Association of Rab3A with Synaptic Vesicles at Late Stages of the Secretory Pathway

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Abstract. Rab3A is a small GTP-binding protein highly concentrated on synaptic vesicles. Like other small GTP-binding proteins it is thought to cycle between a soluble and a membrane-associated state. To determine at which stage of the life cycle of synaptic vesicles rab3A is associated with their membranes, the localization of the protein in neurons and neuroendocrine cells at different developmental and functional stages was investigated. In all cases, rab3A was colocalized with synaptic vesicle markers at the cell periphery, but was absent from the Golgi area, suggesting that rab3A associates with vesicles distally to the Golgi complex and dissociates from vesicle membranes before they recycle to this region. Immunofluorescence experiments carried out on frog motor end plates demonstrated that massive exocytosis of synaptic vesicles is accompanied by a translocation of rab3A to the cell surface. The selective localization of rab3A on synaptic vesicles at stages preceding their fusion with the plasmalemma suggests that the protein is part of a regulatory machinery that is assembled onto the vesicles in preparation for exocytosis.

NEURONS are secretory cells that release neurotransmitters by regulated exocytosis. However, nerve cells have several unique properties, that set them apart from other secretory systems. It is now well established that neurons can secrete two classes of molecules, neuropeptides and classical neurotransmitters, via two different Ca^{2+}-dependent mechanisms (Hökfelt et al., 1984; Smith and Augustine, 1988). Biosynthesis, transport, processing, storage, and release of neuropeptides follow the established route of regulated secretion in other secretory cells. This involves packaging of the peptides in secretory granules (large dense-core vesicles) at the level of the Golgi complex and transport of the fully assembled granules to the sites of release (for reviews see Burgess and Kelly, 1987; De Camilli and Jahn, 1990). In contrast, storage and release of nonpeptide neurotransmitters involves a different type of secretory organelle, the small synaptic vesicle (synaptic vesicle). Synaptic vesicles are loaded with content in nerve terminals where they can be continuously regenerated by local membrane recycling. Exocytosis of large dense-core vesicles and of synaptic vesicles is differentially regulated and takes place at distinct sites of the nerve terminal surface (for review see De Camilli and Jahn, 1990).

The identification and characterization of several membrane proteins specific for synaptic vesicles has greatly advanced our understanding of the biogenesis and life cycle of these organelles (Kelly, 1988; Südhof and Jahn, 1991; Matteoli and De Camilli, 1991). Furthermore, the study of synaptic vesicle proteins has led to the discovery of a hitherto unknown class of related microvesicles in endocrine cells (synaptic-like microvesicles). These vesicles are involved in an exo-endocytotic membrane cycle independent of secretory granules (De Camilli and Navone, 1986; Johnston et al., 1989*a*; Baumert et al., 1989, 1990; Cutler and Kramer, 1990. Clift O-Grady et al., 1990; Cameron et al., 1991).

The life cycle of synaptic vesicles includes biogenesis in the region of the *trans*-Golgi network, transport to nerve terminals, regulated exocytosis, and local recycling by a pathway which probably includes coated vesicles and endosome-like compartments, retrograde transport to the cell body (De Camilli and Jahn, 1990; Südhof and Jahn, 1991). It is clear that each step of this pathway must be controlled by specific proteins interacting with the vesicle surface in an ordered and sequential fashion. Thus, proteins which shuttle between membrane bound and free states during the life cycle of synaptic vesicles are of special interest. One such protein is synapsin (Südhof et al., 1989*b*; Sihra et al., 1989; De Camilli et al., 1990). Recently, low molecular weight GTP-binding proteins (G-proteins) have emerged as strong candidates for such traffic controllers in both exo- and endo-cytic pathways in all eukaryotic cells. A large number of these proteins have been identified. Most of them are present in a soluble and in a membrane-bound pool (Bourne, 1988; Walworth et al., 1989; Hall, 1990; Balch, 1990).

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Each of these proteins appears to be associated with subsets of functionally interconnected membrane systems in a highly specific way. By immunocytochemistry, the yptl protein (Gallwitz et al., 1983) was found to be localized in the Golgi complex (Gallwitz et al., 1983; Segev et al., 1988), rab2 in a putative intermediate compartment between the ER and the Golgi complex (Chavrier et al., 1990*a*, *b*), rab5, and rab7 on organelles of the endocytic pathway (Chavrier et al., 1990*a*, *b*), and rab6 on medial and *trans*-cisternae of the Golgi apparatus (Goud et al., 1990).

Recently it was shown that rab3A, a G-protein selectively expressed in neurons and neuroendocrine tissues (Touchot et al., 1987; Matsui et al., 1988; Ayala et al., 1989; Sano et al., 1989; Zahraoui et al., 1989; Mizoguchi et al., 1990; Fischer v. Mollard et al., 1990), is concentrated on synaptic vesicles in mammalian brain (Fischer v. Mollard et al., 1990). This localization raised the possibility that this G-protein is involved in directing some aspect of synaptic vesicle traffic, a hypothesis that was further supported by the observation that rab3A appears to dissociate from synaptic vesicle membranes after nerve terminal stimulation (Fischer v. Mollard et al., 1991). However, it remained unclear at which stages of the vesicular life cycle rab3 is associating or dissociating from the vesicle membrane.

In the present study we have used monoclonal and polyclonal antibodies and different experimental systems to investigate the association of rab3A with synaptic vesicles at different stages of their life cycle. Our results indicate that rab3A immunoreactivity colocalizes with synaptic vesicles and synaptic-like microvesicles present in the cell periphery. They suggest that rab3A becomes associated with vesicle membranes only after they leave the Golgi complex and dissociates from them at some point after fusion.

