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# SYNAPTIC VESICLE BIOGENESIS

Matthew J. Hannah[1,3], Anne A. Schmidt[2,3], and Wieland B. Huttner[2]

[1]*MRC Laboratory for Molecular Cell Biology, University College London, London WClE 6BT, UK;* [2]*Department of Neurobiology, University of Heidelberg, Im Neuenheimer Feld 364, D-69120 Heidelberg; and Max-Planck-Institute of Molecular Cell Biology and Genetics, Pfotenhauerstr. 110, D-01307 Dresden, Germany;*
*e-mail: whuttner@sun0.urz.uni-heidelberg.de;* [3]*joint first authors*

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■ **Abstract** Synaptic vesicles, which have been a paradigm for the fusion of a vesicle with its target membrane, also serve as a model for understanding the formation of a vesicle from its donor membrane. Synaptic vesicles, which are formed and recycled at the periphery of the neuron, contain a highly restricted set of neuronal proteins. Insight into the trafficking of synaptic vesicle proteins has come from studying not only neurons but also neuroendocrine cells, which form synaptic-like microvesicles (SLMVs). Formation and recycling of synaptic vesicles/SLMVs takes place from the early endosome and the plasma membrane. The cytoplasmic machinery of synaptic vesicle/SLMV formation and recycling has been studied by a variety of experimental approaches, in particular using cell-free systems. This has revealed distinct machineries for membrane budding and fission. Budding is mediated by clathrin and clathrin adaptors, whereas fission is mediated by dynamin and its interacting protein SH3p4, a lysophosphatidic acid acyl transferase.

CONTENTS

# INTRODUCTION

## Classes of Neurosecretory Vesicles

Neurons contain two primary classes of regulated secretory vesicles, synaptic vesicles and secretory granules, which store messenger molecules and release them upon stimulation (De Camilli & Jahn 1990, Kelly 1991, Huttner et al 1995). These two classes of secretory vesicles are distinguished by the means by which the respective messenger molecules are translocated from the cytoplasm into the lumen of the endomembrane system.

As is the case in non-neuronal regulated secretory cells, secretory granules of neurons (often also referred to as large dense core vesicles) mediate the regulated secretion of proteins, specifically neuropeptides. These messenger molecules (or larger precursors) are translocated across the membrane at the level of the endoplasmic reticulum, pass through the Golgi complex, and are packaged into secretory granules. Following secretion of the secretory granule contents, the secretory granule membrane must be recycled to the Golgi complex if it is to be refilled with neuropeptides. Because the site of neuropeptide release at the cell periphery is a distance from the Golgi complex, which is located in the neuronal cell body, this recycling poses certain limitations with regard to the speed with which the secretory granule membrane can be reutilized.

By contrast, synaptic vesicles mediate the regulated secretion of non-proteinaceous messenger molecules, the neurotransmitters, which are transported across the membrane at the level of the synaptic vesicle itself. The ability of the synaptic vesicle to take up neurotransmitters directly from the surrounding cytosoplasm allows the local reutilization of the synaptic vesicle membrane, i.e. independent from the endoplasmic reticulum and Golgi complex machineries.

Although the principal differences in the recycling of the synaptic vesicle and secretory granule membranes are reflected by differences in biogenesis, there are potential links between them. Specifically, given that both classes of vesicles may coexist in the same neuron and be localized in the same axon terminal, some secretory granule membrane constituents, after exocytosis of the secretory granule, have been proposed to be used for synaptic vesicle biogenesis rather than being

recycled to the Golgi complex for reutilization in secretory granule biogenesis (Winkler 1997). In catecholaminergic neurons, the transmembrane uptake system for biogenic amines is found in both the secretory granules and the special synaptic vesicles of these cells, which are referred to as small dense core vesicles (SDCVs) because of the electron dense core seen after certain chemical fixation. The utilization of secretory granule membrane constituents for synaptic vesicle biogenesis is discussed in the context of the intracellular traffic of neurotransmitter transporters.

## Relationship of Synaptic-Like Microvesicles to Synaptic Vesicles

Neuroendocrine cells contain membrane vesicles that are highly related to synaptic vesicles of neurons and therefore are referred to as synaptic-like microvesicles (SLMVs) (Thomas-Reetz & De Camilli 1994). First, several of the integral membrane proteins common to synaptic vesicles irrespective of the type of neurotransmitter stored therein have been found in SLMVs (Jahn & De Camilli 1991). Second, upon electron microscopical analysis and various kinds of subcellular fractionation, SLMVs exhibit the small and uniform size characteristic of synaptic vesicles (Navone et al 1986, Clift-O'Grady et al 1990, Régnier-Vigouroux et al 1991, Bauerfeind et al 1993, Schmidt et al 1997b). Third, SLMVs are capable of taking up and storing neurotransmitters (Reetz et al 1991, Bauerfeind et al 1993, Thomas-Reetz et al 1993, Thomas-Reetz & De Camilli 1994).

However, it is less clear whether SLMVs meet another key criterion defining synaptic vesicles, the ability to fuse with the plasma membrane upon stimulation in a calcium-dependent fashion. A depolarization-induced, calcium-dependent release of the neurotransmitters GABA and acetylcholine from the neuroendocrine cell lines AR42J and PC12, respectively, has been reported and is probably due to the regulated exocytosis of SLMVs (Ahnert-Hilger & Wiedenmann 1992, Bauerfeind et al 1995b). However, it cannot be excluded that this release reflects a consequence of regulated exocytosis of secretory granules, which would result in increased endocytosis and membrane recycling from early endosomes to the plasma membrane. Because early endosomes also take up and contain neurotransmitter (Bauerfeind et al 1993), increased membrane recycling from early endosomes could also cause regulated secretion of neurotransmitter (Bauerfeind et al 1994). SLMVs have been reported to contain synaptotagmin I (Bauerfeind et al 1995b), which is thought to act as a calcium-regulated clamp in exocytotic membrane fusion (Südhof & Rizo 1996). However, SLMVs do not accumulate under the plasma membrane of neuroendocrine cells (as do secretory granules or synaptic vesicles in neurons), which would be expected if their exocytosis were dependent on increases in cytosoplasmic calcium (Thomas-Reetz & De Camilli 1994). Rather, SLMVs are consumed constitutively, presumably by fusion with the plasma membrane (Faúndez et al 1997).

These apparent differences in exocytotic behavior between SLMVs and synaptic vesicles have implications for the membrane traffic events that underlie the

biogenesis of these organelles. We will therefore discuss these aspects of SLMV and synaptic vesicle biogenesis in separate sections.

### Definitions

We use the term biogenesis of SLMVs and synaptic vesicles in a broad sense and include both the formation de novo and the re-formation by recycling in this term.

Formation de novo is defined as the first assembly of an SLMV or synaptic vesicle, i.e. an assembly in which none of the SLMV/synaptic vesicle membrane protein and lipid molecules have previously been part of such vesicles. An example of formation de novo would be the newborn postmitotic neuron that prior to establishing its first synapses, initiates the synthesis of synaptic, vesicle-specific membrane proteins and begins to form synaptic vesicles.

Re-formation by recycling is defined as the formation of an SLMV or synaptic vesicle from membrane protein and lipid molecules that previously had been part of such a vesicle. An example of re-formation by recycling would be the "kiss-and-run" mode of neurotransmitter release in which the synaptic vesicle secretes its lumenal contents into the extracellular space via a transient continuity with the presynaptic plasma membrane, followed by its disconnection from this membrane (see below).

There is an intermediate mode of vesicle formation that combines aspects of formation de novo and re-formation by recycling. In this mode, some of the membrane protein and lipid molecules used to form an SLMV or synaptic vesicle are newly synthesized and have not been part of such vesicles. An example of this mode would be the continuous generation of additional SLMVs in a dividing neuroendocrine cell (see below). We refer to this mode of SLMV/synaptic vesicle biogenesis as formation under renovation.

## BIOGENESIS OF SLMVs—COMPARTMENTS AND TRAFFIC ROUTES

Almost all of the present knowledge about the cellular compartments involved in the biogenesis of SLMVs and the intracellular traffic routes taken by membrane proteins destined to SLMVs comes from studies using a neuroendocrine cell line, the rat pheochromocytoma-derived line PC12. These studies have been complemented by analyzing the intracellular traffic of SLMV membrane proteins upon expression in non-neuronal cells such as fibroblasts and epithelial cells. It is important to bear in mind that essentially all observations made in these studies were obtained with dividing cells, which continuously increase the copy number of SLMVs and precursor organelles.

### Traffic Route of Newly Synthesized SLMV Membrane Proteins

All transmembrane proteins of SLMVs examined are synthesized and inserted into the membrane in the rough endoplasmic reticulum, followed by their transport

to and through the Golgi complex. This holds true also for the v-SNARE synaptobrevin/VAMP (referred to hereafter as synaptobrevin), which lacks a signal sequence and whose transmembrane segment is located near the C terminus (Kutay et al 1995). The question as to which intracellular traffic route is taken by newly synthesized SLMV membrane proteins after their passage through the Golgi complex has been studied by pulse-chase labeling of PC12 cells followed by subcellular fractionation and analysis of synaptophysin, a major SLMV membrane protein (Cutler & Cramer 1990, Régnier-Vigouroux et al 1991, Bauerfeind et al 1993). These studies used not only labeling with radioactive amino acids, which are incorporated into membrane proteins during their synthesis in the rough endoplasmic reticulum, but also labeling with radioactive sulfate, which is incorporated into newly synthesized proteins during their passage through the *trans*-Golgi network (TGN). The latter labeling allows a greater temporal resolution of post-Golgi membrane traffic and hence the dissection of the compartments involved.

Newly synthesized synaptophysin is not detected in mature (Cutler & Cramer 1990) or immature (Régnier-Vigouroux et al 1991) secretory granules but leaves the TGN in constitutive secretory vesicles and appears at the cell surface within 10 to 15 min (Régnier-Vigouroux et al 1991). Following its first cell surface appearance, newly synthesized synaptophysin undergoes cycles of endocytosis-exocytosis that initially are mediated by membrane vesicles larger and denser than SLMVs (Régnier-Vigouroux et al 1991, Bauerfeind et al 1993). Subsequently, newly synthesized synaptophysin appears in SLMVs, with the proportion of newly synthesized synaptophysin that is found at any time in SLMVs versus larger and denser membranes reflecting the steady-state distribution of synaptophysin (Figure 1, *left*).

It remains to be investigated whether other newly synthesized SLMV membrane proteins such as SV2, the vesicular acetylcholine transporter (VAChT), synaptobrevin, or synaptotagmin take the same intracellular membrane traffic

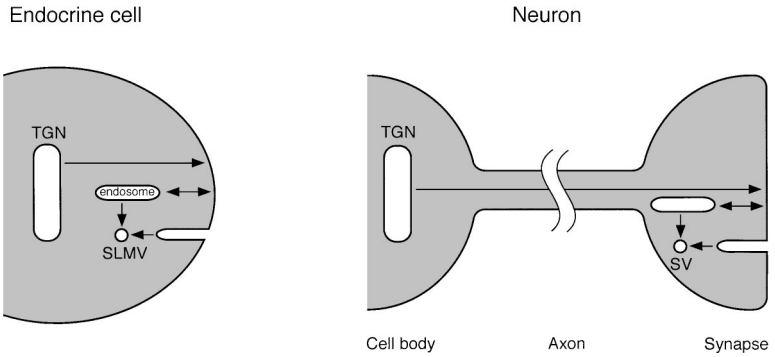


**Figure 1** Schematic representation of the traffic routes of newly synthesized SLMV/synaptic vesicle proteins to SLMVs of endocrine cells and and synaptic vesicles (SV) of neurons.

route to SLMVs as synaptophysin. Also, the available data do not strictly exclude the possibility that the constitutive delivery of newly synthesized synaptophysin from the TGN to the cell surface is not direct but occurs via an early endosomal compartment, as has been reported for the transferrin receptor (Futter et al 1995).

Nonetheless, extrapolating from SLMVs to synaptic vesicles, the data on the intracellular traffic of newly synthesized synaptophysin to SLMVs have several implications for the origin of synaptic vesicles. First, the data do not support the original concept (Winkler et al 1987) that synaptic vesicles are formed from the membrane of secretory granules after exocytosis of the latter. They also do not support the possibility that synaptic vesicles are formed from the membrane that is removed from the maturing secretory granule and apparently delivered to endosomes (Bauerfeind & Huttner 1993, Arvan & Castle 1998). Rather, the data on the traffic of newly synthesized synaptophysin are consistent with the concept (De Camilli & Jahn 1990) that the biogenesis of synaptic vesicles is independent of that of secretory granules.

Second, these observations also do not support the concept (discussed in Holtzman 1992, Zimmermann et al 1993) that synaptic vesicles originate from the TGN. This is not to say that synaptic vesicles cannot be present in the neuronal perikaryon; they can, provided that the donor membrane (see below) and cytoplasmic machinery are present in the perikaryon.

Third, the endocytotic-exocytotic cycling of newly synthesized synaptophysin prior to its appearance in SLMVs suggests that early endosomes, which have long been implicated in synaptic vesicle re-formation by recycling (Heuser 1989), are also somehow involved in synaptic vesicle formation under renovation and, possibly, their formation de novo. That synaptophysin carries targeting information to early endosomes has been demonstrated in several studies in which synaptophysin was expressed in non-neuronal cells. Upon expression in fibroblastic cells, synaptophysin was found to be colocalized with membrane proteins that constitutively cycle between the plasma membrane and early endosomes, such as the transferrin receptor and the LDL receptor (Johnston et al 1989, Linstedt & Kelly 1991b, Cameron et al 1991). These observations also led to the suggestion that synaptic vesicles may have evolved from a recycling pathway common to all cells (De Camilli & Jahn 1990).

