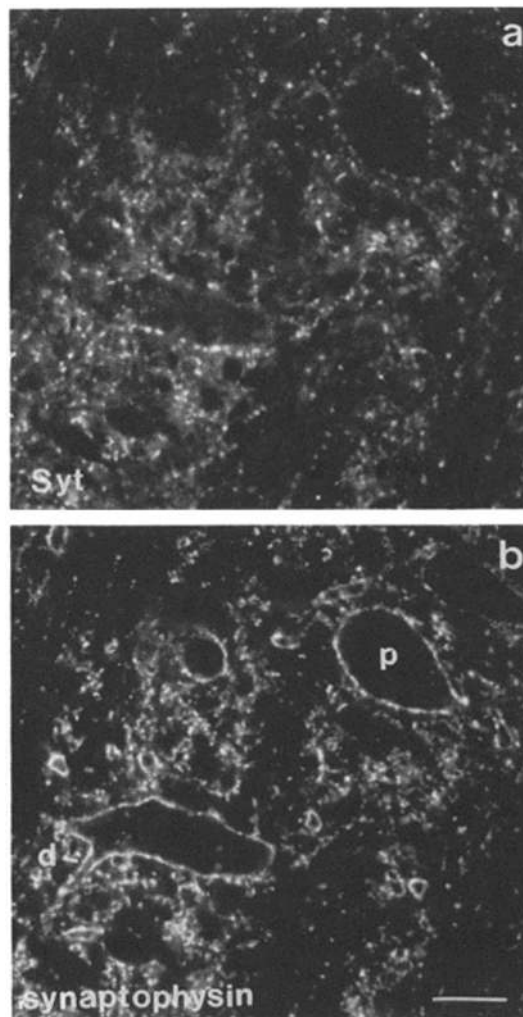


Figure 2. Double-immunofluorescence micrographs of a rat brain stem section showing that Syt_{lum}-Abs produce a nerve terminal pattern of staining at a subpopulation of synapses. The same frozen section was stained with Syt_{lum}-Abs (a) and with mAbs directed against synaptophysin (b). Synaptophysin-positive puncta, which represent individual presynaptic terminals, outline the profiles of perikarya (p) and dendrites (d). Immunoreactivity recognized by Syt_{lum}-Abs precisely corresponds to a subpopulation of synaptophysin immunoreactive puncta. Bar, 22.2 μ m.

heterogeneous expression of Syt I in brain (Wendland et al., 1991; Geppert et al., 1991).

We next investigated whether Syt_{lum}-Abs recognized the native protein in living hippocampal neurons. To this aim, primary cultures of rat hippocampal neurons plated at high density and kept in culture for 15 days (Bartlett and Banker, 1984a,b) were used. As previously demonstrated, these cultures are rich in synaptic contacts, which have the morphological features of synapses in situ (including the characteristic presynaptic accumulation of SVs) (Bartlett and Banker, 1984b; Fletcher et al., 1991) and which are physiologically functional (Goslin and Banker, 1991). If Syt_{lum}-Abs bind to native Syt I, their addition to the culture medium should result in a selective labeling of SV membranes which are exposed to the cell surface by exocytosis at synapses. Cultures were incubated in the presence of Syt_{lum}-Abs for 1 h at 37°C, briefly washed, fixed, and detergent permeabilized.