Materials and Methods

cDNA Cloning and Expression in COS cells

Bovine rab3B was cloned by the polymerase chain reaction exactly as previously described for rab3A (Fischer v. Mollard et al., 1990) except that the oligonucleotide primers used had the sequences GCGAAGCTTCATATGGCTTCAGTGACCGATGGTAA and GCGGATCCTAGCACGAGCAGTTCTGCTG, respectively. The polymerase chain reaction product was cloned into the expression vector pCB1 (Fischer v. Mollard et al., 1990) and the resulting construct was transfected into COS cells as described (Mignery et al., 1990). The construct and the sequence of the polymerase chain reaction product were verified by DNA sequencing after subcloning into M13 vectors using the dideoxy chain termination method (Sanger et al., 1976). SDS-PAGE analysis of the proteins from transfected COS cells followed by immunoblotting were performed as described using peroxidase-labeled secondary antibodies (Johnston et al., 1989*b*).

Expression and Purification of Recombinant Rab3A

Rab3A was expressed in bacteria as described (Fischer v. Mollard et al., 1990) and found to be mostly soluble and actively binding GTP after bacterial expression (data not shown). One liter cultures of expressing bacteria induced for two hours with IPTG were harvested and lysed in 50 mM Hepes-NaOH, pH 7.4, 1 mM EDTA, 0.1 M NaCl, 5 μg/ml leupeptin and pepstatin, 1 mg/ml lysozyme, and 0.2 mM PMSF on ice for 15 min, using 2 ml buffer per gram of bacteria (wet weight). Cells were broken by four 15-s bursts of sonication with intermittent cooling using a Sonifier cell disrupter 350. Cells were then incubated for 30 min at room temperature with 20 μg/ml of DNAse I (Boehringer-Mannheim Biochemicals). Bacterial cytosol was prepared by centrifuging the lysed cells for 1 h at 50,000 rpm in a rotor (Ti70; Beckman Instruments Inc., Palo Alto, CA). Rab3A was purified from the cytosol by two cycles of anion exchange chromatography on a monoQ column. The first chromatography was performed in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM DTT using a gradient from 0.05 to 1.0 M NaCl. Rab3A eluted as a single peak at 0.18 M NaCl and was rechromatographed on a monoQ column in 20 mM BisTris, pH 6.4, 1 mM EDTA, and 1 mM DTT with a gradient from 0.1 and 1.0 mM NaCl. On this column, rab3A eluted almost homogeneous at 0.17 M NaCl and was used for the immunizations.

Antibodies

Polyclonal rabbit antibodies were generated against a 12-mer peptide (Fischer v. Mollard et al., 1990) corresponding to the NH_2-terminus of rat rab3A (Touchot et al., 1987). The serum was affinity purified as described (Südhof et al., 1989) using the same peptide and found by immunoblotting to recognize only rab3A in a total brain homogenate (Fischer v. Mollard, 1990). mAbs directed against rab3A were generated according to standard procedures (Kohler and Milstein, 1975; Jahn et al., 1985) using recombinant rab3A as antigen (Fischer v. Mollard et al., 1990). Analysis of the specificity of the polyclonal and monoclonal antibodies using COS cells transfected with control DNA or rab3A and rab3B expression constructs demonstrated that the polyclonal antibody and one of the mAbs was specific for rab3A, whereas the other mAb recognized both rab3A and rab3B (see Fig. 1). Monoclonal and polyclonal antibodies directed against synaptophysin were prepared and purified as previously described (Navone et al., 1986). The characterization of a mAb to the cytoplasmic domain of synaptotagmin (protein p65) will be described elsewhere. Monoclonal antibodies directed against the *trans*-Golgi integral membrane protein GIMPt were the kind gift of C. A. Suarez-Quian (Georgetown University, Washington, D.C.). Polyclonal antibodies raised against yeast yptl were the kind gift of D. Gallwitz (Göttingen, Germany).

Light Microscopy Immunocytochemistry

Tissue sections. 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. Preparation of frozen sections and fluorescence immunostaining was performed as described (De Camilli et al., 1983*a*; Baumert et al., 1990).

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 (1984). Briefly, hippocampi were dissociated by treatment with trypsin (0.1% 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 supplemented with 2 mM glutamine, 1% HL1 (Ventrex, Portland, ME), 10% horse serum, and 5% FCS at densities ranging from 16,000 cells/cm^2 to 20,000 cells/cm^2. The following day, coverslips were transferred (upside down) to dishes containing a monolayer of cortical glial cells, so that they were suspended over the glial cells but not in contact with them (Bartlett and Banker, 1984). Cells were maintained in MEM without sera, supplemented with 1% HL1, 2 mM glutamine, and 1 mg/ml BSA. Neurons were fixed with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer containing 0.12 M sucrose. They were processed for single or double immunofluorescence as described (De Camilli et al., 1983*a*; Baumert et al., 1990) and mounted in 70% glycerol in PBS containing 1 mg/ml phenylenediamine. Primary bovine chromaffin cells were the kind gift of Dr. Haycock (Louisiana State University Medical Center, New Orleans, LA) and were prepared as described (Haycock et al., 1988). PC12 cells (Greene and Tischler, 1976) were the kind gift of R. Burry (Ohio State University, Columbus, Ohio).

Neuromuscular Preparations. Pairs of cutaneous pectoris nerve muscle preparations were used for control and stimulation experiments. They were dissociated from MS 222 (Sandoz Pharmaceutical, Basel, Switzerland) anaesthetized frogs (Rana pipiens) and soaked in 2 ml of modified Ringer's solution containing 115 mM NaCl, 2.1 mM KCl, 1 mM NaH_2PO_4, 4 mM $MgCl_2$, and 1 mM EGTA, in the presence or in the absence of black widow spider venom, as described in Hurlbut and Ceccarelli, 1979. During the incubation (15 min at room temperature), standard electrophysiological techniques were used to record miniature endplate potentials intracellularly, in order to ascertain that the secretory process induced by the venom corresponded to the one previously described (Hurlbut and Ceccarelli, 1979). Neuromuscular preparations were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, stained using secondary antibodies conjugated to rhodamine and counterstained with fluorescein-conjugated α-bungarotoxin (5 μg/ml) as previously described (Matteoli et al., 1988). For microscopic observations single muscle fibers were teased apart under a dissecting microscope and mounted on glass slides in 95% glycerol.