However, a still unresolved issue is the targeting of synaptophysin observed upon expression in certain other non-neuronal epithelial cell lines (Leube et al 1989, 1994). In these studies, while some synaptophysin was apparently colocalized with endosomal structures, the majority was observed in membranes thought to represent a distinct population of vesicles whose formation is induced by synaptophysin expression (Leube et al 1989, 1994). Resolving this issue will require that the methods of subcellular fractionation and morphological analysis used in the studies with fibroblastic cells be applied to the epithelial cells and vice versa.

## SLMV Donor Membranes

SLMV donor membranes are defined as the membranes from which SLMVs are formed. It has been known for some time that SLMVs can originate by an endocytotic process. SLMVs can be labeled by the extracellular fluid phase marker HRP (Johnston et al 1989, Clift-O'Grady et al 1990, Bauerfeind et al 1993), and SLMV membrane proteins such as synaptophysin appear in SLMVs after labeling at cell the surface (Schmidt et al 1997b). These observations, together with (*a*) the targeting of synaptophysin to the plasmalemmal–early endosomal membrane system (Johnston et al 1989, Linstedt & Kelly 1991b, Cameron et al 1991) and (*b*) the endocytotic-exocytotic cycling of newly synthesized synaptophysin prior to its appearance in SLMVs (Régnier-Vigouroux et al 1991) suggest that the SLMV donor membrane is the plasma membrane, the early endosome, or both (Figure 1, *left*). It is important to stress the possibility that the SLMV donor membrane may be distinct depending on whether SLMV formation occurs de novo or under renovation versus by recycling. In either case, formation of SLMVs implies a neuroendocrine-specific sorting step that segregates the SLMV membrane proteins from the normal residents of the plasma membrane and the early endosome, such as the transferrin receptor and the LDL receptor (Linstedt & Kelly 1991b, Cameron et al 1991, Régnier-Vigouroux et al 1991, Schmidt et al 1997b).

***Early Endosomes*** In PC12 cells at steady state, the pool of synaptophysin in the plasmalemmal–early endosomal membrane system is greater than that in SLMVs (Cameron et al 1991, Norcott et al 1996, Schmidt et al 1997b), which implies a rate-limiting step in SLMV biogenesis from the plasmalemmal–early endosomal membrane system. Because the amount of synaptophysin in early endosomes is much greater than that at the plasma membrane (Cameron et al 1991, Norcott et al 1996, Schmidt et al 1997b), it was reasonable to suggest that the SLMV donor membrane is the early endosome rather than the plasma membrane (Linstedt & Kelly 1991b, Cameron et al 1991, Régnier-Vigouroux et al 1991, Bauerfeind et al 1993). [The greater amount of synaptophysin in early endosomes than at the plasma membrane, however, does not exclude an alternate possibility, i.e. that early endosomes are a storage site of SLMV membrane proteins, with SLMV formation occurring from the plasma membrane (see below).] Evidence supporting the formation of SLMVs from early endosomes has come from two lines of investigation with PC12 cells.

First, when PC12 cells are allowed to take up HRP for 5 min, chased for various times, and subjected to subcellular fractionation, the internalized fluid phase marker is detected in SLMV-containing fractions after 3 h, but not after 7 min of chase (Bauerfeind et al 1993). This suggests that fluid of the early endosome lumen can appear in SLMVs, albeit in a process requiring $>7$ min. If so, a corollary to this, given the fluid phase nature of the HRP tracer, would be that these SLMVs do not undergo constitutive exocytosis.

The second, more compelling line of evidence comes from the detailed characterization of the donor membranes mediating SLMV formation in the cell-free system pioneered by Kelly and colleagues (Desnos et al 1995a, Clift-O'Grady et al 1998, Lichtenstein et al 1998). Upon incubation of PC12 cells at 15°C, these donor membranes accumulate a synaptobrevin mutant labeled by an externally added antibody against a lumenal epitope, but do not form SLMVs. A significant portion (~25%) of this antibody is resistant to acid stripping, consistent with its internalization into endosomes (Desnos et al 1995a). By subcellular fractionation, a subpopulation of the donor membranes can be isolated that retains ~40% of the SLMV formation capacity of the homogenate but contains <2% of a plasma membrane marker (Lichtenstein et al 1998). Finally, upon internalization of HRP-coupled transferrin followed by oxidation of diaminobenzidine, the SLMV formation capacity of the donor membranes is reduced by half, indicating that SLMV formation occurs from a transferrin receptor-containing membrane (Lichtenstein et al 1998). These observations show that the SLMVs containing the synaptobrevin mutant can be formed in vitro from early endosomes. The lack of transferrin receptor from these SLMVs further shows that their formation involves the segregation, in early endosomes, of SLMV membrane proteins from resident proteins cycling in the plasmalemmal–early endosomal membrane system.

The formation of SLMVs from early endosomes was observed with PC12 cells that were not exposed to nerve growth factor, i.e. cells of a more endocrine phenotype. Upon neuronal differentiation of PC12 cells by nerve growth factor, two types of early endosomes can be distinguished that are characteristic of polarized neurons. One is the transferrin receptor-containing, housekeeping type found in the cell body, which corresponds to the somatodendritic early endosomes of neurons and the basolateral early endosomes of epithelial cells (Mundigl et al 1993, Bonzelius et al 1994). The other is the transferrin receptor-lacking, specialized type of early endosome found in the PC12 cell neurites, which corresponds to the axonal early endosomes of neurons and the apical early endosomes of epithelial cells. Synaptic vesicles of neurons are assembled in the axon, and hence if an early endosome is involved in this assembly, it would be that of the specialized axonal type (see below). The observation that in undifferentiated PC12 cells SLMVs can be formed from transferrin receptor-containing early endosomes (Lichtenstein et al 1998) therefore implies either some difference in assembly between these SLMVs and synaptic vesicles and/or the coexistence and intermixing of specialized and housekeeping early endosomes in undifferentiated PC12 cells.

***Plasma Membrane*** Evidence that SLMVs can form from the plasma membrane has been obtained in studies with PC12 cells in which the appearance in SLMVs of synaptophysin, biotinylated at the cell surface, was investigated in intact and perforated cells (Schmidt et al 1997b, Schmidt & Huttner 1998). When cells are incubated with a membrane-impermeable biotinylation agent at 18°C, all the biotinylated synaptophysin remains associated with large non-SLMV membranes.

About 70% of this synaptophysin is accessible to an externally added membrane-impermeant thiol reducing agent, MesNa, which removes the biotin group, indicating that the majority of synaptophysin biotinylated at 18°C resides in the plasma membrane and the rest presumably in endosomes. Upon reversal of the temperature back to 37°C, either in a chase with intact cells (Schmidt et al 1997b, Schmidt & Huttner 1998) or in a reaction with perforated cells (Schmidt & Huttner 1998), some (<20%) of the synaptophysin biotinylated at 18°C appears in SLMVs. Exposure of the cells to externally added MesNa prior to the temperature reversal indicates that essentially all of the biotinylated synaptophysin appearing in SLMVs originates from the plasma membrane (Schmidt et al 1997b, Schmidt & Huttner 1998).

However, it is a specialized domain of the plasma membrane that gives rise to SLMVs. Although accessible to MesNa, a 150-D molecule, this domain is inaccessible to avidin, a 68,000-D protein (Schmidt et al 1997b). This differential accessibility is observed not only for synaptophysin biotinylated at 18°C but also for that pulse-biotinylated at 37°C (Schmidt et al 1997b), demonstrating the physiological significance of this specialized plasma membrane domain. In line with the presence of deep plasma membrane invaginations in synaptic terminals engaged in synaptic vesicle recycling (Takei et al 1996, Gad et al 1998) (see below), the MesNa-accessible but avidin-inaccessible (sequestered) domain of the plasma membrane of PC12 cells that gives rise to SLMVs may be a plasma membrane invagination, largely devoid of transferrin receptor, whose opening is too narrow to allow the passage of avidin (Schmidt et al 1997b) (Figure 1, *left*). The very narrow mouth of caveoli provides a precedent for a plasma membrane invagination whose opening is highly selective with regard to the passage of extracellular molecules (Anderson 1993). By immunogold electron microscopy of PC12 cells, little synaptophysin is observed at the plasma membrane proper, and significantly more is found in a pleiomorphic tubulo-cisternal membrane system directly beneath the plasma membrane (Johnston et al 1989, Schmidt et al 1997b), which we term the perisome (Figure 2). However, membrane continuity between this subplasmalemmal system and the plasma membrane proper has only occasionally been observed (Johnston et al 1989), and the relationship of this subplasmalemmal membrane system to the sequestered domain of the plasma membrane that gives rise to SLMVs remains to be established. Nonetheless, it is interesting to note that plasma membrane invaginations of pleiomorphic appearance have been observed in a wide variety of cells and have been implicated in endocytosis (Pastan & Willingham 1981, 1983).

Alternatively, the sequestered domain of the plasma membrane that gives rise to SLMVs may correspond to the lateral plasma membrane of adjacent PC12 cells (either the lateral plasma membrane or an invagination of the lateral plasma membrane). These cells form tight cell-to-cell contacts with each other that block the diffusion of externally added avidin into the intercellular space and, hence, the access of avidin to the lateral plasma membrane of adjacent cells (MJ Hannah & WB Huttner, unpublished observations). In fact, the lateral plasma membrane

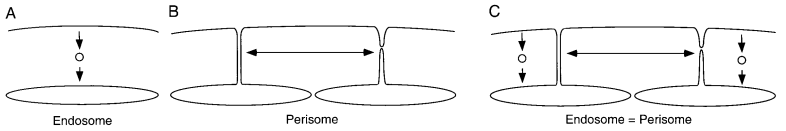


**Figure 2** Possible relationship between early endosome and perisome. (*A*, *B*) early endosomes and perisomes as distinct organelles. (*A*) A traditional early endosome containing transferrin receptor, separate from the plasma membrane, and communicating with it via transport vesicles. (*B*) The perisome, a subplasmalemmal pleiomorphic tubulo-cisternal membrane system lacking transferrin receptor, communicating with the plasma membrane via (transient) tubular membrane connections (Schmidt et al 1997b, Gad et al 1998). (*C*) Early endosome and perisome appearing as "two sides of the same coin."

of adjacent PC12 cells exhibits certain features characteristic of the presynaptic plasma membrane of neurons, such as the accumulation of SAP97 (MJ Hannah & WB Huttner, unpublished observations), a peripheral membrane protein of the presynaptic membrane (Garner & Kindler 1996). A presynaptic-like nature of the SLMV-forming domain of the plasma membrane would also be consistent with its lack of transferrin receptor (Schmidt et al 1997b).

Whatever the exact nature of the plasma membrane domain that gives rise to SLMVs, there are several specific features of the synaptic vesicle membrane protein traffic through this domain. First, not only synaptophysin but also other synaptic vesicle membrane proteins pass through the plasma membrane on their way to SLMVs. SV2 and synaptophysin exhibit a similiar accessibility to MesNa after biotinylation at 18°C (Schmidt et al 1997b). Moreover, Kelly and colleagues, using a modification of their original cell-free system, have shown that a synaptobrevin mutant, labeled at 4°C by addition of an antibody against a luminal epitope, appears in the SLMVs fraction (Shi et al 1998). Second, synaptic vesicle membrane proteins are continuously being delivered into this plasma membrane domain, not only at 37°C but also at 18°C, because biotinylated synaptophysin accumulates with time at 18°C (Schmidt et al 1997b). The source of the latter synaptophysin is not SLMVs but most likely the endosome since at 18°C, SLMV levels remain stable without SLMV formation, i.e. there is little SLMV exocytosis (Schmidt et al 1997b). Third, in contrast to endocytic vesicles, which continue to be formed from the plasma membrane also at 18°C, thereby removing biotinylated transferrin receptor from the cell surface, SLMVs do not form from the plasma membrane at this temperature (Schmidt et al 1997b). This differential temperature requirement points to differences in lipid composition and/or machinery of formation between endocytic vesicles and SLMVs.

***Two Donor Membranes—Only One Type of SLMV?*** The existence of two types of donor membranes that generate SLMVs, the plasma membrane and the early endosome, raises three questions. First, are the machineries mediating SLMV

formation from either donor membrane the same, or are they distinct? Second, are the SLMVs formed from the two donor membranes identical or distinct? And third, do both donor membranes operate in each of the three modes of SLMV formation, i.e. formation de novo, formation under renovation, or re-formation by recycling, or is each donor membrane specific for a certain mode?

As to the first question, evidence indicates that the cytoplasmic machinery mediating SLMV formation from the plasma membrane is distinct from that mediating SLMV formation from the early endosome (see below).