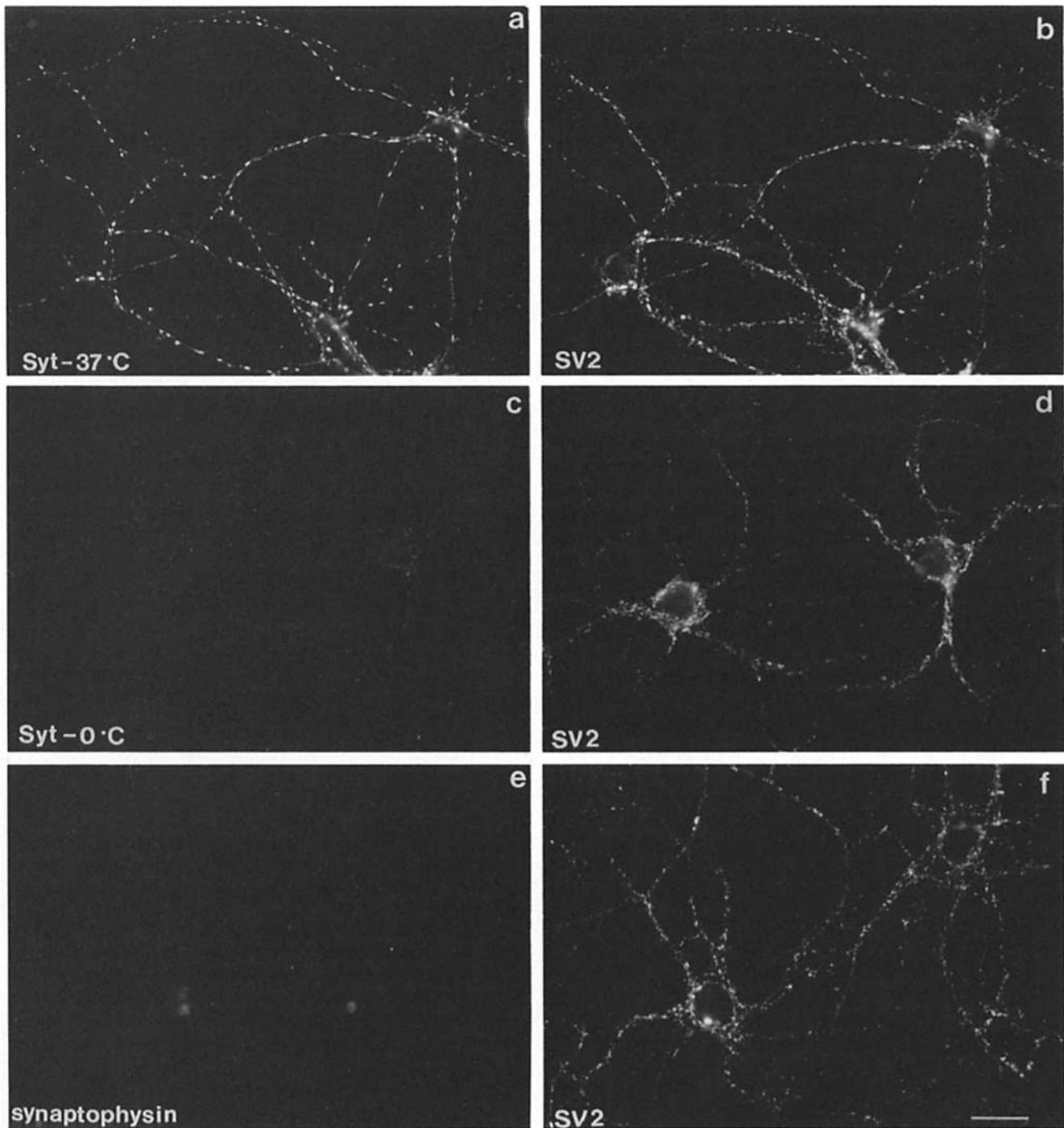


Figure 3. Double-immunofluorescence micrographs showing that Syt_{lum}-Abs label presynaptic nerve terminals when added to living hippocampal cultures in primary cultures. 15 d-old neurons were incubated for 1 h in the presence of Syt_{lum}-Abs at 37°C (*a* and *b*) or 0°C (*c* and *d*), or of rabbit antibodies directed against the cytoplasmic tail synaptophysin at 37°C (*e* and *f*). Following this incubation, neurons were washed, fixed, detergent-permeabilized, reacted with rhodamine-conjugated goat anti-rabbit IgGs (*a*, *c*, and *e*) and counterstained for the SV protein SV2 by mAbs followed by FITC-conjugated goat anti-mouse IgGs (*b*, *d*, and *f*). Puncta of SV2 immunoreactivity represent presynaptic nerve terminals (presynaptic clusters of SVs) which outline perikarya and dendrites (see Fletcher et al., 1991). Syt_{lum}-Abs produce an intense labeling of the same structures when applied at 37°C (*a*) and only a very weak labeling when applied at 0°C (*c*). No labeling was produced by antibodies directed against the cytoplasmic domain of synaptophysin. (*e*). Bar, 23.8 μm.

Cell-bound Syt_{lum}-Abs were then revealed by rhodamine-conjugated secondary antibodies. As shown in Fig. 3 *a*, this protocol resulted in an intense labeling of varicose structures outlining neuronal profiles. This fluorescence pattern was

then compared with the distribution of the entire population of SVs in the same cultures. To this aim, the same cultures were counterstained for another SV protein, SV2 (Buckley and Kelly, 1985) using mouse mAbs followed by secondary

antibodies conjugated to fluorescein (Fig. 3 *b*). These experiments demonstrated a coincidence of the sites positive for Syt_{lum}-Abs with sites where synaptic vesicles are accumulated. These sites were previously shown to represent presynaptic nerve terminals (Fletcher et al., 1991). The presence of many puncta positive for SV2 immunoreactivity, but negative for Syt_{lum}-Abs (Fig. 3, *a* and *b*), is explained by the presence of Syt I only in a subpopulation of neurons and, therefore, of nerve terminals. Only very low labeling was produced by Syt_{lum}-Abs when these were applied at 0°C (Fig. 3, *c* and *d*), indicating that no significant levels of Syt I are present at the cell surface at any given time, and that the staining produced by Syt_{lum}-Abs in living neurons is dependent upon SV exocytosis. Likewise, no staining was obtained when Syt_{lum}-Abs were substituted by a similar concentration of antibodies directed against the cytoplasmic domain of Syt I (Perin et al., 1991a; Matteoli et al., 1991) (not shown) or synaptophysin (Navone et al., 1986; Johnston et al., 1989b) (Fig. 3, *e* and *f*). The latter antibodies were previously shown to recognize the corresponding proteins when used to stain fixed permeabilized cells (Navone et al., 1986; Cameron et al., 1991; Matteoli et al., 1991). Thus, the labeling produced by Syt_{lum}-Abs at 37°C is not due to bulk uptake of the antibody by fluid phase endocytosis or to an artifactual permeabilization of the neurons. These findings validate the use of Syt_{lum}-Abs as markers for the luminal domain of Syt I in living cells.

Exo-endocytosis of Syt I Containing Membranes in Isolated Hippocampal Neurons

We next used Syt_{lum}-Abs to investigate whether SV exocytosis takes place in processes of developing hippocampal neurons before the formation of synaptic contacts. For these experiments we used hippocampal neurons plated at low density and kept in culture for 3–5 d. Most neurons of these cultures were completely isolated from other cells and had the characteristics of stage 3 and stage 4 neurons as defined by Dotti et al. (1988). Cultures were incubated in the presence of Syt_{lum}-Abs for 1 h at 37 or 0°C and then processed for the detection of cell-bound antibodies as described above for mature cell cultures (Fig. 4, *a*, *c*, and *e*). They were then counterstained with mAbs directed against β -tubulin (Fig. 4, *b* and *d*), to visualize all cellular processes, or against the dendritic protein MAP2 (De Camilli et al., 1984) (Fig. 4 *f*), to visualize dendritic processes selectively. Virtually no staining was observed in cultures incubated with Syt_{lum}-Abs at 0°C, suggesting that, as in mature neurons, no significant levels of Syt I are present at the cell surface at any given time (not shown). In cultures incubated at 37°C, Syt_{lum}-Abs produced an intense staining of a subpopulation of cells. The staining had a finely punctate appearance, and, in general, was primarily concentrated in the distal portion of one process, which differed in morphology from the other processes. In general this process was the longest (Fig. 4, *c* and *e*), but in a few cases it was the thickest (Fig. 4 *a*). In stage 4 neurons this process was found to be the axon by its lack of MAP2 immunoreactivity (Fig. 4, *e* and *f*). The peculiar morphology of some of these axons, when compared to the typical axons described in the same type of cultures by Banker and coworkers (Dotti et al., 1988; Goslin and Banker, 1989), may be explained by the presence of Syt I only in a neuronal subpopulation. A moderate degree of dendritic staining was

present in most neurons (Fig. 4 *a*, *c*, and *e*). The pattern of staining in cell processes was consistent with a predominant localization of Syt_{lum}-Abs in intracellular vesicles (see below). A paranuclear region was also stained (Fig. 4, *a*, *c*, and *e*, see also Fig. 6). Staining of this area, which in double-labeling experiments using anti-Golgi complex antibodies was found to correspond to the region of the Golgi apparatus (not shown), was intense at early stages of differentiation and was only very weak in old neurons. No staining was observed when Syt_{lum}-Abs were substituted by similar concentrations of antibodies directed against the cytoplasmic side of Syt I or of other SV antigens (not shown). These findings indicate that in developing neurons Syt I is continuously exposed to the cell surface by exocytosis and rapidly reinternalized by endocytosis.