Microscopic Observation. Preparations were examined with an axiophot microscope (Zeiss; Oberkochen, Germany) equipped with epifluorescence microscopy or with a scanning confocal laser microscope (600 MRC; Bio-Rad Laboratories, Cambridge, MA). In the latter case, cells were illuminated with 488-nm light from the instrument's argon laser through a Zeiss axiovert microscope (Zeiss) using a 63×/1.25 NA Neofluar objective. The confocal aperture in the laser scanning assemblage was kept completely open to maximize the collection of epifluorescent light from the sample. The signal was stored in the frame buffer of the microscope's Nimbus host computer.

Electron Microscopy Immunocytochemistry

Immunogold labeling of agarose embedded tissue fragments of bovine and/or rat hypothalami was performed as described (De Camilli et al., 1983*b*). Fragments were produced by a gentle homogenization in a loose-fitting glass-teflon homogenizer. The homogenization medium contained 0.25 M sucrose in 5 mM phosphate buffer. A lytic fixation (De Camilli et al., 1983*b*) was used to allow antibody penetration in nerve endings resealed after homogenization.

Results

Purification of Recombinant Rab3A and Production of mAbs

Bovine rab3A was expressed in bacteria as described (Fischer v. Mollard et al., 1990) and was purified by ion exchange chromatography to more than 90% purity (data not shown). Purified rab3A was used to produce mAbs, resulting in the production of two different hybridoma lines producing rab3-specific antibodies of the IgG-type. Since immunoblotting of brain extracts demonstrated that these antibodies were specific for a 25-kD band but suggested differences in their epitope specificity (data not shown), we investigated the reactivities of the mAbs with rab3A and rab3B expressed by transfection in COS cells. As shown in Fig. 1, one mAb (clone 42.2) specifically reacted only with rab3A, whereas the second antibody (clone 42.1) equally recognized rab3A and rab3B, suggesting that these two small GTP-binding proteins not only share sequence homology but also contain a common exposed structural motif. For this study antibodies specific to rab3A were used.

Selective Localization of Rab3A on Synaptic Vesicles at the Neuronal Periphery

By double-immunofluorescence antibodies specific for rab3A were found to produce an immunostaining pattern of the rat brain neuropile very similar to that produced by antibodies directed against other synaptic vesicle markers, namely synapsin I (De Camilli et al., 1983*a*) (Fig. 2, *a* and *b*), protein p29 (Baumert et al., 1990) (Fig. 2, *c* and *d*), synaptophysin (Navone et al., 1986), and synaptotagmin (Perin et al., 1990) (not shown). The unit element of the immunostain in all micrographs was represented by puncta of variable size which correspond to individual nerve terminals filled with synaptic vesicles. The close correspondence of puncta positive for rab3A and puncta positive for p29 is demonstrated in Fig. 2, *c* and *d* which show double labeling of a brain stem section at high magnification. A similar pattern of rab3A immuno-

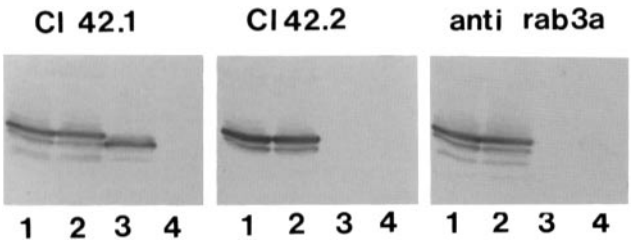

Figure 1. Characterization of polyclonal and monoclonal antibodies directed against rab3. Immunoblots of total homogenates of COS cells which had been transfected with the following cDNA's: lane *1*, bovine rab3A; lane *2*, human rab3A; lane *3*, bovine rab3B; and lane *4*, control DNA. Blot 1 was probed with the mAb 42.1; blot 2 with the mAb 42.2, and blot 3 with rab3 polyclonal antibody.

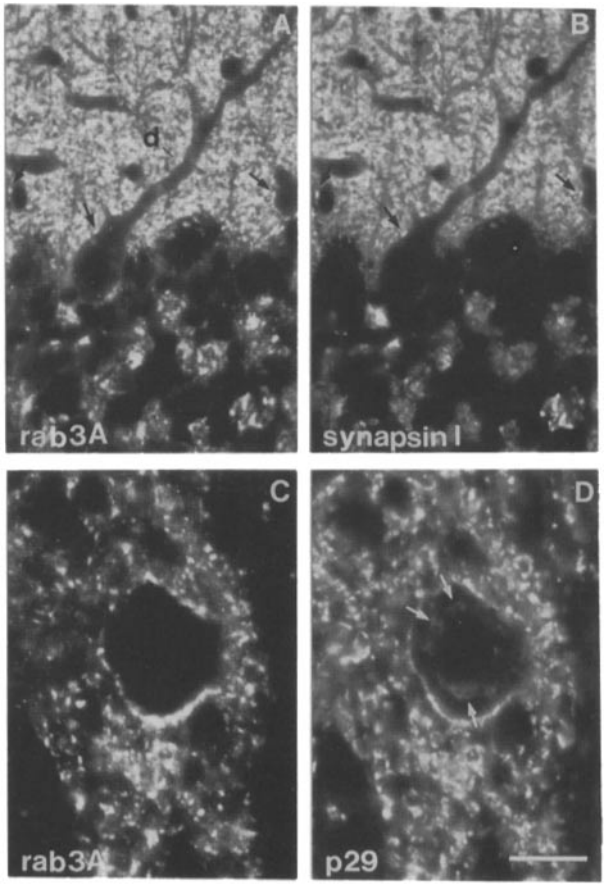

Figure 2. Double-immunofluorescence micrographs showing colocalization of immunoreactivities for rab3A and other synaptic vesicle proteins in rat brain sections. (*A* and *B*) Colocalization of rab3A (*A*) with synapsin I (*B*) in rat cerebellar cortex. In the molecular and Purkinje cell layers, brightly fluorescent dots, which represent single nerve terminals, outline negative images of cell bodies and dendrites (*d*). In the granule cell layer, immunoreactive islands represent nerve terminals of the glomeruli. Virtually all nerve endings positive for rab3 are also positive for synapsin I. Note, for example, endings indicated by small black arrows in the two pairs of pictures. (*C* and *D*) Comparison of the localization of rab3A (*C*) and of protein p29 (*D*) in rat brainstem. In both fields neuronal perykaria and dendrites are outlined by immunoreactive terminals (not all nerve terminal positive for p29 are positive for rab3A, indicating a heterogeneous expression of rab3A by central nervous system neurons). A network of labeled perinuclear particles, representing elements of the Golgi complex, can be observed in sections stained with the antibody directed against p29 (*D, arrows*). These Golgi complex elements are not labeled in neurons stained with antibodies directed against rab3A (*C*). Bars: (*A* and *B*) 25 μm; (*C* and *D*) 19 μm.