Second is the issue of multiple types of SLMVs. Recent evidence (MJ Hannah & WB Huttner, unpublished observations) shows that the synaptophysin-containing SLMVs isolated by glycerol gradient centrifugation (Clift-O'Grady et al 1990) from PC12 cells have at least two populations, those also positive for synaptotagmin and those containing little, if any, synaptotagmin. In addition to these physiologically occurring SLMVs, Kelly and colleagues, have reported the isolation of an SLMV population from PC12 cells transfected with a synaptobrevin mutant that shows enhanced SLMV targeting; these SLMVs contain more synaptobrevin relative to synaptophysin than the SLMVs isolated from untransfected PC12 cells (Grote et al 1995). Thus it appears that the SLMVs isolated by glycerol gradient centrifugation can be made up of multiple populations. However, while it is an intriguing possibility that distinct SLMV populations are generated in a differential manner from the plasma membrane and the endosome, we are not aware of studies that have rigorously investigated whether this is actually the case. This also holds true for the cell-free system established by Kelly and colleagues, in which SLMVs containing the VAMP mutant can be generated from both the endosome and the plasma membrane, because a detailed comparison of the endosome- and plasma membrane–derived SLMVs remains to be reported.

As to the third question, i.e. whether both donor membranes operate in each of the three modes of SLMV formation, it is likely that in the neuroendocrine cell lines used to study SLMV biogenesis such as PC12 cells, formation de novo probably does not exist because these cells a priori contain SLMVs and generate new cells from SLMV-containing cells, and any newly synthesized SLMV membrane protein is likely to intermix, prior to its incorporation into SLMVs, with SLMV membrane proteins that have already been part of an SLMV (see below). An interesting perspective, however, is the isolation of PC12 variant clones lacking SLMVs (Corradi et al 1996); these clones may be developed into a model for the study of the de novo formation of SLMVs once the requirements to induce SLMV biogenesis in these PC12 variants have been identified. With regard to the donor membranes mediating SLMV formation under renovation and re-formation by recycling, the passage of newly synthesized synaptophysin through the plasma membrane and early endosomes before its appearance in PC12 cell SLMVs (Régnier-Vigouroux et al 1991, Bauerfeind et al 1993) likely implies its intermixing with synaptophysin that has already been incorporated into SLMVs. The probability of intermixing presumably correlates with a given protein's pool size in a given compartment [for synaptophysin lower at the plasma membrane (relatively small pool) than

once it has been internalized from the plasma membrane into early endosomes (relatively large pool)]. We therefore consider only the plasma membrane as the donor membrane mediating SLMV re-formation by recycling, and conversely the early endosome as a donor membrane mediating SLMV formation under renovation. However, the latter mode of SLMV formation also operates at the plasma membrane because the appearance in SLMVs of newly synthesized sulfate-labeled synaptophysin biotinylated at the plasma membrane at 18°C is sensitive to externally added MesNa when added at the end of the 18°C biotinylation and prior to chase at 37°C (MJ Hannah & WB Huttner, unpublished observations).

Consideration of these modes of SLMV formation and the synaptophysin pools in the plasma membrane and early endosomes also helps to explain an apparent paradox in the kinetics of synaptophysin traffic to SLMVs. Newly synthesized, sulfate-labeled synaptophysin is rapidly transported from the TGN to the plasma membrane but cycles with a $t_{1/2}$ of about 1 h between the cell surface and the early endosome before appearing in SLMVs (Régnier-Vigouroux et al 1991). In contrast, synaptophysin biotinylated at the cell surface appears in SLMVs much more quickly, with the plateau being reached after 10 min (Schmidt et al 1997b). The kinetics of this appearance are rapid because SLMV formation occurs directly from the plasma membrane, and the proportion of the cell surface-biotinylated synaptophysin that is not incorporated into SLMVs disappears because it becomes diluted in the larger endosomal pool of non-biotinylated synaptophysin. By contrast, the relatively long $t_{1/2}$ of appearance of the newly synthesized, sulfate-labeled synaptophysin in SLMVs is presumably caused by it first intermixing with the much larger pool of pre-existing synaptophysin that cycles between the plasma membrane and the early endosome, from which it is then withdrawn for SLMV formation under renovation.

## BIOGENESIS OF SYNAPTIC VESICLES—COMPARTMENTS AND TRAFFIC ROUTES

### Mature Neurons

***Transport of Newly Synthesized Synaptic Vesicle Membrane Proteins to the Synapse*** In their classical study (Tsukita & Ishikawa 1980), Tsukita and Ishikawa demonstrated that the membrane carriers accumulating on the proximal site of an axonal transport block were largely comprised of sausage-shaped, vesiculotubular structures of 50–80 nm, rather than 50 nm spheres (i.e. the size and shape of synaptic vesicles). This key observation made the important point that in mature neurons, synaptic vesicles are not formed in the perikaryon and hence do not originate from the TGN. Rather, synaptic vesicle membrane proteins are transported down the axon in membrane carriers that are larger than, and by definition precursors to, synaptic vesicles. Although these membrane carriers have not been characterized in detail, they most likely are the containers mediating constitutive membrane

transport from the TGN to the plasma membrane, in analogy to the traffic of newly synthesized SLMV membrane proteins (Figure 1, *right*) (Régnier-Vigouroux et al 1991, Régnier-Vigouroux & Huttner 1993). In support of this, Hirokawa and colleagues (Nakata et al 1998), using GFP fusion proteins expressed in dorsal root ganglion neurons in culture, have shown that TGN-38, a transmembrane protein of the TGN known to constitutively traffic to the plasma membrane and back to the TGN (Reaves & Banting 1992), is transported down the axon in tubulovesicular membrane structures indistinguishable from those carrying plasma membrane proteins and synaptic vesicle proteins. The sausage-like shape of the tubulovesicular anterograde axonal membrane carriers is not a specific neuronal feature; recent imaging data on the constitutive transport of GFP fusion proteins from the TGN to the plasma membrane in non-neuronal cells have revealed similarly shaped membrane carriers (Hirschberg et al 1998).

The anterograde axonal transport of synaptic vesicle membrane proteins in membrane structures distinct from synaptic vesicles implies that the latter are assembled in the presynaptic terminal. Independent evidence for this conclusion comes from the observations of Hirokawa and colleagues showing that the axonal membrane carriers of synaptic vesicle proteins not only differ in size and shape from synaptic vesicles but that there are various types of these carriers with distinct cargo (Okada et al 1995, Hirokawa 1996). Specifically, the characterization of the motor proteins of the kinesin superfamily, which mediate anterograde membrane transport, has revealed that the axonal membrane carriers associated with KIF1A contain some, but not all, of the membrane proteins common to synaptic vesicles, i.e. synaptophysin and synaptotagmin but not SV2 (Okada et al 1995). SV2 therefore must be transported down the axon in membranes distinct from those carrying synaptophysin and synaptotagmin, which implies that the incorporation of these three membrane proteins into the same synaptic vesicle occurs at the synapse.

Observations that apparently are inconsistent with the concept of synaptic vesicle assembly occurring at the synapse, i.e. at the cell periphery, and that seem to support an origin of synaptic vesicles from the perikaryal Golgi complex, have been reported and discussed in Holtzman (1992) and Zimmermann et al (1993). These observations include (*a*) the presence, in the perikaryon of certain neurons, of small circular membrane profiles immunoreactive for synaptic vesicle proteins (Holtzman 1992); and (*b*) the presence, in the axons innervating the Torpedo electric organ, of vesicles with a size resembling that of synaptic vesicles (Zimmermann et al 1993). In our opinion, however, these observations are not compelling, for two reasons. First, the small vesicular profiles observed in the perikaryon have not been shown to be synaptic vesicles; they could represent, for example, cross-sectioned membrane tubules or *cis*-Golgi-derived transport vesicles (Rothman & Orci 1992). However, even if they were synaptic vesicles, they would not necessarily have to originate from the Golgi complex but could be formed from the perikaryal plasma membrane or early endosomes, in line with

the synaptic vesicle donor membranes in synapses (see below). Second, in the case of Torpedo, it should be noted that the synaptic vesicles of the electric organ (Whittaker 1987) are larger than mammalian synaptic vesicles (Peters et al 1991), which makes it difficult to distinguish them on purely morphological grounds from transport vesicles containing synaptic vesicle proteins.

There are obvious advantages for the neuron in placing the site of synaptic vesicle assembly at the synapse and in using sausage-shaped membrane carriers to deliver synaptic vesicle proteins to this site. Such carriers are more economical than spherical ones in that they can transport more membrane constituents per single container. Synaptic vesicle assembly at the synapse implies that formation de novo, formation under renovation, and re-formation by recycling all take place at the same site, which allows for an efficient utilization of the machinery involved.

Neurons are highly polarized cells, and constitutive membrane traffic from the TGN to the plasma membrane follows two principal routes, an axonal and a somatodendritic one (Bradke & Dotti 1998). Newly synthesized membrane proteins destined for incorporation into synaptic vesicles at the synapse must therefore be sorted into the axonal rather than the somatodendritic TGN-derived constitutive membrane carriers. Indeed, in polarized hippocampal neurons in culture (the classical model system for such studies) (Bradke & Dotti 1998), a chimeric protein consisting of the entire cytoplasmic domain of synaptobrevin fused to the N terminus of the transferrin receptor, which normally shows an exclusive somatodendritic distribution, is found at presynaptic sites in the axon (though not in synaptic vesicles) (AE West et al 1997). This observation documents that (*a*) a synaptic vesicle membrane protein contains axonal sorting information that is dominant over somatodendritic sorting information; (*b*) in the case of synaptobrevin, this axonal sorting information is located in the cytoplasmic domain; and (*c*) the axonal sorting information in the cytoplasmic domain of synaptobrevin is insufficient to result in the incorporation of the protein into synaptic vesicles, suggesting that the transmembrane and/or the (very small) lumenal domain of this protein are involved in synaptic vesicle targeting (see sorting signals below).

The exact nature of the axonal sorting signal in the synaptobrevin cytoplasmic domain, and the generality of a cytoplasmic localization of this signal for the other synaptic vesicle membrane proteins, remain to be determined. However, it is interesting to note that in the case of the amyloid precursor protein (APP), axonal sorting information has been shown to reside in the transmembrane-ectodomain rather than the cytoplasmic tail (Bradke & Dotti 1998). Because APP is also an axonally destined membrane protein but, in contrast to synaptobrevin, not a typical synaptic vesicle protein, the differential localization of axonal sorting information in APP versus synaptobrevin is consistent with the finding (Okada et al 1995) that presynaptic plasma membrane proteins and synaptic vesicle membrane proteins are transported down the axon in distinct membrane carriers. [For an in-depth discussion of axonal versus somatodendritic sorting, the reader is referred to the comprehensive article by Dotti and colleagues (Bradke & Dotti 1998)].

***Synaptic Vesicle Formation at the Synapse*** The majority of data on the formation of synaptic vesicles in mature neurons concern their recycling at the synapse. We therefore here discuss the pathways of synaptic vesicle recycling and the donor membranes involved, and address the incorporation of newly synthesized membrane proteins into synaptic vesicles, i.e. formation under renovation and formation de novo, in the context of immature neurons.

*Recycling Pathways and Donor Membranes* Ever since the pioneering work of Heuser & Reese (1973) and Ceccarelli et al (1973), there have been two views of synaptic vesicle recycling. Neither mutually exclusive when first proposed in 1973, nor now, these two views nonetheless have served as conceptual poles, and we use them to discuss the different principles underlying the various modes of synaptic vesicle recycling.

Kiss-and-run: The kiss-and-run mode of synaptic vesicle exocytosis-endocytosis was originally proposed by Ceccarelli and colleagues (Ceccarelli et al 1973, Fesce et al 1994). In this mode, the synaptic vesicle forms a transient pore with the presynaptic plasma membrane through which the neurotransmitter is released and recycles by simply closing this pore and disconnecting from the plasma membrane (Figure 3*A*). From a purist point of view, synaptic vesicle exocytosis-endocytosis via kiss-and-run can be regarded as a mode of recycling that does not require any sorting of synaptic vesicle membrane proteins and lipids from plasma membrane proteins and lipids because there is no intermixing during neurotransmitter exocytosis in the first place.

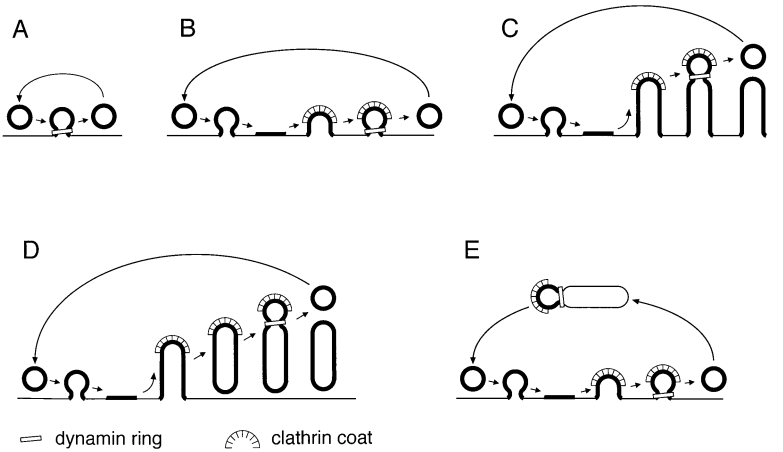


**Figure 3** Modes of synaptic vesicle recycling. (*A*) Fission-only, kiss-and-run mode; (*B-E*) budding-plus-fission modes from either the planar presynaptic plasma membrane (*B*), a plasma membrane invagination (*C*), an internal vacuole derived from a plasma membrane invagination (*D*), or an early endosome (*E*). Thick lines, synaptic vesicle membrane; thin lines, plasma membrane and early endosomal membrane.