Syt_{lum}-Abs Are Retained in SVs and Redistribute with SVs during Synaptogenesis

The fluorescent pattern of labeling produced by Syt_{lum}-Abs taken up by isolated neurons during 1 h of incubation appeared to be very similar to the distribution of SV proteins in these neurons as assessed by previous studies (Fletcher et al., 1991). In fact, when cells previously exposed to Syt_{lum}-Abs for 1 h were counterstained for SV2 after fixation and detergent permeabilization, the distributions of cell-bound Syt_{lum}-Abs and of SV2 were found to be identical at this level of resolution (Fig. 5, *a* and *b*). This observation suggests that internalized Syt_{lum}-Abs are retained in SVs and that at least a significant fraction of all SVs undergo exocytosis during 1 h.

To further investigate the fate of Syt_{lum}-Abs after internalization, neurons were kept in normal neuronal medium for various times after the removal of antibodies and before fixation and permeabilization. Subsequently, cells were processed to visualize cell-bound Syt_{lum}-Abs and SV2 as described above. A striking colocalization of bound Syt_{lum}-Abs (Fig. 5, *a*, *c*, and *e*) and SV2 (Fig. 5, *b*, *d*, and *f*) was observed not only when neurons were fixed within few minutes from the end of incubation (see above) but also when cells were fixed after 2 (Fig. 5, *c* and *d*), 4 (not shown), or 6 d (Fig. 5, *e* and *f*). After 2 d, axons had greatly elongated and both immunoreactivities were still concentrated in their distal portion although now they were spread over a longer segment (Fig. 5, *c* and *d*). After 4 and 6 d a large number of synaptic contacts had formed (Fig. 5, *e* and *f*) as demonstrated by the large puncta of SV2 immunoreactivity which outlined the profiles of perikarya and dendrites. It was previously shown (Fletcher et al., 1991) that during synaptogenesis SVs become clustered at presynaptic varicosities, the large SV2 immunoreactive puncta visible in Fig. 5 *f*, and are depleted from intervaricose axonal segments. Syt_{lum}-Abs were colocalized with SV2 at synaptic varicosities and depleted from intervaricose axonal segments. At all developmental stages the distribution of SV2 in neurons which had been incubated with Syt_{lum}-Abs was the same as that of SV2 in control neurons not incubated with antibodies, indicating that internalized Syt_{lum}-Abs did not modify the overall distribution of SVs.

Taken together these observations suggest (*a*) that Syt/Syt_{lum}-Abs complexes have a long half life; (*b*) that these complexes are retained in SVs and are not sorted away from SVs membranes for targeting to a degradative pathway; and