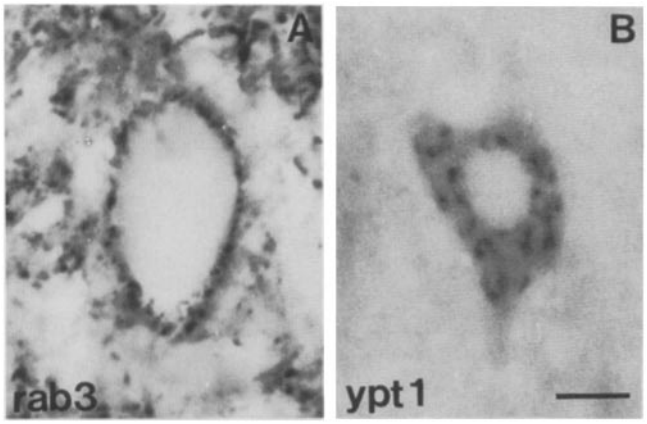

Figure 3. Bright field light microscopy micrographs showing a comparison of the distribution of rab3A (*A*) and of YPT1 (*B*) in rat brain. Immunoperoxidase staining of brain stem sections (immunoreactivity appears black). Two similar neurons of the same regions are shown. Rab3A immunoreactivity is present in axon terminals, many of which outline the perikaryon of the neuron which is completely devoid of rab3A immunoreactivity. YPT1 (rabl) immunoreactivity is present only in cytoplasmic particles which have the typical distribution of the Golgi complex in neurons (see De Camilli et al., 1985). Bar, 8 μm.

reactivity was observed at all of a variety of central nervous systems regions examined which included cerebellum (Fig. 2 *a*), cerebral cortex, hippocampus, caudatus, putamen, brainstem (Fig. 2 *c*), and spinal cord. Furthermore, similar patterns of immunostaining were obtained with polyclonal antibodies specific for rab3A and mAbs that recognize both rab3A and rab3B (not shown).

It was demonstrated previously that significant immunoreactivity for intrinsic membrane proteins of synaptic vesicles is also found in neuronal Golgi complexes (Navone et al., 1986; Baumert et al., 1990) (see also Fig. 2 *d*). Such immunoreactivity represents newly synthesized synaptic vesicle membrane proteins and synaptic vesicle membranes recycled from the nerve terminal (De Camilli and Jahn, 1990; M. Matteoli, M. Perin, T. C. Südhof, and P. De Camilli, unpublished observations). No rab3A immunoreactivity was detected under any condition of immunostaining in the Golgi complex region of any neurons in brain tissue (Figs. 2 *c* and 3 *a*). However, the region of the neuronal Golgi complex was stained intensely by an antibody directed against another small GTP-binding protein, YPT1 (Fig. 3 *b*) which was previously shown to be localized in the Golgi complex region of other cell types (Segev et al., 1988).

The subcellular localization of rab3A in nerve terminals was investigated by immunogold labeling of agarose-embedded tissue fragments using polyclonal antibodies. Gold particles were found to be selectively localized in the presynaptic compartment. As shown in Fig. 4, within nerve terminals they were almost exclusively associated with the membranes of synaptic vesicles. Only scattered gold particles were sometimes observed on other organelles such as mitochondria. No gold labeling was found on the plasmalemma, as previously shown for other synaptic vesicle proteins in resting nerve terminals (Navone et al., 1986; Baumert et al., 1990).

An association of rab3A with synaptic vesicles was also observed at stages preceding synapse formation. This was shown in hippocampal neurons in primary culture. In immature isolated neurons synaptic vesicles are scattered throughout neuronal processes and are particularly concentrated in axons, primarily the distal axon (Fletcher et al., 1991). Furthermore, at this developmental stage, the accumulation of synaptic vesicle marker proteins in the Golgi complex area is more prominent than in older cultures or in adult brain tissue (Fletcher et al., 1991). Fig. 5 shows isolated hippocampal neurons double stained for rab3A and for the intrinsic protein of synaptic vesicles synaptotagmin (protein p65). As shown by the figure, rab3 colocalizes with synaptotagmin in the processes, but not in the Golgi area. The site of the Golgi complex in these neurons was determined by double labeling for synaptotagmin and for GIMPt, a marker antigen for this organelle (Yuan et al., 1987) (*insets* of Fig. 5 *c*). In older cul-