Clathrin-mediated endocytosis: This concept, originally proposed by Heuser and Reese (Heuser & Reese 1973, Heuser 1989), is that the synaptic vesicle undergoes full fusion with the presynaptic membrane, collapsing into the plasmalemma, and its subsequent recycling follows the pathway of clathrin coat-mediated endocytosis, with the synaptic vesicle membrane being retrieved from the plasma membrane via a clathrin-coated endocytic vesicle and delivered to an endosomal membrane from which a synaptic vesicle re-forms (Figure 3*E*). This mode of recycling allows for the sorting of synaptic vesicle membrane proteins and lipids from plasma membrane proteins and lipids at the level of the plasma membrane and/or the endosomal membrane. It should be pointed out, however, that although allowing for sorting, this mode of recycling does not necessarily require sorting if, for example [and in analogy to the raft concept (Simons & Ikonen 1997)], the synaptic vesicle membrane proteins and lipids were assembled into a membrane microdomain whose components do not intermix with those of the plasma membrane and the endosomal membrane.

Endosomes versus plasma membrane invaginations: The Heuser-Reese concept of synaptic vesicle recycling has been modified by De Camilli and colleagues. In view of the presence of clathrin-coated buds not only on the presynaptic plasma membrane proper but also on deep invaginations in continuity with the presynaptic plasma membrane, synaptic vesicles are proposed to recycle in a single, clathrin coat–mediated budding and dynamin-mediated fission step that can occur from the presynaptic plasma membrane (Figure 3*B*) as well as from deep plasma membrane invaginations (Figure 3*C*) (Takei et al 1996, Gad et al 1998). In the classical Heuser-Reese concept, the clathrin coat–mediated vesicle forms an endocytic vesicle destined to fuse with an endosomal membrane, whereas in the De Camilli modification of the original concept, the clathrin coat–mediated event directly produces a synatic vesicle. Like the original Heuser-Reese concept, the modified concept allows for the sorting of synaptic vesicle membrane proteins and lipids from plasma membrane proteins and lipids.

In the original Ceccarelli and Heuser-Reese concepts, the donor membrane from which the recycling synaptic vesicle originates is the presynaptic plasma membrane (Figure 3*A*) and an endosomal membrane not in continuity with the plasma membrane (Figure 3*E*), respectively. The De Camilli modification of the original Heuser-Reese concept represents a merge in that the donor membrane can be the presynaptic plasma membrane (Figure 3*B,C*) as well as an internal membrane (Figure 3*D*) because the clathrin-coated, bud-bearing presynaptic vacuoles that are not in continuity with the plasma membrane are thought to be derived from the pinching off of deep invaginations from the presynaptic plasma membrane (Takei et al 1996). An unresolved issue is the membrane composition and fate of those regions of the deep invaginations and internal vacuoles that appear to lack clathrin-coated buds. Is the membrane composition in these regions similar to that of synaptic vesicles, i.e. does most of a deep invagination correspond to multiple units of synaptic vesicle membrane in the process

of internalization, and is a deep invagination and the internal vacuole derived therefrom eventually broken down into synaptic vesicles by successive rounds of clathrin coat–mediated vesicle formation? Or do these regions correspond to synaptic vesicle membrane intermixed with presynaptic plasma membrane, from which the synaptic vesicle membrane constituents segregate into the clathrin-coated buds?

Whatever the answers, a large body of evidence supports the notion that synaptic vesicles recycle directly from the presynaptic plasma membrane proper as well as from deep plasma membrane invaginations and internal presynaptic vacuoles (Figure 1, *right*). For excellent reviews on this subject see Heuser 1989, Fesce et al 1994, Südhof 1995, De Camilli & Takei 1996, Cremona & De Camilli 1997.

A study on synapses of hippocampal neurons using the fluorescent membrane dye FM1-43, a powerful tool to investigate membrane endocytosis and exocytosis in living cells (Betz & Wu 1995), shows that the amount of dye internalized per endocytic vesicle equals that released per exocytic vesicle (Murthy & Stevens 1998). This not only shows that the endocytic vesicle is of the same size as the exocytic vesicle, i.e. the synaptic vesicle, but also that the endocytosed synaptic vesicle membrane does not intermix with an internal membrane prior to exocytosis, as would be expected if the former were to fuse with an endosome-like membrane during the synaptic vesicle cycle. These observations are consistent with synaptic vesicle re-formation by recycling being mediated by a single budding/fission step that occurs from the presynaptic plasma membrane or from a membrane invagination in continuity with the presynaptic plasmalemma.

Budding versus fission: The biochemical and genetic dissection of the cytoplasmic machinery mediating synaptic vesicle recycling, which is discussed in detail below, has revealed that the machinery mediating synaptic vesicle budding, i.e. the clathrin coat, is distinct from that mediating synaptic vesicle fission, i.e. the dynamin ring (Takei et al 1995). Synaptic vesicle recycling directly from the presynaptic plasma membrane appears to occur in two ways: by fission only (Figure 3*A*) or by budding plus fission (Figure 3*B*). The fission only type of recycling is suggested by the phenotype of the temperature-sensitive *Drosophila* mutant *shibire*, which carries a mutation in the dynamin gene; at the restrictive temperature, synaptic vesicle recycling from the presynaptic membrane is blocked at the stage of collared pits, which apparently lack a clathrin coat but contain dynamin at the neck connecting the vesicle with the plasma membrane (for reviews, see Warnock & Schmid 1996, Schmid et al 1998). The endocytic vesicles in non-neuronal cells of *shibire* flies are blocked in the form of coated pits (Kessell et al 1989), consistent with the known role of clathrin in the budding of endocytic vesicles (Hirst & Robinson 1998). Unless one assumes that in neurons the clathrin coat depolymerizes from a bud that has a mutant dynamin collar, the accumulation of clathrin-lacking collared pits at *shibire* synapses suggests that clathrin coat-mediated budding is not an obligatory step in synaptic vesicle recycling, at

least not under the conditions of the relatively short temperature shift used to induce the *shibire* phenotype. This in turn suggests that the fission only type of synaptic vesicle recycling does exist, as originally proposed in the kiss-and-run model of Ceccarelli and colleagues, which is supported by a recent study (Ales et al 1999).

On the other hand, the presence of clathrin-coated, synaptic vesicle–sized pits at the presynaptic membrane, as originally observed by Heuser & Reese and in numerous subsequent studies (Heuser 1989), strongly suggests that synaptic vesicle recycling from the plasma membrane can also involve budding prior to fission, consistent with the concept that a budding step is required to re-form the synaptic vesicle if it has collapsed into the plasma membrane during exocytosis (Figure 3*B*). The same requirement presumably holds true if the re-formation of synaptic vesicles does not occur from the presynaptic plasma membrane but from invaginations of the presynaptic membrane (Figure 3*C*) and from internal vacuoles (Figure 3*D*), both of which form clathrin-coated buds (Takei et al 1996, De Camilli & Takei 1996, Cremona & De Camilli 1997, Gad et al 1998).

Two synaptic vesicle recycling pathways: Synaptic vesicle re-formation by recycling can be classified not only into the fission only versus the budding plus fission type, but also into the active zone versus the nonactive zone pathway, which co-exist in the same synapse but differ morphologically, kinetically and in their sensitivity to $Ca^{2+}/Mg^{2+}$ (Koenig & Ikeda 1996). By studying the recovery of synaptic vesicle membrane endocytosis in *shibire* synapses, Koenig & Ikeda have shown that synaptic vesicle membrane recycling from the active zone of the plasmalemma is fast and involves no membrane intermediates, whereas that from the plasma membrane regions outside the active zone occurs more slowly and involves membrane tubules and cisternae. When the recovery of membrane recycling was investigated at a semi-restrictive temperature, the active zone pathway showed thin, unbranched membrane invaginations, whereas the nonactive zone pathway was characterized by slightly thicker, branched membrane tubules.

It is tempting to relate the distinct morphologies of the active and nonactive zone pathways to the fission only and budding plus fission types of synaptic vesicle recycling. As is discussed further below, dynamin induces the formation of thin membrane tubules, and clathrin that of membrane buds. Bud formation on a growing membrane tubule will presumably lead to its branching. It is therefore conceivable that the unbranched membrane invaginations emanating from the active zone of a *shibire* synapse at the semi-restrictive temperature reflect the predominant action of dynamin, but not clathrin, in this pathway of membrane retrieval, and the branched membrane tubules of the nonactive zone pathway reflect the concerted action of both clathrin and dynamin. In other words, a view of two co-existing pathways of synaptic vesicle re-formation by recycling emerges: (*a*) the one from the active zone follows the kiss-and-run concept of Ceccarelli and colleagues and occurs largely via the fission only type of membrane retrieval mediated by the dynamin machinery; and (*b*) the one from the nonactive zone operates predominantly in the clathrin plus dynamin-mediated, budding plus fission type of retrieval

of synaptic vesicle membrane that has collapsed into, and perhaps intermixed with, the plasma membrane, in line with the Heuser-Reese concept.

Two pools of synaptic vesicles: Are the two pathways of synaptic vesicle membrane recycling elicited in a differential manner, and do the re-formed synaptic vesicles exhibit differential exocytotic behavior? It has long been known that there are two pools of synaptic vesicles, the readily releasable pool and the reserve pool. Neurotransmitter release from synapses in response to relatively mild stimulation is thought to involve only the readily releasable pool, while the response to stronger, or longer lasting stimulation involves both that pool and the reserve pool (Kuromi & Kidokoro 1998). Koenig & Ikeda (1996) propose that the active zone pathway of synaptic vesicle recycling replenishes the readily releasable pool, and the nonactive zone pathway the reserve pool. This is a very plausible proposition because it may provide a simple mechanistic link between the two pathways of synaptic vesicle recycling and the differential mobilization of the two pools of synaptic vesicles in response to mild versus strong stimulation. Under mild stimulation, the rate of fusion with the presynaptic plasma membrane of synaptic vesicle membrane, mobilized only from the readily releasable pool, does not exceed the endocytic capacity of the dynamin machinery at the active zone, and hence membrane retrieval occurs instantly after fusion, before the synaptic vesicle membrane collapses into the plasmalemma, i.e. in the fission only, kiss–and–run mode via the active zone recycling pathway. Under stronger stimulation, mobilization of the reserve pool in addition to the readily releasable pool leads to a rate of fusion of synaptic vesicle membrane with the plasma membrane that exceeds the endocytic capacity of the dynamin machinery at the active zone, resulting in synaptic vesicle membrane collapse into, and perhaps intermixing with, the presynaptic plasma membrane; in this case, a clathrin plus dynamin-mediated budding plus fission type of membrane retrieval via the nonactive zone recycling pathway is required.

## Immature Neurons

Most neurons of the vertebrate central nervous system originate from the neuroepithelial cells in the ventricular zone (Jacobson 1991, McConnell 1995, Huttner & Brand 1997). An in situ hybridization study during early neuronal development shows that the expression of mRNAs encoding synaptic vesicle proteins, which is lacking in the dividing neuroepithelial cells, is induced in young postmitotic neurons prior to neurite extension and synapse formation (Marazzi & Buckley 1993). Monitoring exo-endocytosis by labeling with an extracellularly added antibody against the ectodomain of synaptotagmin I reveals that in developing neurons in culture, both constitutive (Matteoli et al 1992) and regulated (Kraszewski et al 1995) exo-endocytosis takes place via small vesicles prior to synaptogenesis and, after synapse formation, it is not necessarily confined to the synapse. It is unclear to which extent this exo-endocytosis reflects the recycling of synaptic vesicle precursor membranes, recycling of bona fide synaptic vesicles, or a combination of both. The first scenario implies that synaptic vesicle formation de novo,

which occurs prior to the establishment of synaptic contacts, involves constitutive exo-endocytosis, indicating an intriguing parallel to the biogenesis of SLMVs discussed above. Extrapolating from SLMV biogenesis, synaptic vesicle formation de novo, in contrast to synaptic vesicle reformation by recycling, may not (or not only) occur from the plasma membrane, plasma membrane invaginations, and internal vacuoles that result from the pinching-off of invaginations from the plasma membrane, but (also) from distinct organelles, i.e. the specialized early endosomes found in the axon, which differ from those found in the dendrites and the perikaryon (Mundigl et al 1993, Kelly 1993, Bonzelius et al 1994).

As a corollary, synaptic vesicle formation could be mediated by distinct, plasma membrane versus endosomal-type cytoplasmic machineries. Indeed, it was recently reported (Zakharenko et al 1999) that neurotransmitter secretion along the developing axon, but not that at the nerve terminal, is blocked by brefeldin A, indicative of an involvement of ARF. This suggests that synaptic vesicle formation in developing (and, perhaps, mature) neurons, similar to SMV formation in PC12 cells, can occur from either early endosomes, via a mechanism involving ARF and the AP3 adaptor complex, or from the plasma membrane (including invaginations and pinched-off vacuoles), via the AP2 adaptor complex and clathrin. The AP2/clathrin-mediated synaptic vesicle formation seems to be more important than that mediated by ARF/AP3, given that in *Drosophila*, mutation of the $\alpha$-adaptin subunit of AP2 results in synapses depleted of synaptic vesicles and in death, whereas in *mocha* mice, which lack functional AP3 adaptors, the neurological phenotype is relatively mild, with apparently unaltered numbers and morphology of synaptic vesicles (although a subpopulation lacks the ZnT-3 zinc transporter) (Kantheti et al 1998).