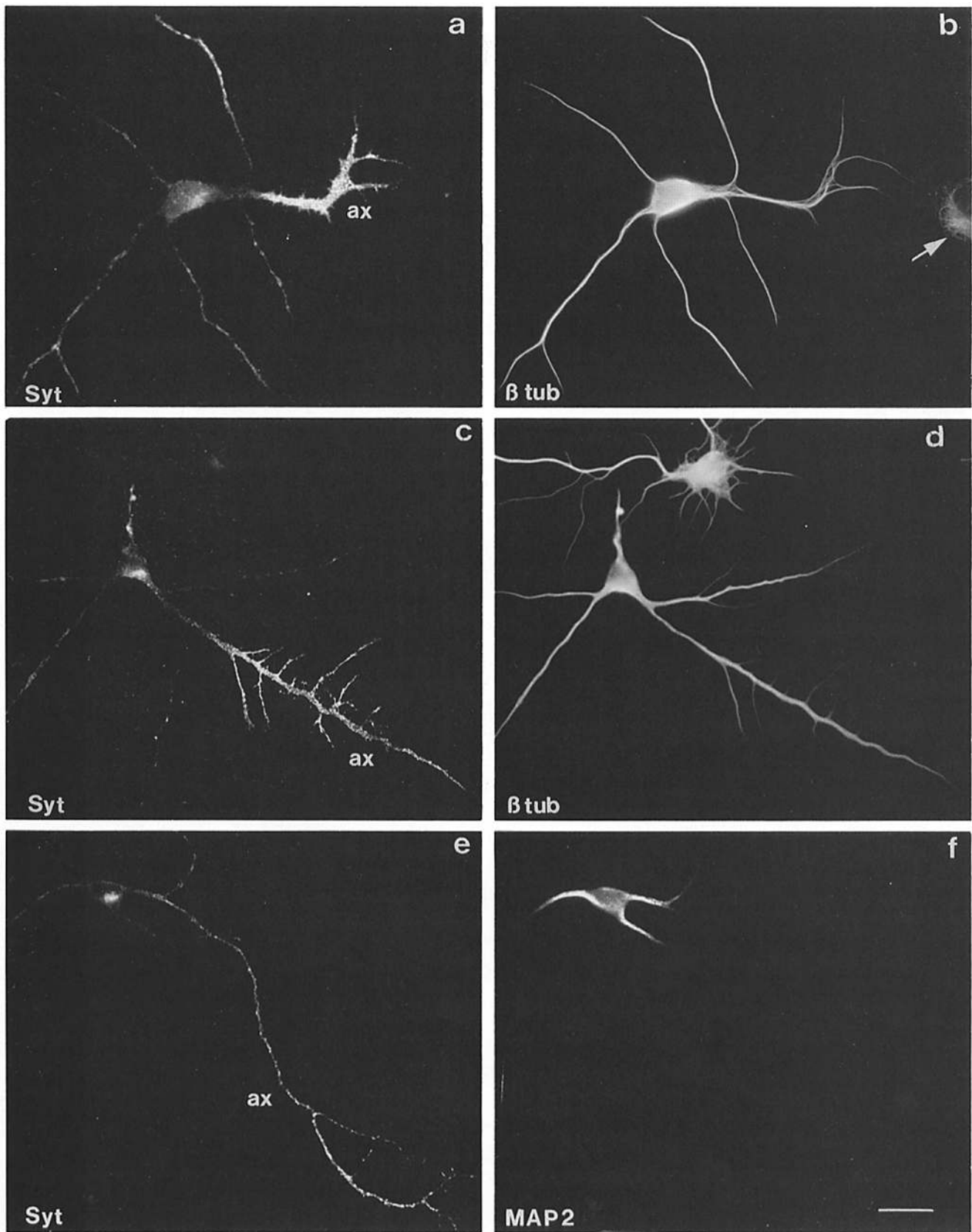


Figure 4. Uptake of Syt_{lum}-Abs by living hippocampal neurons grown in isolation. 3- (*a-d*) and 5- (*e-f*) d-old neurons were incubated in the presence of Syt_{lum}-Abs for 1 h at 37°C, briefly washed, fixed, detergent-permeabilized, reacted with rhodamine-conjugated goat anti-rabbit IgGs (*a*, *c*, and *e*) and counterstained for β -tubulin (*b* and *d*) or for MAP2 (*f*) by mAbs followed by FITC-conjugated goat anti-mouse IgGs. Syt_{lum}-Abs produced a bright labeling of a subpopulation of neurons (note in *c* and *d* a neuron which is positive for β -tubulin and unstained by Syt_{lum}-Abs) but not of astrocytes. An astrocyte in *b* is indicated by an arrow. Immunoreactivity recognized by Syt_{lum}-Abs has a finely punctate appearance. It is present in all processes but is particularly concentrated in one of them, the axon (*ax*), which is clearly different in overall morphology from the other processes. *e* and *f* show that this process is negative for MAP2 immunoreactivity. The accumulation of Syt_{lum}-Abs visible in the perikaryon corresponds to the area of the Golgi complex. Bar: (*a* and *b*) 15.8 μ m; (*c-f*) 25 μ m.

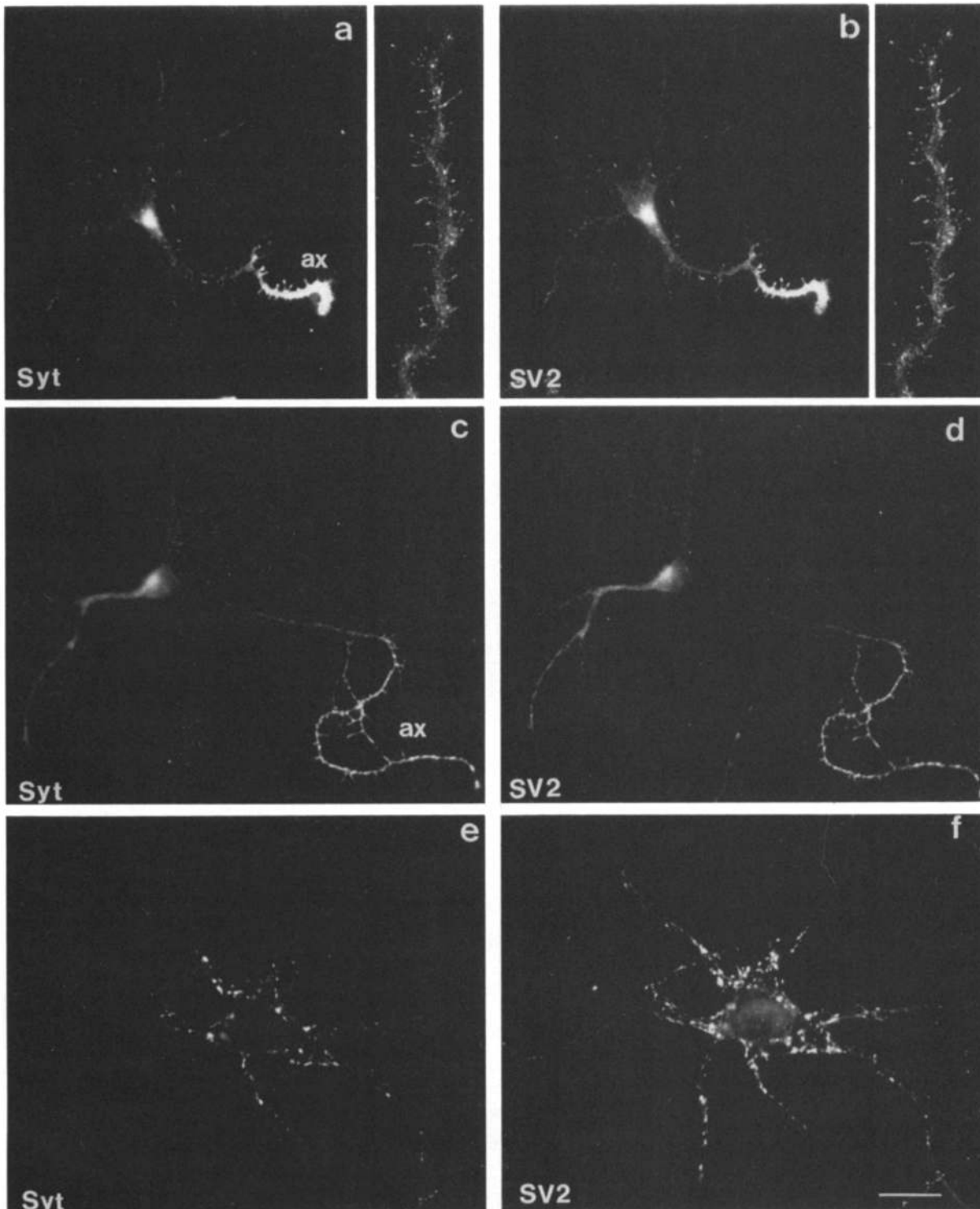


Figure 5. Colocalization of Syt_{lum}-Abs with SV2 in cultured hippocampal neurons at various times following the antibody incubation. 3-d-old neurons were incubated for 1 h at 37°C in the presence of Syt_{lum}-Abs and then, 15 min (*a* and *b* and corresponding inserts), 2 d (*c* and *d*), and 6 d (*e* and *f*) following this incubation, they were fixed, detergent-permeabilized, reacted with rhodamine-conjugated goat anti-rabbit IgGs (*a*, *c*, and *e*) and counterstained for SV2 (*b*, *d*, and *f*). Immunoreactivity puncta visible in *e* and *f* represent axon terminals of neuronal perikarya not shown in the field which make synaptic contacts with the neuron shown in the field. Only a subpopulation of SV2-positive nerve terminals are positive for Syt_{lum}-Abs in *e* and *f*. With this exception Syt_{lum}-Abs and SV2 are precisely colocalized in the three pairs of figures. The insets of *a* and *b* show at high magnification the axon of a neuron fixed at 15 min. Note the identity of the two fluorescent patterns and the intense staining of filopodia. *ax*, axon. Bar: (*a* and *b*) 16 μm; (*c-f*) 20 μm; (*insets*) 13.2 μm.