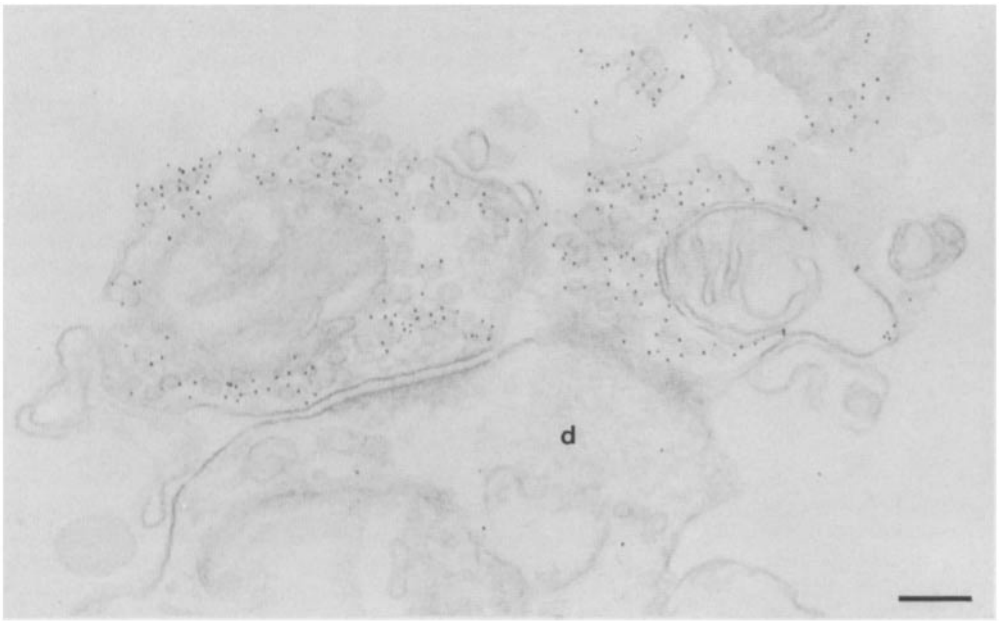

Figure 4. Electron micrograph showing the selective accumulation of rab3A on synaptic vesicles in nerve terminals. Immunogold labeling of agarose-embedded fragments of bovine brain. Axon endings still attached to portions of a dendrite (*d*) via the synaptic junction are visible. Gold particles are primarily concentrated in nerve terminals on the membranes of synaptic vesicles. The presynaptic plasmalemma is unlabeled. Bar, 138 nm.

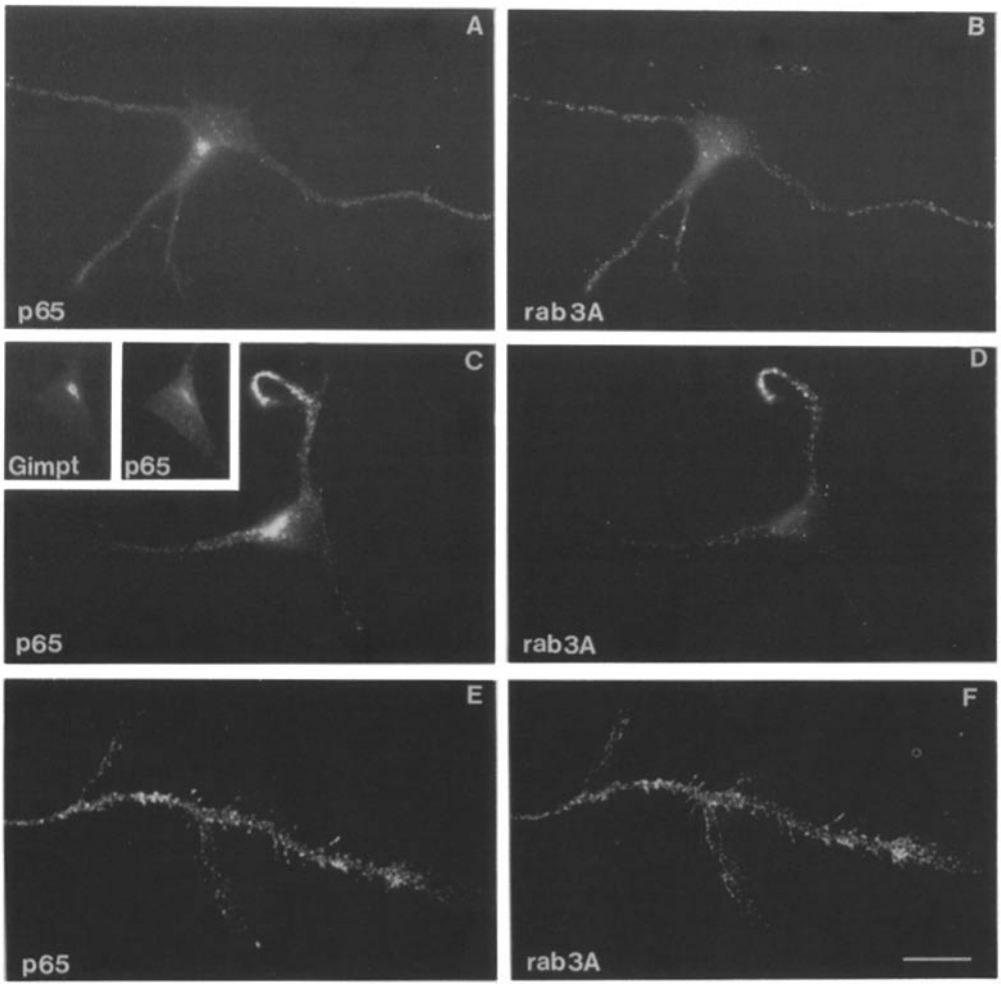

Figure 5. Double-immunofluorescence micrographs illustrating the distribution of rab3A and of the intrinsic protein of synaptic vesicles synaptotagmin (protein p65) in cultured hippocampal neurons. (*A–D*) Rab3A, *B* and *D*, and synaptotagmin, *A* and *C*, have a similar distribution in neuronal processes of a neuron after 3 d in vitro. Synaptotagmin immunoreactivity, but not rab3A immunoreactivity, is present in the Golgi complex region. The two insets of *C* show double labeling of a same neuron for GIMPt, an integral membrane protein of the "*trans*-Golgi" (Yuan et al., 1987), and for synaptotagmin. They show that paranuclear accumulation of synaptotagmin corresponds to the Golgi complex. (*E* and *F*) Colocalization of rab3A and synaptotagmin in a same isolated axon of a neuron maintained in culture for 5 d. Both proteins have an identical punctate distribution throughout the distal axonal arbours. (*A–D*) 8.5 μm; *E* and *F*, 7 μm; *Inset*, 9 μm.

tures, as synaptic contacts were established, rab3 was found to cluster as presynaptic sites in parallel with other synaptic vesicle markers (not shown) (Fletcher et al., 1991).