## SORTING AND ASSEMBLY OF SYNAPTIC VESICLE MEMBRANE CONSTITUENTS

Most of the major proteins of mammalian synaptic vesicles are probably now known. An exhaustive description of the protein composition of synaptic vesicles is beyond the scope of this article, and numerous reviews have been written on this subject (Jahn & De Camilli 1991; Jahn & Südhoff 1993, 1994; Linial & Parnas 1996; Südhof 1999). Herein we concentrate on the implications of the membrane protein composition for synaptic vesicle biogenesis and on the protein-protein interactions, which we feel may be relevant to synaptic vesicle formation. In the context of these two issues, we also briefly address the lipid composition of synaptic vesicles and protein-lipid interactions potentially relevant for their biogenesis.

In the following discussion, a synaptic vesicle protein is regarded as any membrane protein found in synaptic vesicles, irrespective of whether it (*a*) is thought to be synaptic vesicle specific (e.g. synaptophysin); (*b*) occurs in synaptic vesicles because it is required for synaptic vesicle function but is also found and operates in other organelles (e.g. the proton pump); or (*c*) is recycled via the synaptic vesicle to

the presynaptic membrane where it likely exerts its primary function (e.g. syntaxin) (Walch-Solimena et al 1995). The amount of membrane protein a synaptic vesicle can contain is physically limited to that which can be incorporated into the lipid bilayer of a ~50 nm spherical vesicle (Jahn & Südhoff 1993). Synaptic vesicles purified from whole brain have a distinct and fairly simple protein composition (Huttner et al 1983, Jahn & De Camilli 1991, for references), which is consistent with the restricted capacity of a small vesicle and also suggests a certain degree of biochemical homogeneity (see below). Also, the fact that synaptic vesicles contain a very restricted set of the membrane proteins a neuron synthesizes must be the result of sorting. In this context, the sorting of synaptic vesicle membrane proteins is defined as their segregation (active or passive) from non-synaptic vesicle membrane proteins in the plane of the membrane, be it during the formation of a vesicle from a donor membrane, or during the formation of distinct membrane microdomains within a donor membrane. The assembly of the synaptic vesicle membrane is defined as the initiation of interactions (both homophilic and heterophilic) between synaptic vesicle membrane proteins that persists at least until the synaptic vesicle has been formed, i.e. fissioned from its donor membrane. It should be noted that the sorting and assembly as defined here is not restricted to the donor membrane from which the synaptic vesicle is formed, nor are sorting and assembly mutually exclusive (as assembly may be a means of sorting).

## Implications of Synaptic Vesicle Protein Composition for Biogenesis

Most of the proteins found in synaptic vesicles are members of larger gene families containing multiple isoforms (Jahn & Südhoff 1993, Südhof 1999). In many cases different neuronal populations express distinct isoforms, so that synaptic vesicles from different areas of the brain will have distinct compositions. However, it is generally assumed that every synaptic vesicle contains at least one member of each of the gene families (Jahn & Südhof 1993) and, additionally, that all the synaptic vesicles from a particular neuron will have the same composition (Jahn & Südhof 1993). It should be stressed that although synaptic vesicles within a given nerve terminal are morphologically homogeneous, the premise of compositional uniformity has not to our knowledge been rigourously tested. In fact, as we discuss below, there is now considerable evidence that at least with regard to the concentration of neurotransmitter transporters, there can be considerable variability in the individual synaptic vesicle content. Whether synaptic vesicles within a particular synaptic bouton are biochemically uniform, has a significant bearing on the possible mechanisms that could have been used to form them.

***Uniform Composition*** The small size of a synaptic vesicle means that each vesicle contains relatively few copies of each of its constituent proteins, with some probably represented only once or twice in each vesicle (Jahn & Südhof 1993, Südhof 1999). Therefore, the production of vesicular homogeneity would require

that the formation is tightly controlled at the single protein level. How could such regulation be achieved? A possibility would be if each synaptic vesicle protein could be individually sorted and assembled into the synaptic vesicle by its own adaptor complex. The combination of adaptor complexes for all the synaptic vesicle proteins would in essence form a template for the synaptic vesicle membrane. However, for either the plasmalemmal or endosomal donor membrane, only one type of adaptor complex (AP2 or AP3) involved in the formation of synaptic vesicles has been described (see below), and therefore such a template does not appear to exist. It is possible (though improbable) that every synaptic vesicle protein binds to the same adaptor complex, but even if this binding existed, it would only ensure that the proteins were sorted into the nascent vesicle; it could not ensure that every vesicle would contain the same ratio of proteins.

In the absence of a template for the synaptic vesicle membrane, an alternative way of generating homogeneous synaptic vesicles would be if all the synaptic vesicle membrane constituents spontaneously pre-assembled within the plane of the lipid bilayer into a (huge) protein complex with the correct stoichiometry. Only a single member of this complex would then have to interact with the adaptor complex during the formation of the vesicle. Multimeric, heterogeneous complexes of synaptic vesicle membrane proteins have been described within such a context (Bennett et al 1992), and these are discussed more fully below.

***Variable Composition*** If it is assumed that some microheterogeneity exists among the synaptic vesicles of a particular neuron, then the degree of control required during vesicle formation itself becomes much less stringent. In fact, if the donor membrane from which synaptic vesicles are formed contains only synaptic vesicle proteins (see below), then no additional sorting steps are required during the formation of the synaptic vesicles themselves. In this case the stoichiometry of the particular proteins within the synaptic vesicle is determined by the relative concentrations of the constituent proteins in this donor membrane. The formation of the synaptic vesicle itself could theoretically proceed as a default budding step from the highly pre-sorted donor membrane. The obvious biosynthetic advantage of this scenario compared with mechanisms that would be required to produce uniform vesicles is its simplicity and potential speed. Recently, data obtained from transgenic overexpression of the vesicular acetylcholine transporter (VAChT) (Song et al 1997) and knockout of the neuronal vesicular monoamine transporter (VMAT2) (Takahashi et al 1997, Fon et al 1997, Wang et al 1997) have provided strong in vivo evidence that at least for neurotransmitter transporter proteins, copy number in synaptic vesicles is not tightly controlled but is indeed the consequence of expression levels (Song et al 1997, Fon et al 1997; see also Reimer et al 1998, Williams 1997). Analysis of the synaptic transmission in cultured neurons taken from transgenic mice overexpressing VAChT showed that the size of the neurotransmitter quanta (which corresponds to the neurotransmitter content of a single vesicle) were significantly larger than in neurons from wild-type animals (Song et al 1997). It was therefore concluded that increased expression of

VAChT leads to the synthesis of synaptic vesicles containing more copies of the transporter molecules and these vesicles are therefore able to accumulate a higher concentration of neurotransmitter (Song et al 1997, Williams 1997). In addition to simplifying the process of vesicle formation, a certain degree of flexibility in the composition of the synaptic vesicle membrane within a neuron is potentially beneficial at another level, as it gives the opportunity for regulation of synaptic transmission (Song et al 1997, Fon et al 1997). An increase in biosynthesis of a neurotransmitter transporter molecule induced, for example, by activity of the neuron, would lead to an increase in the concentration of the protein in the synaptic vesicle donor membrane and subsequently in the synaptic vesicles themselves. The increased size of the neurotransmitter quanta released by these vesicles would presumably lead to some kind of chronic enhancement in synaptic transmission (Song et al 1997, Fon et al 1997). It has been shown that VMAT content of chromaffin granules is elevated by stimulation of the cell (Desnos et al 1995b). Additionally, analysis of the VMAT2 knockout mouse heterozygotes has provided evidence for significant influence of reduced transporter number on behavior (Takahashi et al 1997, Fon et al 1997, Wang et al 1997).

The abundance of some synaptic vesicle proteins suggests that they are represented only a maximum of once or twice within the vesicle membrane. Therefore, an inevitable consequence of allowing the composition of the synaptic vesicle membrane to be determined primarily by the composition of the donor membrane is that on statistical grounds there are likely to be vesicles completely lacking some proteins. Because two of the low copy number proteins in synaptic vesicles are thought to be the proton pump and neurotransmitter transporters (Südhof 1999, Parsons et al 1993), this senario predicts the formation of a certain proportion of synaptic vesicles that are unable to accumulate neurotransmitter and therefore unable to play any (conventional) part in neurotransmission. Although, initially it may seem unlikely that any such random element would be tolerated in a physiological process as important as neurotransmission, it is known that synaptic transmission is a probabilistic rather than deterministic process, with the likelihood of an action potential in a neuron leading to synaptic transmission being significantly less than one. This is generally taken as evidence that not all action potentials are able to give rise to fusion of the synaptic vesicle with the plasma membrane. This may be true, but most of these studies have been carried out electrophysiologically using the postsynaptic potential as evidence of presynaptic vesicle fusion. Consequently, a synaptic vesicle that fuses normally but contains no neurotransmitter (because it contains no transporter or no proton pump) would go unnoticed in such studies. Indeed, simultaneous measurement of capacitance increase and acetylcholine release from PC12 cells showed a large discrepancy between the number of rapid fusion events and the detectable release of neurotransmitter (Ninomiya et al 1997). One (though certainly not the only) interpretation of this would be that many of the SLMVs fusing with the plasma membrane contained no neurotransmitter (Ninomiya et al 1997). Data consistent with the existence of neuronal synaptic vesicles unable to take up neurotransmitter

have been obtained in the analysis of VAChT overexpressing mice (Song et al 1997), as it was found that VAChT overexpression leads to increases not only in the amplitude (see above), but also in the frequency of neurotransmitter release (Song et al 1997).

A potentially dangerous consequence of not tightly regulating the composition of each synaptic vesicle would be the synthesis of vesicles unable to fuse with the plasma membrane. Such vesicles would have the potential to rapidly accumulate in an active nerve terminal. It is probably no coincidence that synaptobrevin, which has been shown to be part of the minimal machinery for membrane fusion, in conjunction with syntaxin and SNAP-25, (Schiavo et al 1992, Söllner et al 1993, Weber et al 1998, Jahn & Südhof 1999), is the most abundant synaptic vesicle protein (on a molar basis) (Walch-Solimena et al 1995), and therefore the likelihood of generating a synaptic vesicle devoid of this protein and hence incapable of fusing with the plasma membrane is very small.

## Protein-Protein Interactions

Although many interactions between synaptic vesicle proteins have been described, very little is known regarding a possible role of these interactions in synaptic vesicle biogenesis. In most cases such interactions have been functionally characterized with respect to a role in the regulated exocytosis of the synaptic vesicle rather than its formation (Jahn & Südhof 1994). This is understandable from a practical point of view as the final readout in most in vivo assays of synaptic vesicle protein function is regulated release of neurotransmitter. However, it should be stressed that in many cases it is not trivial to distinguish between an interaction that is essential for the biogenesis (including recycling) of the synaptic vesicle from one that is essential for regulated exocytosis of the vesicle, as both are required for neurotransmitter release.

The protein-protein interactions playing a role in synaptic vesicle biogenesis could be operating at two levels, which are not mutually exclusive. First, interaction could occur during the segregation of the synaptic vesicle membrane proteins from the non-synaptic vesicle proteins. For example, assuming that synaptophysin molecules are actively sorted into axonal vesicles at the level of the TGN (see above), then from a naive point of view it would obviously be more efficient to sort *n* hexamers of synaptophysin than it would be to deal individually with 6*n* monomers. Likewise, if proteins A, B, and C are to be included in the synaptic vesicle membrane while D, E, and F are to be excluded, then a complex of ABC (and/or DEF) would significantly simplify the sorting process.

Second, interaction could occur during the assembly of the synaptic vesicle components into a functional organelle. In the most extreme example of this type of interaction, the entire contents of a presumptive synaptic vesicle membrane could spontaneously assemble into a hetero-oligomeric complex (as mentioned above). Less extensive interactions could also play a role by ensuring that certain

functionally important combinations of proteins remain together during the formation of the vesicle membrane. An example of such a functional assembly is the Vo subunit of the proton pump, which is a hetero-oligomeric complex made up of at least three different proteins (Stevens & Forgac 1997). This complex is assembled in the ER and once assembled probably stays together during its subsequent membrane trafficking (Stevens & Forgac 1997). The interactions involved in proton pump assembly are not described here (see Stevens & Forgac 1997).

***Homo-Oligomeric Complexes*** Many of the major synaptic vesicle proteins form homo-oligomers, including synaptophysin (Johnston & Südhof 1990, Hannah et al 1998), SCAMP (Wu & Castle 1997), synaptobrevin (Calakos & Scheller 1994, Laage & Langosch 1997), and synaptotagmin (Perin et al 1991). The functional significance (if any) of these interactions is unknown. As discussed above for synaptophysin, the tendency of a protein to homo-oligomerize may be used as an aid to sort the protein away from non-synaptic vesicle proteins. Recent work performed in our laboratory (Hannah et al 1998, Hannah et al 1999) suggests that in vivo homo-oligomerization of synaptophysin in PC12 cells occurs at the level of the TGN concomitant with the formation of the constitutive secretory vesicles in which the protein is transported to the plasma membrane (Régnier-Vigouroux et al 1991). Accordingly, kinetic analysis of the oligomerization of newly synthesized synaptophysin shows that it does not correlate with arrival of the protein in SLMVs [$t_{1/2}$ of 45 min and 1.5 h (Régnier-Vigouroux et al 1991), respectively]. In addition, synaptophysin exogenously expressed in fibroblasts, which do not contain SLMVs or most of the other synaptic vesicle proteins, also oligomerizes in the TGN. These data are therefore consistent with synaptophysin homo-oligomerization not playing a role primarily in the formation of the synaptic vesicle membrane per se, but in the sorting of the protein into a distinct, presumably axonal, vesicle at the level of the TGN.