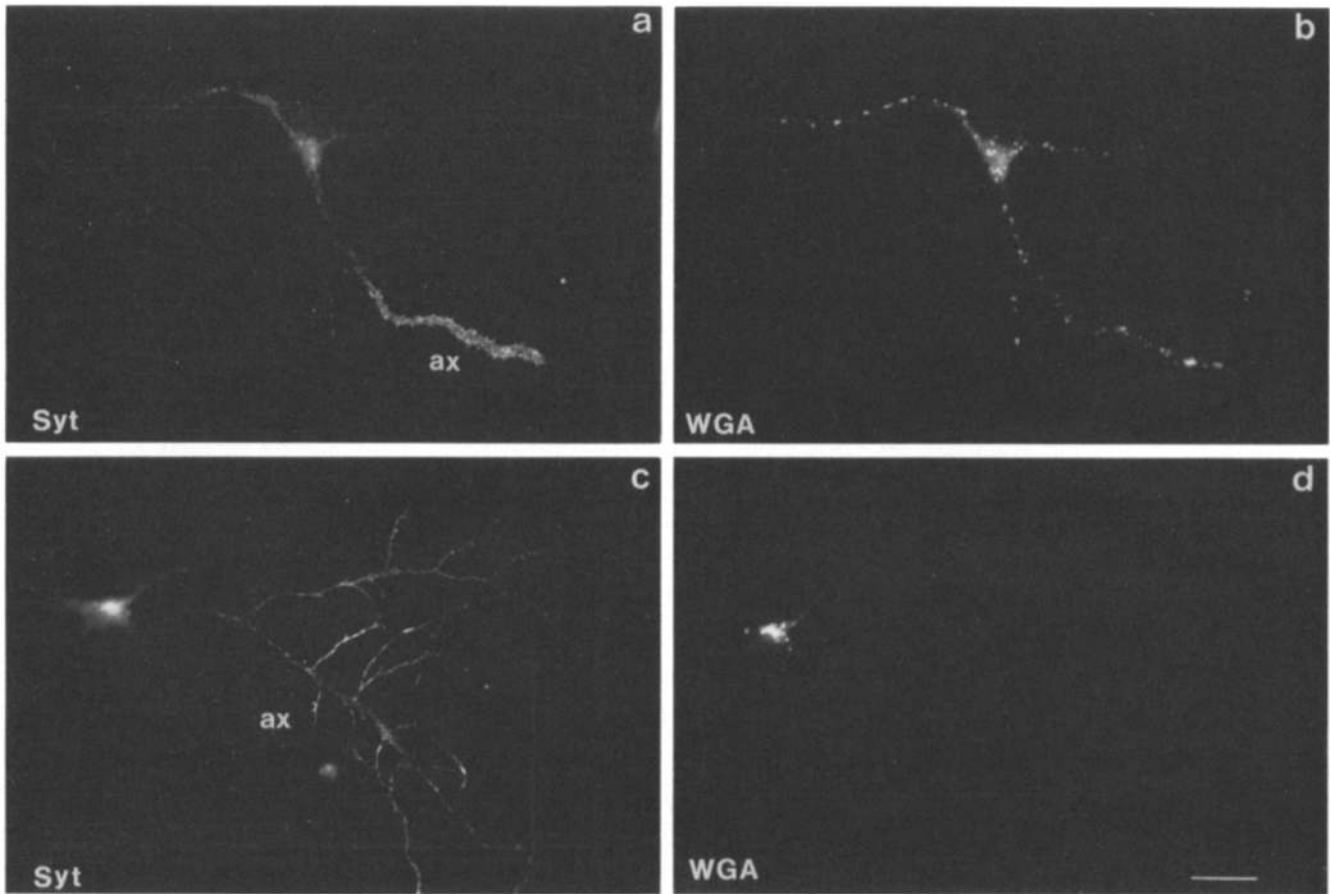


Figure 6. Differential sorting of Syt_{lum}-Abs and WGA after internalization. 3-d-old cultures were incubated simultaneously for 1 h at 37°C with Syt_{lum}-Abs and with FITC-conjugated WGA. 15 min (*a* and *b*) or 2 d (*c* and *d*) following this incubation, they were fixed, detergent-permeabilized, and reacted with rhodamine-conjugated goat anti-rabbit IgGs to visualize cell-bound Syt_{lum}-Abs. Double-fluorescence micrographs demonstrating Syt_{lum}-Abs (*a* and *c*) and WGA (*b* and *d*) are shown. In both pairs of figures the distribution of the two probes is very different. In both cases Syt_{lum}-Abs label primarily the axon (*ax*) which is much more elaborated in *c*. WGA is equally taken up by the axon and by dendrites (*b*). After 2 d virtually all WGA has been transported in a retrograde direction to the perikaryon (*d*). Bar: (*a* and *b*) 15.8 μm; (*c* and *d*) 23.8 μm.

(*c*) that the clustering of SVs at synapses, and in general the traffic of SVs, is not affected to any significant extent by the presence of Syt_{lum}-Abs bound to the luminal domain of Syt I.

Different Fates of Syt_{lum}-Abs and WGA after Internalization

We next investigated whether, in contrast to Syt_{lum}-Abs, other proteins internalized by absorptive endocytosis could be rapidly transported to the cell body. To this aim, 3-d old cultures were incubated for 1 h at 37°C in the presence of both Syt_{lum}-Abs and FITC-conjugated WGA (Stoeckel et al., 1977). Cells were then fixed 15 min, 2 d, or 4 d after this incubation and reacted with secondary antibodies to reveal Syt_{lum}-Abs. Fig. 6 shows the distribution of the two tracers 15 min (Fig. 6, *a* and *b*) and 2 days (Fig. 6, *c* and *d*) after the end of the incubation. Already at the earlier time point (Fig. 6, *a* and *b*) the two patterns of staining were very different. Syt_{lum}-Abs produced the typical staining seen in the absence of WGA (Fig. 6 *a*). WGA staining occurred in the form of large puncta which were present at similar concentration in the axons and dendrites, suggesting a similar uptake of the lectin by both processes (Fig. 6 *b*). After 2 d (Fig. 6, *c* and *d*) and 4 d (not shown), Syt_{lum}-Abs were still

primarily localized in the distal axonal arbour (Fig. 6 *c*). In contrast, all WGA was clustered in the perikaryal region (Fig. 6 *d*).