Rab3A Selectively Colocalizes with Peripheral Vesicles in Cultured Chromaffin Cells

Endocrine cells contain a population of recycling microvesicles distinct from secretory granules (synaptic-like microvesicles) which are biochemically similar to neuronal synaptic vesicles (De Camilli and Jahn, 1990). They are scattered in the cytoplasm of these cells and are particularly concentrated in their Golgi-centrosomal area (Navone et al., 1986; Baumert et al., 1990). A significant proportion of the synaptic-like microvesicles, including those in the Golgi centrosomal area, can be labeled by extracellular tracers suggesting their endocytic origin (Johnston et al., 1989; M. Matteoli, A. Metcalf, M. Perin, T. C. Südhof, and P. De Camilli, unpublished results). Rab3A was shown to selectively copurify with synaptophysin-positive microvesicles in bovine adrenal medulla (Fischer v. Mollard et al., 1990). It was therefore of interest to determine whether rab3A is associated with recycling membranes of synaptic-like microvesicles at all stages or whether, as in neurons, rab3A is absent from vesicles in the area of the Golgi complex.

To address this question, the distribution of rab3A immunoreactivity was compared with the distribution of synaptophysin, a major intrinsic membrane protein of synaptic-like microvesicles, in cultured bovine chromaffin cells and in PC12 cells, a cell line derived from rat chromaffin cells. Fig. 6, *a* and *b* shows a bovine chromaffin cell with a single short process. Synaptophysin immunoreactivity is very intense in the paranuclear region and at the end of the cellular process. Rab3 immunoreactivity is colocalized with synaptophysin in the process but is absent from the synaptophysin-positive structures in the cell body. Fig. 6, *c* and *d* shows double-stained undifferentiated PC12 cells visualized by confocal laser microscopy. Only synaptophysin is visible in the area of the Golgi complex. Both synaptophysin and rab3A immunoreactivity are colocalized in the peripheral cytoplasm, in particular at areas of cell contacts. In the case of synaptophysin, this peripheral immunoreactivity was previously shown by EM immunocytochemistry to be represented by subplasmalemmal vesicles (Johnston et al., 1989). In PC12 cells with processes, both rab3A and synaptophysin were localized at the tip of the processes, but only synaptophysin was present in the Golgi complex area (not shown).

Redistribution of Rab3A in Nerve Terminal upon Massive Exocytosis

The results reported above support the hypothesis that rab3A associates with vesicles membranes distally to the Golgi complex and dissociates from vesicles before their return to

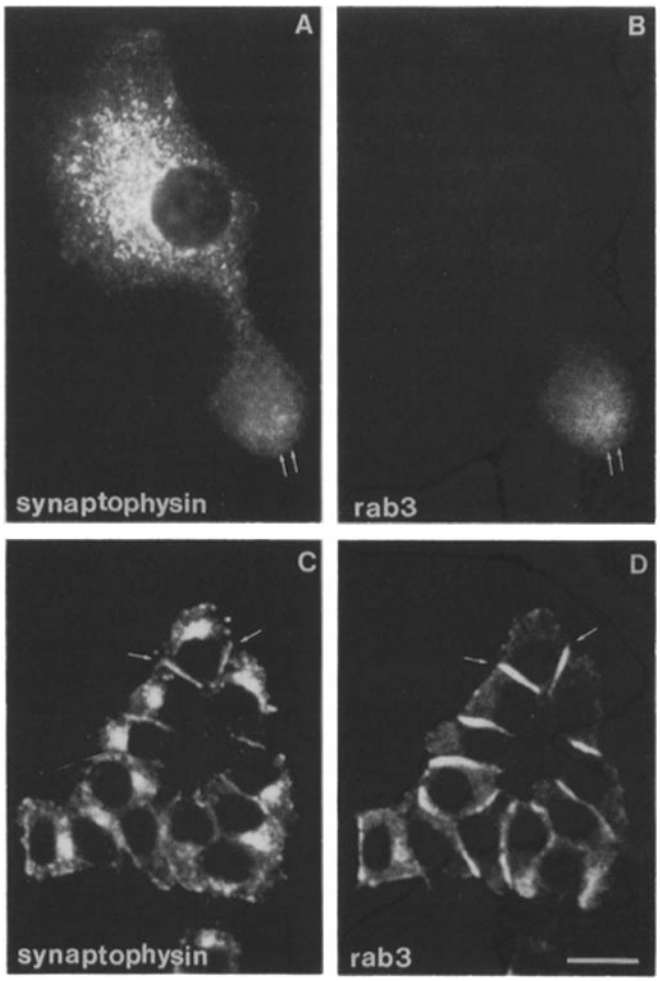

Figure 6. Double-immunofluorescence micrographs showing comparisons of the distribution of synaptophysin and of rab3 in cultured bovine chromaffin cells and in PC12 cells. (*A* and *B*) Cultured bovine chromaffin cell double stained for synaptophysin (*A*) and rab3 (*B*). Puncta of synaptophysin immunoreactivity are present throughout the cell but are particularly concentrated in the cell process and at a paranuclear location that corresponds to the Golgi/centrosomal region. Rab3A is present only in the cell process and is not detectable in the Golgi/centrosomal region. Within the cell process most rab3-positive puncta precisely correspond to synaptophysin-positive puncta (for example, *double arrows*). (*C* and *D*) Confocal laser micrograph showing PC12 cells double labeled for synaptophysin (*C*) and for rab3A (*D*). Both rab3A and synaptophysin are colocalized at the cell periphery (arrows indicate their accumulation at regions of cell contacts). Rab3A immunoreactivity is not detectable in the Golgi area, where synaptophysin is concentrated. Bar: *A–D*, 12.5 μm.

the Golgi/centrosomal area. We next investigated whether rab3A dissociates from synaptic vesicle membranes before or after their fusion with the plasmalemma. To this aim we used the frog neuromuscular junction as the experimental system. This synapse is a particularly suitable model for these morphological experiments because of its very large size and of the thorough characterization of experimental conditions that lead to synaptic vesicle depletion (Ceccarelli and Hurlbut, 1980). Massive exocytosis coupled to a block of endocytosis can be obtained at this synapse by stimulation with the black widow spider venom in the absence of extracellular Ca^{2+}. The result is a complete depletion of synaptic vesicles with a concomitant increase of the plasma-membrane area and a redistribution of synaptic vesicle antigens in the plasmamembrane (Torri-Tarelli et al., 1989).