***Hetero-Oligomeric Complexes*** Bennett et al (1992) were the first to systematically study the interactions between synaptic vesicle proteins with respect to the possibility of hetero-oligomer involvement in vesicle formation. They concluded that a detergent-resistant, multimeric complex exists in rat synaptic vesicles consisting of SV2, synaptotagmin, synaptophysin, synaptobrevin, and the 39-kDa subunit of the vesicular proton pump. It was further proposed that this complex might represent a sorting/assembly unit for the majority of the synaptic vesicle membrane (approximately three copies per vesicle) (Bennett et al 1992) similar to that postulated above as a prerequisite for the formation of biochemically uniform synaptic vesicles. However, with respect to this proposal, it should be noted that the molar ratios of the proteins in the detergent-resistant complex were different from that in the intact synaptic vesicles (Bennett et al 1992), and therefore this complex could not be responsible for the composition of the whole synaptic vesicle membrane.

It is unclear what role the extensive, multimeric complex proposed by Bennett may have in synaptic vesicle biogenesis. Extrapolation from detergent-solubilized extracts to the physiological situation is difficult because interactions between membrane proteins are often influenced (both positively and negatively) by the conditions used to solubilize them. Indeed, the multimeric complex containing SV2, synaptotagmin, synaptophysin, synaptobrevin, and the proton pump subunit was seen only in CHAPS-solubilized extracts, with different interactions being more prevalent if other detergents were used (Bennett et al 1992). In addition, evidence for a lack of functional in vivo interaction (at least in fibroblasts) between synaptotagmin, synaptophysin, and SV2 was obtained by Feany et al (1993), who found that these three proteins when expressed together in Chinese hamster ovary (CHO) cells, were localized in different subcellular compartments (Feany et al 1993). The differential localization indicates that any interactions the three proteins may have had with each other within the biosynthetic pathway were not in themselves sufficient to form a multimeric sorting complex. However, the in vivo studies of Feany et al (1993) were performed in a fibroblastic cell line, whereas the in vitro interactions of Bennett et al (1992) were demonstrated using neuronal material. Therefore possible causes of the discrepancy between the observations could be the distinct lipid environment of neuronal membranes (see below) or differences in post-translational modification of the proteins expressed in fibroblasts compared with neurons. With repect to this latter point, it has recently been shown that the (only) well-characterized protein-protein interaction within the synaptic vesicle membrane, between synaptobrevin and synaptophysin (see below), is indeed dependent on an unidentified post-translational modification specific to mature neurons (Becher et al 1999). Finally, if the sorting complex proposed by Bennett et al (1992) does exist in neurons in vivo, it must be a post-axonal vesicle phenomenon (i.e. not acting as a sorting complex at the level of the TGN), since SV2 has been shown to travel down the axon in a vesicle that is distinct from that carrying most of the other synaptic vesicle membrane proteins (Okada et al 1995).

***Synaptobrevin-Synaptophysin-Proton Pump*** Although the number of proteins in the complex originally invisaged by Bennett et al (1992) is possibly an overestimate, the existence of a smaller subcomplex also observed by these authors, consisting of synaptophysin, synaptobrevin, and subunits of the proton pump, has been supported by data from a number of groups. Synaptophysin can be cross-linked within synaptic vesicles to synaptobrevin (Johnston & Südhof 1990, Calakos & Scheller 1994, Edelmann et al 1995, Washbourne et al 1995). The interaction of synaptobrevin with synaptophysin in detergent extracts of synaptosomes is mutually exclusive to the interaction of synaptobrevin with another protein, syntaxin I (Edelmann et al 1995, Galli et al 1996). There is considerable evidence showing that an interaction of synaptobrevin with syntaxin is vital for the exocytotic fusion event (for a recent review, see Jahn & Südhof 1999), and therefore the mutually exclusive interaction of synaptobrevin with synaptophysin

suggests a possible regulatory role in exocytosis (Edelmann et al 1995), but it also does not rule out a role in vesicle formation. Observations from at least two groups have indicated that the detergent-resistant complexes containing synaptophysin and synaptobrevin also contain subunits of the vesicular proton pump (Bennett et al 1992, Galli et al 1996). In these cases it is not clear whether the interactions of the proton pump subunits are with synaptobrevin, synaptophysin, or both, although a direct interaction between synaptophysin and the 39-kDa subunit of the proton pump (in this context originally called physophilin) has been suggested by the Betz group (Thomas & Betz 1990, Siebert et al 1994). It has been speculated that the synaptobrevin-synaptophysin-proton pump complex may play a role in recruiting the proton pump to synaptic vesicles (Galli et al 1996).

## Implications of Synaptic Vesicle Lipid Composition for Biogenesis

The lipid compositions of synaptic vesicle preparations from a variety of sources and of varying purities have been reported (see Westhead 1987). The most striking and consistent feature of these analyses is the enrichment, compared with the membranes of most tissues, in the polyunsaturated fatty acids, particularly docosahexanoic acid (22:6), which constitutes approximately 30% of the phosphatidylethanolamine (PE) and phosphatidylserine (PS) phospholipids (Westhead 1987). However, the high levels of polyunsaturated fatty acids are a characteristic feature of neuronal membranes in general and not just of synaptic vesicles (Breckenridge et al 1972, Sastry 1985). Synthesis of docosahexanoic acid is dependent on the dietry intake of linolenic acid, and although there is a large body of literature concerning the neurological effects of essential fatty acid deficiency (see Gibson & Blass 1999), no relationship between fatty acid composition and the biogenesis of synaptic vesicles has been established.

With respect to possible lipid sorting related to synaptic vesicle biogenesis, the studies of Breckenridge et al (1972, 1973) are of particular relevance. They compared the lipid composition of rat brain synaptosomal plasma membranes (excluding synaptic vesicles) with that of the purified synaptic vesicles. Although the lipid compositions of the two membranes were very similar (Breckenridge et al 1972, 1973), gangliosides, which were present at quite high levels (about 13% of the lipid by weight) in the synaptosome plasma membrane (Breckenridge et al 1972), were almost completely excluded from the synaptic vesicles (Breckenridge et al 1973). The proportion of phospholipids in the two membranes was relatively constant at about 75% of total lipid weight, and it appears that the absence of gangliosides in the synaptic vesicle membrane was compensated for by an increase in the cholesterol content (Breckenridge et al 1972, 1973). A higher than normal ratio of cholesterol to phospholipid has consistently been observed by a number of groups studying synaptic vesicles (Westhead 1987), and therefore this probably represents a genuine characteristic of synaptic vesicles.

It is not clear how the gangliosides are excluded from the synaptic vesicle membrane. The simplest explanation is that the gangliosides become trapped in the dense material or "extracellular fuzz" (Breckenridge et al 1973) that is observed at the active zone (Peters et al 1991). There is evidence that the active zone is rich in glycoconjugates (Peters et al 1991), but the precise mechanisms by which these could become retained are not known. With regard to the above-mentioned enrichment of cholesterol in synaptic vesicles (Breckenridge et al 1973, Westhead 1987), it is generally assumed that cholesterol in membranes interacts with sphingolipids (sphingomyelin and glycosphingolipids) through hydrogen bonding of the sphingosine base with cholesterol (see Allan & Kallen 1993). The importance of this interaction for the retention of cholesterol has been demonstrated in living cells by the enzymatic digestion of sphingomyelin at the cell surface, which causes the loss of cholesterol from the exterior leaflet of the plasma membrane (Slotte & Bierman 1988). Therefore the fact that synaptic vesicles are enriched in cholesterol compared with synaptosomal plasma membranes and at the same time relatively depleted of the sphingolipids (with gangliosides being absent and sphingomyelin not enriched) with which cholesterol normally interacts, suggests that there may be other components in the synaptic vesicle membrane that are able to retain cholesterol. It could be that cholesterol tends to associate more with polyunsaturated fatty acids, although this would not explain the difference in cholesterol levels between synaptosomal plasma membrane and synaptic vesicles, as the fatty acid content of the two was virtually identical (Breckenridge et al 1972, 1973). Alternatively, it could be that cholesterol associates with membranes of high curvature, especially in the case of synaptic vesicles where there could be a significant mismatch between the curvature present in the vesicles due to their size and the curvature that would normally be induced by the phospholipids present (Westhead 1987, Deutsch & Kelly 1981).

***Protein-Lipid Interactions*** Another possible explanation to account for relative enrichment of cholesterol is that one of the protein components of the synaptic vesicle binds to cholesterol. Recent evidence obtained in our laboratory, using a novel photoactivatable cholesterol, has shown that synaptophysin is one of the major membrane proteins in PC12 cells labeled with this reagent (Thiele et al 1999). Therefore, it is possible that a function of synaptophysin is to sort cholesterol to synaptic vesicles or even to induce or stabilize a particular protein-lipid assembly, which could recruit other synaptic vesicle membrane components. Such a putative synaptophysin-cholesterol microdomain would be analogous to the glycosphingolipid-cholesterol rafts that have been proposed to sort proteins to the apical surface of epithelial cells (Simons & Ikonen 1997). However, in contrast to such raft proteins (Simons & Ikonen 1997), synaptophysin is very soluble in Triton X-100 at low temperatures and therefore such a microdomain, if it exists, does not lead to the formation of Triton X-100–resistant complexes analogous to the detergent-insoluble glycosphingolipid complexes (DIGs or DICs), which have been used to help characterize the protein

components of glycosphingolipid-cholesterol rafts (Brown & Rose 1992). The absence of a detectable phenotype in mice lacking synaptophysin (Eshkind & Leube 1995, McMahon et al 1996) could be due to the ability of other synaptic vesicle proteins to perform the same function, especially as there are many synaptic vesicle proteins with a proposed membrane topology similar to that of synaptophysin (synaptogyrin, SCAMP, synaptoporin). Alternatively, the phenotype may have been missed. The composition of the synaptic vesicles was carefully examined with respect to its protein compostion (Eshkind & Leube 1995, McMahon et al 1996) but as far as we are aware, the lipid compostion of the synaptic vesicles from the synaptophysin knockout mice has not been studied.

## Sorting Signals

Sorting events responsible for the specific protein composition of synaptic vesicles probably occur at a number of distinct stages during their biogenesis. The segregation of axonal from somatodendritic proteins at the level of the TGN in the perikaryon has been discussed (see above). Here we review the evidence for sequence determinants present in synaptic vesicle proteins that are involved in endocytosis and targeting to synaptic vesicles.

***Signals for Endocytosis*** Synaptic vesicle proteins are retrieved by endocytosis. Accordingly, many synaptic vesicle proteins when expressed individually in heterologous systems are found in endocytic compartments. This is true for synaptophysin (Johnston et al 1989, Linstedt & Kelly 1991b, Leube et al 1994), SV2 (Feany et al 1993), synaptobrevin (Grote & Kelly 1996), and the neurotransmitter transporters VMAT2 and VAChT (Liu & Edwards 1997, Tan et al 1998). Importantly, although all endocytic in nature, these compartments are not necessarily the same. Synaptophysin and SV2 are found in different endosomes when expressed together in CHO cells (Feany et al 1993), and synaptophysin is found in morphologically and compositionally distinct endosomes depending on whether it is expressed in CHO cells (Johnston et al 1989, Linstedt & Kelly 1991b) or certain non-neuroendocrine epithelial cells (Leube et al 1994). It is not known whether this differential distribution is due to sorting within a common endocytic compartment or to differential routes of internalization.

Interestingly, synaptotagmin, which is the one synaptic vesicle protein known to directly interact with the plasma membrane endocytic machinery of AP2 and clathrin (Zhang et al 1994), appears to be retained at the plasma membrane of transfected fibroblasts (Feany et al 1993). This suggests that while the other synaptic vesicle proteins can be constitutively endocytosed, the recycling of synaptotagmin may be regulated. This observation may have a significant bearing on the formation of synaptic vesicles in neurons because synaptotagmin has been directly implicated in synaptic vesicle recycling at a mature synapse in vivo (Jorgensen et al 1995). [The binding of another synaptic vesicle protein (synaptobrevin) to

a different adaptor complex (AP3), which has also been implicated in synaptic vesicle biogenesis in PC12 cells (Salem et al 1998), is not thought to be relevant for endocytosis from the plasma membrane but for vesicle formation from an endosome (Faúndez et al 1997, Salem et al 1998).]

The endocytosis signals used by the various synaptic vesicle proteins have yet to be completely delineated, although it is becoming apparent that there will not be a single, common motif used by all. It has been demonstrated that the long, cytoplasmic, C-terminal tail of synaptophysin is neccesary for endocytosis (Linstedt & Kelly 1991a) and also reported that it is sufficient to restore internalization of a tail-less mutant of the low-density lipoprotein receptor (see Linstedt & Kelly 1991a). The sequence(s) within the cytoplasmic tail responsible for rapid internalization of synaptophysin have not been determined. It contains a number of tyrosine-containing repeats (Südhof et al 1987, Leube et al 1987) that do not conform to either the NPXY or YXXØ consensus sequences for adaptor protein binding (Marks et al 1997), although they do bear a strong resemblance to the internalization and lysosomal targeting sequences found at the extreme C terminus of the lysosomal membrane proteins LAMP-1 and LAMP-2 (Marks et al 1997). However, the structurally related proteins pantophysin and SCAMP do not contain these same repeats and yet are also endocytosed (Brand & Castle 1993, Haass et al 1996).