Exo-endocytotic Recycling of Syt I

The experiments described above suggested that in isolated neurons a significant fraction of total Syt I becomes surface exposed at least once during 1 h. In addition, the persistence of Syt_{lum}-Abs in cell processes of developing neurons for several days suggested a half-life of the Syt I/Syt_{lum}-Abs complex in the order of days. These considerations raised the possibility that in developing neuronal processes, like at synapses, Syt I and SVs undergo a very active exo-endocytotic recycling. To address this question, we used EM immunocytochemistry. We had shown earlier that at any given time the amount of Syt I present at the cell surface is very low. If Syt I and the Syt I/Syt_{lum}-Abs complex undergo recycling, addition of protein A-gold particles to neurons which had been previously exposed to Syt_{lum}-Abs for 1 h should result in the internalization of gold particles mediated by antibody-containing vesicles which are reexposed at the cell surface as a result of recycling. Neuronal cultures were incubated with Syt_{lum}-Abs for 1 h, washed for 15 min, incubated for

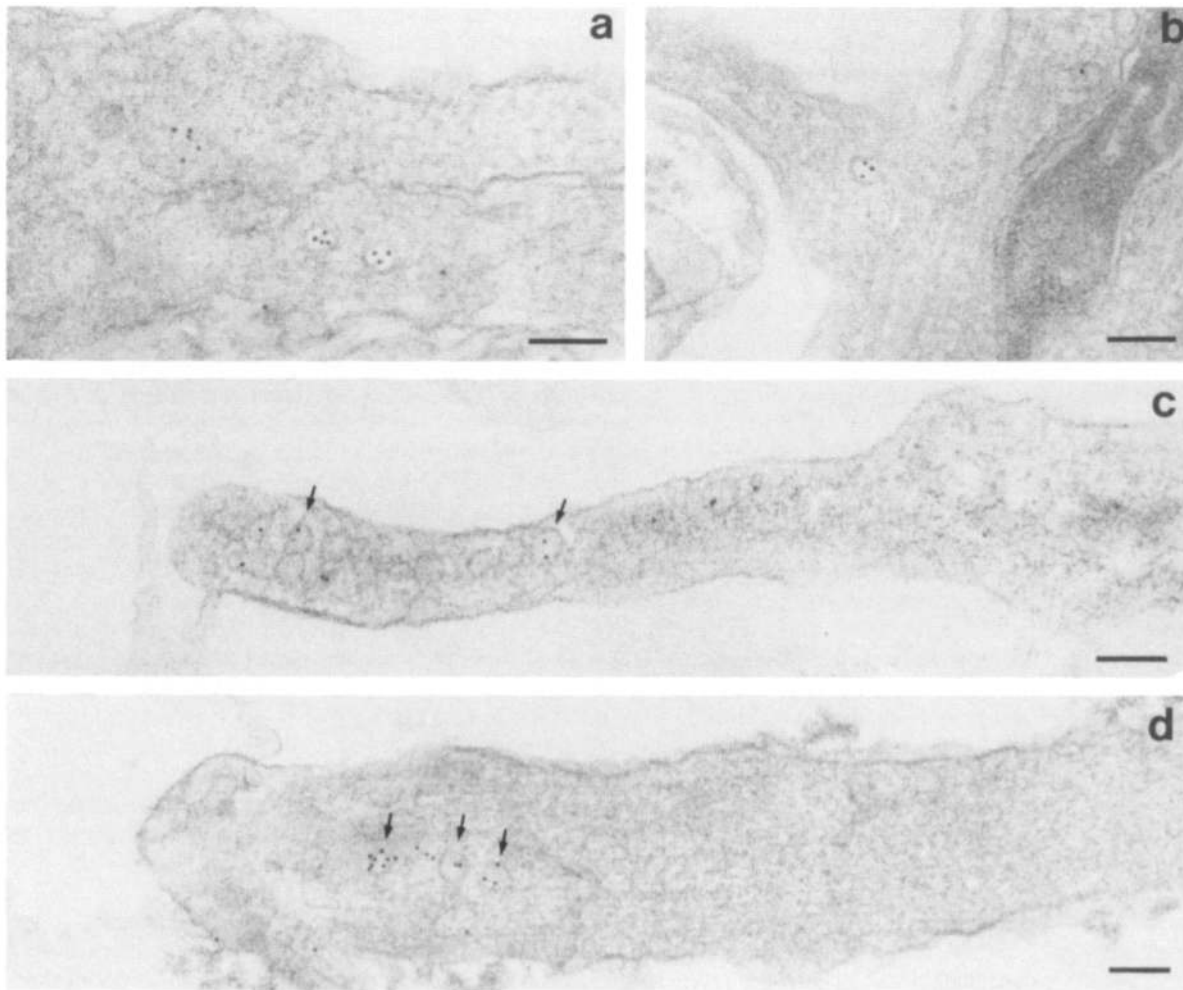


Figure 7. Internalization of Syt_{lum}-Abs by cultured hippocampal neurons revealed by EM immunocytochemistry. Neurons were first incubated for 1 h at 37°C with Syt_{lum}-Abs, then, 15 min later, for one additional hour with protein A-gold particles. A gallery of electron micrographs showing internalized gold particles is shown. Particles are localized in the lumina of small vesicles, many of which (*arrows*) are visible in axonal filopodia cut along a plane parallel to the substrate (*c* and *d*). No gold particles are present on the plasma membrane. Bars, 100 nm.

1 h with protein A-gold particles (6-nm diameter), briefly washed, and finally processed for plastic embedding and EM. A large number of internalized gold particles were observed. Most of these particles were localized within small vesicular structures, many of which were in the same size range as SVs (Fig. 7). These vesicles were often arranged in small clusters. Gold-labeled vesicles were particularly enriched in axonal filopodia (Fig. 7), which were also heavily stained by Syt_{lum}-Abs in immunofluorescence experiments (see for example *insets* of Fig. 5, *a* and *b*). These observations suggest that axonal filopodia may represent a preferential site for SV exocytosis. Virtually no gold particles were observed at free cell surfaces (Fig. 7). A few gold-labeled tubulo-vesicular structures and some multivesicular bodies were also observed. The former may represent intermediate stages in the reformation of SVs (Heuser and Reese, 1973), while the latter may reflect a pool of Syt I being targeted to the cell body. Practically no gold particles were observed within intracellular vesicles when the 1-h incubation with Syt_{lum}-Abs was substituted with a 1-h incubation with antibodies directed against the cytoplasmic domain of synap-

tophysin (not shown). These experiments confirm a rapid reinternalization of surface-exposed Syt I and a localization of internalized Syt I in SVs. In addition, they suggest a recycling of SVs in growing neuronal processes similar to the well established recycling of SVs in mature nerve endings (Ceccarelli et al., 1973; Heuser and Reese, 1973).