We investigated whether in neuromuscular synapses stimulated by black widow spider venom the redistribution previously observed for synaptophysin and synapsin I by immunofluorescence could also be observed for rab3A. In preliminary experiments the frog motor end plate was found to contain rab3A immunoreactivity detectable by our antibodies raised to mammalian rab3A (Fig. 7, *a* and *b*). In resting nerve terminal rab3A immunoreactivity had the same stripped distribution (Fig. 7 *d*) observed for other synaptic vesicle markers (Fig. 7 *e*). Stripes are determined by segments of the terminals densely populated by vesicles alternating with segments devoid of vesicles which correspond to nerve ending portions embraced by Schwann cell processes (Valtorta et al., 1988; Matteoli et al., 1988).

After treatment with black widow spider venom, rab3A immunoreactivity was found to have a very different pattern. It outlined the profile of the nerve terminal that, as expected, had increased in width (Fig. 7 *F*). An identical redistribution was observed for synapsin I (Fig. 7 *G*) and synaptophysin (not shown) immunoreactivities. This redistribution was previously shown by electron microscopy to reflect translocation of synaptic vesicle proteins to the plasmalemma (Torri Tarelli et al., 1989). These findings suggest that the bulk of rab3A remains associated with the synaptic vesicle membranes during exocytosis.

Discussion

In recent years, strong evidence has suggested a central role for small GTP-binding proteins in the targeting and fusion of cellular membranes (reviewed in Bourne, 1988; Hall, 1990; Balch, 1990). Rab3A (also referred to as Smg25a) is a small GTP-binding protein that is expressed in neurons and some endocrine cells (Touchot et al., 1987; Matsui et al., 1988; Ayala et al., 1989; Sano et al., 1989; Zharaoui et al., 1989; Mizoguchi et al., 1990; Fischer v. Mollard et al., 1990). Subcellular fractionation studies suggested that rab3A is localized on synaptic vesicles to which it is bound, probably via a posttranslational hydrophobic modification (Fischer v. Mollard et al., 1990). In isolated synaptosomes (pinched-off nerve endings), the amount of rab3A recovered on synaptic vesicle membranes was decreased as a consequence of neurotransmitter release (Fischer v. Mollard et al., 1991), suggesting a function for this small GTP-binding protein in the trafficking of synaptic vesicles.

To follow the localization of rab3A in whole neurons at different stages of the synaptic vesicle pathway, we have now used antibodies directed against rab3A to investigate its localization by immunocytochemistry in a variety of cellular systems. Light and electron microscopy experiments demonstrated that in adult brain, rab3A is preferentially found on synaptic vesicles. However, in contrast to intrinsic membrane proteins of synaptic vesicles (Navone et al., 1987; Baumert et al., 1990) and similar to what had been previously observed for the extrinsic synaptic vesicles protein synapsin I (De Camilli et al., 1983; Fletcher et al., 1991), rab3A could never be detected at any developmental stage in the region of the Golgi complex of neurons. In contrast, high concentrations of another small GTP-binding protein, yptl, were