Recently, a di-leucine endocytosis motif has been shown to be responsible for the endocytosis of VAChT and the VMATs (Tan et al 1998). Di-leucine based trafficking signals are not specific to synaptic vesicle proteins (Marks et al 1996) and at present there is no evidence that this motif is involved in the endocytosis of any other synaptic vesicle protein (Tan et al 1998).

Mutations within the cytoplasmic domain of synaptobrevin have been found that affect its rate of endocytosis (Grote & Kelly 1996). Mutants with reduced endocytosis are also less efficiently targeted to SLMVs in PC12 cells (Grote et al 1995, Grote & Kelly 1996), which is consistent with the concept that synaptic vesicle membrane proteins pass through an endosome prior to their appearance in SLMVs (Régnier-Vigouroux et al 1991). However, mutants with increased rates of endocytosis are not neccessarily enriched in SLMVs (Grote et al 1995, Grote & Kelly 1996), suggesting an additional level of control beyond endocytosis.

***Synaptic Vesicle Targeting Signals Versus Synaptic Vesicle Exclusion Signals***
The synaptic vesicle targeting index used by Grote and colleagues (see below) reflects the proportion of a given synaptic vesicle protein that is found in synaptic vesicles relative to that present in the total cell homogenate (Grote et al 1995). Accordingly, a synaptic vesicle targeting signal (SVTS) (Grote & Kelly 1996) is any sequence determinant that increases the synaptic vesicle targeting index. It should be borne in mind that by this definition, an SVTS will include not only (*a*) determinants that increase the packaging of a synaptic vesicle protein from

the donor membrane(s) into synaptic vesicles (e.g. sorting signals mediating the interaction with a putative cytoplasmic sorting machinery), but also (*b*) determinants that allow a synaptic vesicle protein to reach the donor membrane(s) from which synaptic vesicles are formed (e.g. signals required for anterograde transport to the plasma membrane and endocytosis signals), and (*c*) determinants that interfere with the removal of a synaptic vesicle protein from the donor membrane(s) into a membrane compartment other than synaptic vesicles (e.g. interference with transport from the plasma membrane and/or early endosome to lysosomes for degradation) (Figure 4).

The work of Grote et al (Grote et al 1995, Grote & Kelly 1996) on the trafficking of synaptobrevin mutants in PC12 cells is to date the most comprehensive study undertaken on the sequence determinants involved in the targeting of a synaptic vesicle protein. They have constructed an extensive panel of deletion and point mutants of an epitope-tagged synaptobrevin called VAMP-TAg. From these mutants, sequences involved in both endocytosis (see above) and targeting to SLMVs in PC12 cells have been determined.

The data suggest that an SVTS, which is distinct from an endocytosis signal, exists in residues 31–38 of the synaptobrevin sequence, because a deletion mutant lacking these amino acids was endocytosed normally but excluded from SLMVs (Grote et al 1995). In addition the residues 41–50 also contain an SVTS as the deletion of these amino acids prevented endocytosis and appearance in SLMVs. Point mutants of residues 31–38 have not been reported, but alanine scanning mutagenesis of residues 41–50 showed that this region had an effect on both endocytosis and SLMV targeting (Grote et al 1995, Grote & Kelly 1996). Remarkably, the influence of these mutations on targeting of VAMP-TAg to SLMVs was both negative and positive, with mutations of two of the amino acids (aspartate 44 and asparagine 49) producing significant increases in the proportion of protein found in SLMVs. In fact, the gain-of-targeting mutations were quantitatively more striking than the loss-of-targeting mutations, with the asparagine mutation (N49A) producing a protein that was targeted to SLMVs 200-fold better than the non-mutated VAMP-TAg (Grote et al 1995). Significantly, in the cells expressing VAMP-TAg N49A, there was no increase in the proportion of cellular synaptophysin found in SLMVs (Grote et al 1995). This means that the ratio of VAMP-TAg to synaptophysin in the SLMVs was altered by the mutation, giving further support to the concept that the stoichiometry of proteins within synaptic vesicle membranes is not tightly regulated.

The authors' interpretation of the mechanism by which the N49A mutation increases SLMV targeting is that the change from a polar (asparagine) to hydrophobic (alanine) residue within the context of an amphipathic helix has a positive influence on an interaction (direct or indirect) between VAMP-TAg and the putative SLMV sorting machinery (Grote et al 1995). However, in our opinion, one cannot exclude the possibility that the mutation actually has a negative influence on an interaction between VAMP-TAg and a putative machinery that competes with

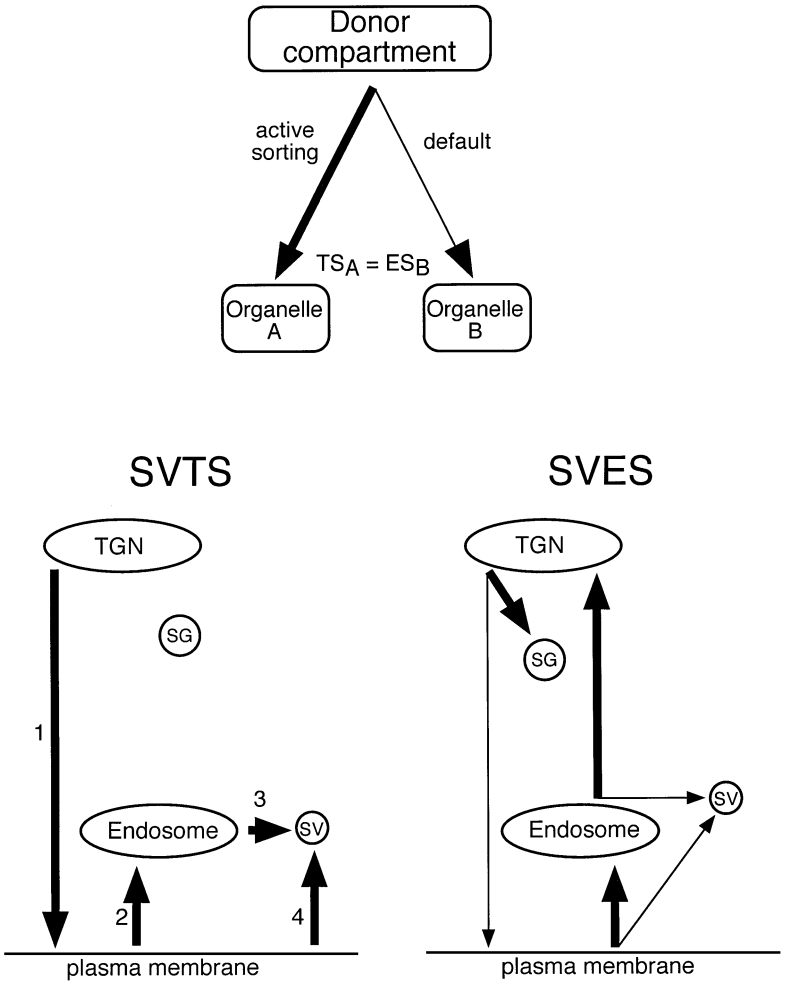


**Figure 4** Synaptic vesicle targeting signal (SVTS) versus synaptic vesicle exclusion signal (SVES). *Top*: If a molecule has two possible destinations from a donor compartment, a targeting signal (TS) mediating active sorting to organelle A (thick arrow) will constitute an exclusion signal (ES) for the default entry into organelle B (thin arrow). *Bottom left*: A SVTS scheme depicting membrane traffic steps at which synaptic vesicle proteins could be actively sorted (thick arrows), resulting in increased synaptic vesicle targeting; 1, TGN to axonal transport vesicle; 2, plasma membrane to endosome; 3, endosome to synaptic vesicle (SV); 4, plasma membrane to synaptic vesicle. *Bottom right*: A SVES scheme depicting membrane traffic steps at which synaptic vesicle proteins could be actively sorted (thick arrows), resulting in decreased synaptic vesicle targeting (thin arrows); SG, secretory granule.

its sorting to SLMVs. If one makes this assumption, i.e. that N49A is a loss-of-interaction mutation, then we would propose that the N49 residue should be called a synaptic vesicle exclusion signal (SVES) (Figure 4). Following this nomenclature, additional SVESs could also be present in residues 60–80, since deletion of amino acids 61–70 or 71–80 produced a 50-fold or 10-fold increase, respectively, in targeting to SLMVs. The postulated existence of multiple and dominant SVESs in the synaptobrevin sequence is consistent with the fact that only a small proportion (2%) of the non-mutated VAMP-TAg was found in PC12 SLMVs (Grote et al 1995, Grote & Kelly 1996).

What could be the nature of a putative machinery that competes with VAMP-TAg sorting to SLMVs? Synaptobrevin is a normal constituent not only of SLMVs but also of secretory granules in PC12 cells (Chilcote et al 1995, Papini et al 1995), and therefore one potential SVES is a secretory granule targeting signal operating at the level of the TGN. At the plasma membrane, there are probably signals that determine which endocytic route the protein takes; in the SLMV donor compartment (irrespective of whether this is the plasma membrane or an early endosome), additional SVESs could include signals to go to lysosomes, back to the TGN, or to another endosome (Figure 4). The proposal that increased SLMV targeting of a protein can be achieved by inhibiting its exclusion from the donor membrane is consistent with the postulate that the concentration of the individual proteins in synaptic vesicles is not tightly regulated, but rather is the consequence of the concentration of that protein in the donor membrane.

Whichever interpretation is nearer the truth (SVTS versus SVES), the data suggest that the trafficking of synaptobrevin is mediated by a complicated relationship of both positive and negative elements within its primary sequence that independently regulate both endocytosis and targeting to SLMVs. One factor contributing to the complexity of synaptobrevin sorting may be the large number of proteins with which it interacts. These include synaptophysin (Johnston & Südhof 1990, Calakos & Scheller 1994, Edelmann et al 1995, Washbourne et al 1995), syntaxin/SNAP-25 (Söllner et al 1993), and the recently discovered VAMP-associated proteins (Skehel et al 1995, Nishimura et al 1999). An analysis of the interaction between the VAMP-TAg mutants and syntaxin or SNAP-25 suggests a possible relationship between v-SNARE/tSNARE binding and sorting to SLMVs, although as with the endocytosis and trafficking data this relationship does not appear to be simple (Hao et al 1997a).

It has recently been shown that synaptobrevin is not just sorted to SLMVs but is physically involved in the formation of the vesicles by an AP3- and ARF-dependent process (Salem et al 1998). It then becomes difficult to know whether the increased appearance of the mutant VAMP-TAg proteins in SLMVs is due to increased sorting of the protein into the vesicles or increased generation of vesicles induced by the protein. Maybe there are VAMP-TAg–containing vesicles induced by expression of mutant VAMP-TAg, analogous to the GLUT-4–containing small vesicles seen when that protein is expressed (Herman et al 1994, Wei et al 1998).

## Sorting of Neurotransmitter Transporters

Four classes of synaptic vesicle neurotransmitter transporter have been characterized so far in the mammalian nervous system: they are those for monoamines, acetylcholine, GABA/glycine, and glutamate (Edwards 1992). The vesicular transporters for monoamines (VMAT1 and VMAT2) (Erickson 1992, Liu 1992), acetylcholine (VAChT) (Roghani et al 1994, Erickson et al 1994), and GABA (VGAT) (McIntire et al 1997) have been cloned.

The monoamine transporter is of particular interest for two reasons. First, the synaptic vesicles, which contain monoamines in the central and peripheral nervous system, are morphologically distinct from the synaptic vesicles found elsewhere (Thureson-Klein 1983, Peters et al 1991). Second, monoamine transport activity is not exclusively localized to these small vesicles, it is also associated with secretory granules (Thureson-Klein 1983, Thureson-Klein & Klein 1990). The synaptic vesicles present in monoaminergic terminals are slightly bigger than in most other terminals, ranging in size from 40–60 nm rather than the more typical 40–50 nm (Peters et al 1991). They also have an electron dense core after certain types of fixation (Klein et al 1982) and hence are known as small dense core vesicles (SDCVs). Secretory granules and SDCVs are usually found in the same nerve terminals, and it was thought that SDCVs represent the remnants of a secretory granule membrane that are retrieved after exocytosis (Winkler et al 1987). Although the biogenesis of SDCVs may not be completely resolved (Winkler 1997), it seems likely that the SDCV is a hybrid vesicle made up of membrane components of both secretory granules and synaptic vesicles (Bauerfeind et al 1995a).