Discussion

In this study we have used antibodies directed against the luminal domain to Syt I, a major SV intrinsic membrane protein, to study SV exocytosis and recycling in living neurons by an immunocytochemical approach. Addition of the antibodies to the culture medium of cultured hippocampal neurons was found to be an effective way to monitor the exo-endo-cytotic traffic of SVs. In preliminary experiments carried out on mature cultures rich in synaptic contacts we demonstrated that nonpermeabilized presynaptic nerve terminals were labeled by Syt_{lum}-Abs under conditions at which exocytosis can take place, i.e., at 37°C, but not at 0°C. This finding validated the use of Syt_{lum}-Abs to detect exocytosis

and confirmed previous data suggesting lack of significant intermixing between SV membranes and the presynaptic plasmalemma during SV exocytosis. Experiments carried out at the motor end plate had demonstrated that even in conditions of sustained neurotransmitter release, no detectable levels of the SV membrane protein synaptophysin accumulate in the presynaptic plasmalemma if exocytosis is balanced by compensatory endocytosis (Valtorta et al., 1988; Torri-Tarelli et al., 1990).

Application of Syt_{lum}-Abs to developing isolated neurons demonstrated that SVs do undergo exocytosis in the processes of these neurons. In these immature cultures as well, no significant levels of Syt_{lum}-Abs became cell bound at 0°C, but a bright labeling was observed after 1 h of incubation at 37°C. These findings, which were made in the absence of any added stimulus, demonstrate that Syt I-containing membranes are continuously exposed to the cell surface by exocytosis and imply the occurrence of a very active compensatory endocytosis. Antibody labeling was found throughout the cytoplasm of neuronal processes and in the Golgi complex area, consistent with a rapid internalization of Syt I after surface exposure. The pattern of labeling was found to be coincident with that of the entire population of SVs as shown by counterstaining the same neurons after fixation and permeabilization with mAbs directed against the synaptic vesicle markers synaptophysin and SV2. The striking colocalization of Syt_{lum}-Abs with SVs indicated not only that the traffic of Syt I, as revealed by Syt_{lum}-Abs, reflects the traffic of SV membranes, but also that at least a significant fraction of the total SV population becomes labeled by Syt_{lum}-Abs during 1 h, i.e., undergoes exocytosis.

Some investigators have proposed that release of nonpeptide neurotransmitters from SVs at nerve terminals may occur via a protein channel rather than by full fusion of the SV membrane with the plasmalemma (Israël et al., 1986). Our findings, which show binding of antibody molecules to a luminal epitope of SVs both in developing and in mature axons, add an additional piece of evidence to the already strong evidence (Heuser and Reese, 1973; Ceccarelli et al., 1973; Torri-Tarelli et al., 1985) supporting a full fusion of the two membranes.

Another conclusion of our study is that in developing neuronal processes Syt I, and therefore SVs, undergo an active exo-endocytotic recycling similar to that which takes place at mature nerve endings (Ceccarelli et al., 1973; Heuser and Reese, 1973). This conclusion was indirectly suggested by the long half-life of the Syt I/Syt_{lum}-Ab complex (6 d after the incubation with Syt_{lum}-Abs no substantial decline in the fluorescence produced by Syt_{lum}-Abs was observed and Syt_{lum}-Abs were still precisely colocalized with SV markers), combined with the observation that a substantial fraction of Syt I becomes surface exposed during 1 h. A direct demonstration that Syt I can be surface exposed at least twice was provided by experiments in which cells were first incubated for 1 h with Syt_{lum}-Abs, then briefly washed to removed unbound antibodies, and finally incubated for another hour with protein A-gold conjugates. EM observation of these preparations revealed that a large number of vesicles with the size range of SVs were labeled by gold particles. This demonstrated not only recycling of the Syt I/Syt_{lum}-Ab complex, but also indicated that recycling of the complex reflects recycling of SVs. Presence of Syt_{lum}-Abs within the

lumen of SVs did not appear to interfere with their traffic because the overall distribution of SV marker proteins was the same in sister cultures which had or had not been exposed to Syt_{lum}-Abs as long as 6 d after the incubation.

A few gold-labeled multivesicular bodies were observed in the EM experiments. The localization of a small fraction of the gold label in multivesicular bodies may be related to the multivalent properties of protein A-gold conjugates which may divert a pool of the Syt/Syt_{lum}-Abs complex to a degradative pathway. In other experimental systems the formation of multimeric complexes by bivalent ligands was found to target for lysosomal degradation intrinsic membrane proteins that otherwise recycle between the plasmalemma and endosomes (Mellman et al., 1984; Mellman and Plutner, 1984). In fact, the distribution of Syt_{lum}-Abs, as assessed by immunofluorescence, was drastically different when rhodamine-conjugated secondary antibodies (goat anti-rabbit IgGs) were applied to living neurons at 37°C, rather than after fixation and permeabilization. In this case the distribution of Syt_{lum}-Abs was found to differ from that of other SV markers (our unpublished observations). This suggests that the formation of larger immune complexes can divert Syt I from its normal recycling pathway. Perhaps, problems of steric hindrance within the vesicle lumen prevent Syt_{lum}-Abs from cross-linking Syt I molecules. Secondary antibodies were able to change the localization of Syt_{lum}-Abs as long as at least 6 d after the primary antibody incubation, further supporting the conclusion that the Syt/Syt_{lum}-Abs complex is reexposed at the cell surface by exo-endocytotic recycling for several days.

The fate of internalized Syt_{lum}-Abs differed from that of internalized WGA (Gonatas et al., 1978; Harper et al., 1980) suggesting the existence in growing axons of sorting mechanisms which segregate molecules destined to local recycling from molecules destined to the cell body. It will be of interest to determine whether WGA and Syt_{lum}-Abs are initially targeted to the same population of sorting endosomes.