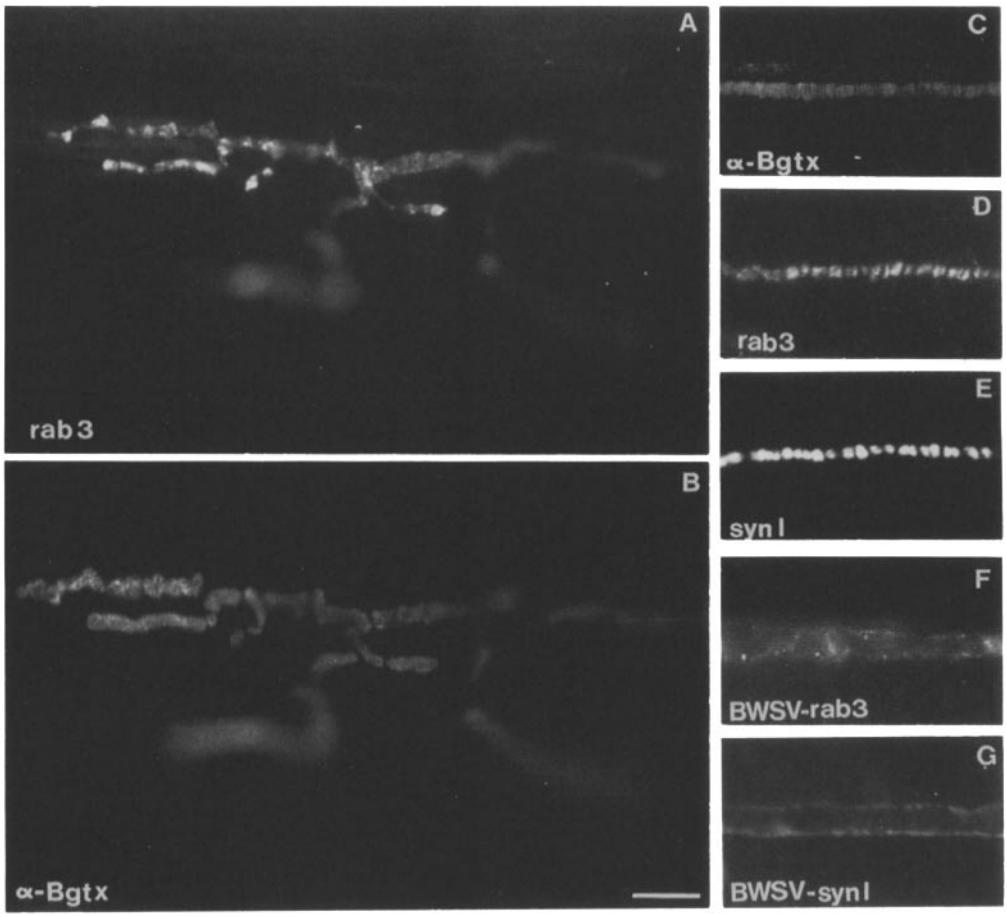

Figure 7. Immunofluorescence micrographs illustrating the distribution of rab3A in control and stimulated frog motor nerve terminals. (*A* and *B*) Double staining of a control neuromuscular junction with antibodies directed against rab3A (rhodamine) (*A*) and with fluoresceine-conjugated α-bungarotoxin (*B*). The toxin binds to acetylcholine receptors and serves as a marker of the synaptic region. A close correspondence between the two staining patterns can be observed, indicating that rab3 immunoreactivity is present throughout the entire nerve terminal arborization. (*C–E*) High power view of segments of control synaptic regions stained with a-bungarotoxin (*C*), antibodies directed against rab3 (*D*) and against synapsin I (*E*). The striped pattern visible in *C* is due to the localization of the acetylcholine receptor on the shoulders of post-synaptic infoldings. The wider stripes visible both in *D* and *E* are due to the clustering of synaptic vesicles in segments of nerve terminal digita which are not surrounded by Schwann cell processes. (*F* and *G*) High power view of segments of nerve terminals immunostained for rab3 (*F*) and for synapsin I (*G*) after stimulation with black widow spider venom. In these stimulated terminals both immunoreactivities are localized at the nerve terminal surface and outline its entire profile. Bars: *A* and *B*, 12 μm; *C–G*, 9 μm.

present in the neuronal Golgi region (Segev et al., 1988; Bacon et al., 1989) but absent from synaptic vesicles.

Lack of rab3A immunoreactivity in the region of the Glgi/centrosomal area was particularly striking in bovine chromaffin cells and in the rat chromaffin cell-derived PC12 cells. These were previously shown to contain rab3A (Sano et al., 1988; Fischer v. Mollard et al., 1990) that was found to be selectively associated with synaptic-like microvesicles by subcellular fractionation (Fischer v. Mollard et al., 1990). In these cells synaptophysin and other synaptic-like microvesicles membrane proteins are present at very high concentration in the Golgi/centrosomal region.

Synaptic vesicle proteins localized in the Golgi area of neurons and endocrine cells reflect transit of newly synthesized proteins as well as recycling of synaptic vesicle membranes from the cell periphery (Johnston et al., 1989; Clift O-Grady et al., 1990; Cameron et al., 1991; M. Matteoli, M. Perin, T. Sudhof, and P. De Camilli, unpublished observations). Thus, lack of rab3A from the Golgi/centrosomal region both in neurons and in chromaffin cells indicates that rab3A binds to vesicle membranes only after they leave the Golgi complex and dissociates from them prior to their return to this region from the cell periphery.

The precise stage distal to the Golgi complex at which rab3A becomes colocalized with other synaptic vesicle proteins remains to be elucidated. Our results obtained in primary neuronal cultures indicate that the association of rab3 with synaptic vesicles is not dependent upon the previous clustering of synaptic vesicles at synaptic sites, i.e., upon the previous formation of a presynaptic specialization. A colocalization of rab3A with synaptophysin is already observed in the axons of neurons grown in isolation, a stage at which synaptic vesicles are sparse throughout most of the axonal arbor. This finding is consistent with the property of most of the axonal surface at this developmental stage to sustain exocytosis (Sun and Poo, 1987; Matteoli et al., 1991) and to be a potential site for synapse formation (Buchanan et al., 1989). Presence of rab3A on synaptic vesicles before synapse formation is consistent with an important role of rab3A in synaptic vesicle exocytosis independent from exocytosis occurring at a synapse. This observation is also in agreement with the presence of rab3A on synaptic-like microvesicles of chromaffin cells, i.e., cells which do not form synapses.

As far as the fate of rab3A upon vesicle exocytosis, our experiments carried out at the frog neuromuscular synapse suggest that, at least after the massive stimulation such as that produced by black widow spider venom, the bulk of

rab3A is translocated to the cell surface in parallel with synaptic vesicle membranes. Although we have shown that the protein is no longer present on membranes which return to the Golgi complex, our analysis did not allow us to determine whether the protein dissociates from membranes which undergo local recycling in nerve terminals. Such a dissociation, however, is suggested by recent experiments carried out on brain synaptosomes. These have shown that stimulation of synaptic vesicle exocytosis by depolarizing agents results in the decrease of the amount of rab3A associated with synaptophysin-positive microvesicles (Fischer v. Mollard et al., 1991). Since our present data suggest that rab3A, like synaptophysin and synapsin I (Torri Tarelli et al., 1989), is translocated to the plasmalemma with exocytosis, it can be argued that dissociation of rab3A from synaptic vesicles follows the fusion event and that the rab3A-negative vesicles observed by Fischer v. Mollard and co-workers represent vesicles newly reformed by endocytosis (Fischer v. Mollard et al., 1991).

In many ways the information presented here for rab3A, is very similar to the information available for sec4, the best characterized low molecular weight G-protein associated with secretory organelles. Sec4, which is thought to be involved in the constitutive exocytosis of yeast secretory vesicles, appears to associate with vesicle membranes after their transit through the Golgi complex but before exocytosis, and to be translocated to the plasmalemma during exocytosis together with the secretory vesicle membrane (Goud et al., 1988; Walworth et al., 1989). It has been suggested that the same pool of sec4 can be used for several rounds of exocytosis, each cycle being tightly coupled to GTP hydrolysis (Walworth et al., 1989). In yeast, exocytosis of secretory organelles results in plasmalemma expansion and is not balanced by compensatory endocytosis. Thus, a reutilization of sec4 implies by definition a cycling through a soluble pool. In the case of synaptic vesicles (Heuser and Reese, 1973; Ceccarelli and Hurlbut, 1973) and of synaptic-like microvesicles of chromaffin cells (Johnston et al., 1989; Clift O-Grady, 1990), exocytosis is followed by compensatory endocytosis, and the reuse of rab3A could, in principle, be accounted for by membrane recycling rather than by a recycling of rab3A through a soluble pool. Our findings, together with the findings of Fischer v. Mollard et al. (1991), suggest a dissociation of rab3A from membranes at some step after exocytosis and support the hypothesis that rab3A undergoes a cycle of association–dissociation with membranes similar to that of sec4.

In conclusion, rab3A is a protein which is recruited to the synaptic vesicle surface in preparation for exocytosis. It is likely to be an important component of a machinery which makes synaptic vesicles competent for fusion with the plasmalemma and which therefore control the neurotransmitter release.

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