It was previously shown that expression of SVs protein by hippocampal neurons in culture precedes differentiation of axons and dendrites (Fletcher et al., 1991). When an axon first differentiates (stage 3), SV proteins become concentrated in this process and eventually restricted to this process. We have found here that labeling by Syt_{lum}-Abs matches the distribution of SV proteins even at early developmental stages, indicating that, at this stage, SVs undergo exo-endocytosis in all cellular compartments including dendrites. Dendritic uptake of Syt_{lum}-Abs was observed also when microtubules had been previously depolymerized by nocodazole treatment (our unpublished observations). This result indicated that dendritic labeling was not dependent upon uptake of antibodies in the axon followed by retrograde transport along microtubules. The occurrence of SV exocytosis in dendrites of isolated hippocampal neurons in culture is consistent with the presence of the protein rab3A on SVs both in dendrites and in axons of the same neurons (Matteoli et al., 1991). Rab3A is a low molecular weight G protein which is thought to play an important role in exocytosis of SVs but which is associated with SV membranes only at those stages which directly precede exocytosis (Fisher v. Mollard et al., 1990, 1991; Matteoli et al., 1991).

Earlier studies carried out by electrophysiological techniques on motor neurons in primary culture have shown that

the neurotransmitter acetylcholine is released from developing axons before the formation of synaptic contacts. More specifically, muscle cells or excised patches of muscle cell membranes attached to a recording pipette were used to demonstrate that acetylcholine is released from most of the entire length of the growing axon (Hume et al., 1983; Young and Poo, 1983; Sun and Poo, 1987; Evers et al., 1989). Young and Poo (1983) had also noted a pulsatile nature of the release and had suggested that it may be a vesicular release. We complement these previous data by demonstrating that developing axons are equipped with the exocytotic machinery which is involved in the release of nonpeptide neurotransmitters at mature nerve terminals. In agreement with these previous data, we show that SV exocytosis takes place along most of the entire axonal surface, with the possible exception of its most proximal portion. Thus, as can be predicted, SV exocytosis does not appear to play any special role in the function of the growth cone, where constitutive exocytotic processes involving other types of vesicles may take place (Lockerbie et al., 1991).

An open question is whether and how SV exocytosis in developing neurons is regulated. In the experiments carried out so far we did not apply any stimuli. Preliminary experiments (not shown) have suggested that exocytosis of SVs as revealed by binding and internalization of Syt_{lum}-Abs, occurs also in Ca⁺⁺-free media. It is of interest that release of Ach from developing axons, as demonstrated by patch clamp techniques (Young and Poo, 1983; Hume et al., 1983; Xie and Poo, 1986; Sun and Poo, 1987; Evers et al., 1989), was not found to be dependent on extracellular Ca⁺⁺. At mature synapses, depolarization-evoked release of neurotransmitters is crucially dependent on extracellular Ca⁺⁺, while basal release is only minimally affected by lack of extracellular Ca⁺⁺ (Fatt and Katz, 1952; Hubbard et al., 1968). SV exocytosis may occur constitutively from developing neurons, and regulatory mechanisms tightly controlled by elevations in cytosolic Ca⁺⁺ may develop only after the formation of a presynaptic specialization, or of presynaptic-like specializations (i.e., SV clusters) in the case of neurons which do not have a "bona fide" postsynaptic site, such as sympathetic neurons. Alternatively, in developing neurons, as in fully differentiated neurons, constitutive and regulated exocytosis may coexist, although maturation of presynaptic nerve endings may coincide with some change in the fine regulation of synaptic vesicle exocytosis. Clearly, our findings, together with those of previous studies (Young and Poo, 1983; Hume et al., 1983; Xie and Poo, 1986; Sun and Poo, 1987; Evers et al., 1989), raise the possibility that release of neurotransmitter from developing neurons may play an important role in the development of neuronal networks and of the proper interactions between axons and innervated nonneuronal tissues. They suggest an important trophic role of nonpeptide neurotransmitters beside the well known role in the fast, point-to-point signaling which occurs at synapses.

The occurrence of SV exocytosis at stages that precede synapse formation is not surprising if one considers recent results that have emerged from the study of neuroendocrine cells. These cells contain a population of microvesicles (synaptic-like microvesicles) that resemble bona fide SVs in a number of biochemical and functional properties (Navone et al., 1986; Wiedenmann et al., 1988; Reetz et al., 1991;

Linstedt and Kelly, 1991; De Camilli, 1991). Synaptic-like microvesicles, like SVs, undergo exocytosis and recycling (Navone et al., 1986; Johnston et al., 1989a; Cameron et al., 1991; Linstedt and Kelly, 1991). Thus, the property to undergo exo-endocytotic recycling appears to be a general property of SVs and related organelles, irrespective of their clustering in nerve endings. The property of SVs to undergo an active recycling before synaptogenesis, further supports the suggestion that SVs may have evolved from a constitutively recycling vesicular organelle present in all cells (Cameron et al., 1991).

Concluding Remarks: Syt_{lum}-Abs as Powerful Tools to Study SV Traffic and Synaptogenesis in Living Neurons

Syt_{lum}-Abs were used here to study the traffic of Syt I and, more generally, the traffic of SVs in developing neurons. Syt_{lum}-Abs are the first available antibodies selectively directed against a luminal epitope of SVs which recognize the native protein in living cells. An unexpected finding was the extremely long half-life of the Syt/Syt_{lum}-Abs complex and the property of this complex to remain localized within SVs for at least several days. Furthermore, presence of the antibodies within SVs did not appear to modify their localization or their properties to undergo exo-endocytosis. These characteristics made Syt_{lum}-Abs antibodies an optimal tool for our study. Because of these properties, Syt_{lum}-Abs promise to be a powerful tool in a variety of other experimental protocols. For example, since clustering of SVs at synaptic sites represent a key step of synaptogenesis, Syt_{lum}-Abs can be used as an *in vivo* marker for synapse formation. Conjugation of Syt_{lum}-Abs to appropriate fluorochromes should allow morphological monitoring of the onset of synaptogenesis in living neurons. Finally, the availability of antibodies specifically directed against luminal epitopes of SVs may allow one to set up quantitative assays to monitor SV exocytosis in intact cells and in cell-free systems. These assays are crucially required to advance our understanding of molecular mechanisms involved in neurotransmitter release.

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