Although PC12 cells contain monoamine transport activity, they do not make SDCVs. The PC12 cell vesicular monoamine transporter (VMAT) is found on secretory granules and in the endosome, but it is mostly excluded from the SLMVs (Bauerfeind et al 1993, 1995a; Tao-Cheng & Eiden 1998; Y Liu et al 1994). When the first VMAT was cloned from a PC12 cell library (Liu 1992) it was found that the mRNA for this transporter was absent from neuronal monoamine populations. Instead these cells express a closely related but distinct gene product (Liu 1992). The striking tissue specificity of VMAT isoform gene expression in the rat correlates with the subcellular localization of transporter activity. rVMAT1 (originally called CGAT, for chromaffin granule amine transporter) is expressed in adrenal medulla chromaffin cells (Peter et al 1995) (and PC12 cells), where it is found almost exclusively on the secretory (chromaffin) granules (Liu 1992, Y Liu et al 1994). rVMAT2 (originally called SVAT, for synaptic vesicle amine transporter) is expressed in central and peripheral monoaminergic neurons (Peter et al 1995), where it is found on secretory granules and on the small (synaptic vesicle-like) SDCVs (Nirenberg et al 1995). This correlation suggests that the VMAT2 sequence contains synaptic vesicle sorting information, a possibility made even more tenable with the observation that bovine adrenal medulla chromaffin cells which (in contrast to the rat) have monoamine transport activity in their SLMVs (Annaert et al 1993) express mostly VMAT2 (Krejci et al 1993, Howell et al 1994). The apparent differential sorting of the VMAT isoforms therefore

appears to be a useful model system with which to investigate the nature of synaptic vesicle sorting information.

However, when VMAT2 was expressed in PC12 cells, it was not found in SLMVs, but in the same places as VMAT1, i.e. secretory granules and endosomes (Tao-Cheng & Eiden 1998, Varoqui & Erickson 1998). An interpretation of this apparent missorting of VMAT2 in PC12 cells is that a monoaminergic neuron-specific component or modification of the protein (not present in PC12 cells) is required to sort VMAT2 to the small, synaptophysin-positive, synaptic vesicle–like SDCVs (Tao-Cheng & Eiden 1998, Reimer et al 1998). However, it should be remembered that VMAT2, similar to synaptobrevin, is also normally a component of secretory granules and hence the protein must contain information that allows it to be segregated from exclusively synaptic vesicle proteins (such as synaptophysin) at the level of the TGN. Therefore, an alternative and equally plausible explanation in light of the discussion of the VAMP-TAg sorting in PC12 cells (see above) is that the unexpected VMAT2 trafficking in PC12 cells is not due primarily to the absence of the appropriate synaptic vesicle sorting mechanisms, but to the dominance within PC12 cells of a secretory granule targeting signal (SGTS), i.e. a synaptic vesicle exclusion signal (SVES) that VMAT2 contains (Figure 4). The fact that VMAT2 is sorted into the synaptophysin-positive SDCVs in the nerve terminal of sympathetic neurons but into secretory granules at the level of the TGN in the same cells can then be elegantly explained by proposing that the putative SVES is not active at the nerve terminal because the machinery that recognizes it is present only in the perikaryon. The PC12 cell in contrast does not have the extensive compartmentalization present in a mature neuron and therefore the VMAT2 SGTS $=$ SVES is dominant throughout the cell.

In addition to the monoamine transporter, PC12 cells also endogenously express a vesicular acetylcholine transporter (VAChT) which, unlike VMAT1, is found in SLMVs (Bauerfeind et al 1993, Liu & Edwards 1997, Tao-Cheng & Eiden 1998). VAChT has been cloned and was found to be homologous to the VMATs, especially within the TM domains (Roghani et al 1994, Erickson et al 1994). Both monoamine and acetylcholine transport activities are demonstrable in the endosome of PC12 cells together with synaptophysin (Bauerfeind et al 1993), and therefore it seems likely that VAChT contains some kind of sorting information that targets it to SLMVs (Bauerfeind et al 1993). Recent studies using PC12 cells transfected with either VAChT (Liu & Edwards 1997, Tao-Cheng & Eiden 1998, Varoqui & Erickson 1998) or VMAT2 (Tao-Cheng & Eiden 1998, Varoqui & Erickson 1998) have confirmed the differential sorting of these two transporters. Additionally, from analysis of the subcellular localization of chimeric transporter proteins, it has been concluded that the cytoplasmic C-terminal region of VAChT contains a SVTS (Varoqui & Erickson 1998). This conclusion is based on the observation that a mutant of VMAT2 with its C-terminal cytoplasmic tail exchanged with that of VAChT was sorted to SLMVs in PC12 cells (Varoqui & Erickson 1998). The importance of this region for SLMV sorting was corroborated by the

reduction in targeting to SLMVs of a mutant VAChT with its C-terminal cytoplasmic tail exchanged with that of VMAT2 (Varoqui & Erickson 1998) [this mutation did not completely abrogate the sorting to SLMVs (Varoqui & Erickson 1998) and therefore if the VAChT cytoplasmic tail does contain a SVTS it cannot be its only one]. Although the data are consistent with this interpretation, they can also be explained by the presence of a dominant SVES (Figure 4) within the cytoplasmic tail of VMAT2 combined with subordinate SLMV targeting information elsewhere in the VMAT2 sequence. In this view, the VMAT2 mutant expressing the VAChT tail is not actively sorted to SLMVs by virtue of the VAChT SVTS, but rather it is not excluded from SLMVs because it has lost the VMAT2 SVES. Conversely, VAChT expressing the VMAT2 cytoplasmic domain is actively excluded from SLMVs due to the dominant SVES this region contains.

## CYTOSOLIC MACHINERY

Two principal experimental approaches have been used to dissect the cytoplasmic machinery mediating the biogenesis of synaptic vesicles and SLMVs: cell-free systems and living cells manipulated with respect to the protein of interest by transfection or microinjection.

### Cell-Free Systems

***Cell-Free Systems Derived From the Neuroendocrince Cell Line PC12*** Two types of cell-free systems reconstituting SLMV formation have been established, both of which use PC12 cell membranes and rat brain cytosol. One type, developed by Kelly and colleagues, is based on the use of either deleted (del 61–70) (Grote et al 1995, Desnos et al 1995a) or mutated (N49A) (Grote et al 1995; Faúndez et al 1997, 1998; Clift-O'Grady et al 1998; Shi et al 1998) forms of VAMP/synaptobrevin tagged with T antigen (VAMP-TAg) epitope and stably transfected into PC12 cells. After binding of iodinated KT3 monoclonal antibody against the TAg epitope, either at 15°C (Desnos et al 1995a; Faúndez et al 1997, 1998; Clift-O'Grady et al 1998) or at 4°C (Shi et al 1998), a PC12 cell homogenate is incubated at 37°C in the presence of cytosol and ATP, and the SLMVs generated are isolated on a glycerol velocity gradient, monitoring the iodinated antibody bound to VAMP-TAg. This cell-free system reconstitutes SLMV formation from distinct donor membranes, depending on whether antibody binding is performed at 15 or 4°C. After binding at 15°C, SLMV formation is thought to be reconstituted from early endosomes (see above) (Desnos et al 1995a, Lichtenstein et al 1998). After binding at 4°C, a temperature at which the antibody has access only to the VAMP-TAg present at the cell surface, SLMV formation is reconstituted from the plasma membrane, consistent with the results obtained with the other PC12 cell-derived cell-free system described below.

The other type of cell-free system reconstituting SLMVs formation was developed in our laboratory. This system uses cell-surface biotinylated synaptophysin as

a marker (Schmidt et al 1997b, Schmidt & Huttner 1998) and perforated PC12 cells (Schmidt & Huttner 1998). PC12 cells are biotinylated using cell-impermeable analogs of biotin at 18°C, a temperature at which the formation of SLMVs is blocked but the recycling of synaptophysin still occurs. The PC12 cells, perforated by mechanical scraping that allows the wash-out of cytosolic components, are subsequently incubated at 37°C, in the presence of brain cytosol and ATP. The SLMVs formed in the perforated-cell assay and containing biotinylated synaptophysin are separated from the donor membranes by differential centrifugation followed by either a glycerol velocity gradient as described by Kelly and colleagues (Clift-O'Grady et al 1990) or a single step of centrifugation on a glycerol cushion (Schmidt & Huttner 1998). As with intact PC12 cells, MesNa treatment after cell surface biotinylation and prior to the perforated-cell reaction abolishes the recovery of biotinylated synaptophysin in SLMVs, indicating that this perforated-cell system reconstitutes SLMV formation from the plasma membrane and/or a membrane invagination in continuity with the plasmalemma rather than the early endosome (Schmidt et al 1997b).

The use of these two cell-free systems has revealed that the cytoplasmic machineries mediating SLMV formation from early endosomes and from the plasma membrane are distinct. It is unresolved, however, why the cell-free system based on labeling VAMP-TAg at 15°C predominantly reconstitutes SLMV formation from early endosomes (Clift-O'Grady et al 1998), whereas that based on labeling synaptophysin at 18°C reconstitutes SLMV formation from the plasma membrane (Schmidt & Huttner 1998). We find it unlikely that this difference is due to the different temperatures used in the labeling step. Rather, this difference may reflect the nature of the SLMV protein used as marker and/or the type of SLMV formed.

***Cell-Free Systems Derived From Synaptosomes*** To gain insight into the cytoplasmic machinery mediating synaptic vesicle re-formation by recycling, De Camilli and colleagues developed a system using lysed rat brain synaptosomes (isolated nerve terminals) and derived membrane fractions to study the membrane recruitment of cytosolic proteins (Takei et al 1995, 1996). The major feature of this system is the freezing of the process of synaptic vesicle membrane recycling at the level of membrane intermediates by using GTP$\gamma$S (a non-hydrolyzable analog of GTP), followed by electron microscopic analysis. Strikingly, the single vesicle budding step from a membrane invagination in continuity with the presynaptic plasma membrane proposed by DeCamilli and colleagues (Takei et al 1996) is fully consistent with the model of SLMV formation from a specialized plasma membrane domain in PC12 cells (Schmidt et al 1997b). Further consistent are the observations that the cytosolic proteins implicated in synaptic vesicle recycling on the basis of their membrane recruitment (Takei et al 1995, 1996) are indeed key players in SLMV formation in the perforated PC12 cell system (Schmidt & Huttner 1998).

***Cell-Free Systems Using Liposomes*** Several groups have used protein-free liposomes to study the role in budding and fission of cytosolic proteins implicated in vesicle formation (Matsuoka et al 1998, Sweitzer & Hinshaw 1998, Takei et al 1998, Bremser et al 1999). Two of these studies (Sweitzer & Hinshaw 1998, Takei et al 1998) have investigated cytosolic proteins implicated in synaptic vesicle formation, i.e. recombinant dynamin I and the clathrin coat purified from brain. With this approach the lipid composition can be varied and, hence, the role of the various lipids in the budding, tubulation and fission steps mediated by the clathrin coat and dynamin machinery can be determined.

***Molecular Dissection of the Protein Machinery*** The cell-free systems described above have revealed that the cytoplasmic components mediating synaptic vesicle formation can be classified into two principal groups. One group consists of the proteins implicated in budding, with clathrin and the adaptor proteins AP2 and AP3 being the major components. Clathrin/AP2 operate at the plasma membrane and plasma membrane–derived invaginations; AP3 operates (apparently without clathrin) at early endosomes. The other group consists of the proteins implicated in fission, with dynamin and its associated lipid modifying enzyme SH3P4 as key players. Dynamin/SH3P4 operate at the plasma membrane and plasma membrane–derived invaginations, either without or in concert with clathrin/AP2. In the following, we discuss, for each of the protein components, the evidence for a role in SLMV and synaptic vesicle biogenesis (Table 1).

## Clathrin and Adaptors

***Clathrin*** Although there are ample morphological and biochemical data that suggest a role of clathrin in synaptic vesicle recycling, studies providing direct functional evidence are surprisingly scarce. The morphological data, starting with the seminal observations of Heuser & Reese (1973) and including the studies on the recruitment of clathrin to presynaptic membranes (Takei et al 1995, 1996) have been reviewed above. The biochemical data originate from the purification of clathrin-coated vesicles (Maycox et al 1992) and synaptic vesicles (Huttner et al 1983) from brain. In essence, clathrin dissociates from synaptic vesicles in the course of their purification (Huttner et al 1983). Conversely, when clathrin-coated vesicles are purified using synaptosomes as starting material, virtually all of the coated vesicles obtained are synaptic vesicles with regard to the membrane that is being recovered in coated form (Pfeffer & Kelly 1985, Maycox et al 1992).

Direct functional evidence for a role of clathrin has been obtained in the two PC12 cell–derived systems reconstituting SLMV formation from the plasma membrane. Depletion of clathrin from rat brain cytosol (Shi et al 1998) or addition of the anti-clathrin heavy chain antibody X22 (Schmidt & Huttner 1998) impairs the formation of SLMVs.

***AP2 Adaptor Complex*** The action of clathrin in the budding of synaptic vesicles from the presynaptic plasma membrane and from membrane invaginations is mediated by the AP2 adaptor complex. Interestingly, the binding site for AP2 on the synaptic vesicle membrane has been shown to be one of the C2 domains in the cytoplasmic portion of the integral membrane protein synaptotagmin I (Zhang et al 1994). This observation provides an intriguing potential link between synaptic vesicle recycling and synaptic vesicle membrane assembly on the one hand and synaptic vesicle exocytosis on the other. Synaptotagmin I appears to exist in a complex with other synaptic vesicle membrane proteins, and hence the interaction of AP2 with synaptotagmin I during budding may promote the re-assembly of the synaptic vesicle in the recycling process. Also, synaptotagmin I is thought to be a calcium sensor and to mediate the calcium dependence of synaptic vesicle exocytosis; it can therefore be regarded as a target of choice for the nucleation of the AP2-mediated clathrin coat. Consistent with this, *Caenorhabditis elegans* mutants deficient in *snt-1* function, the homolog of mammalian synaptotagmin, which show impaired neurotransmission (Nonet et al 1993), exhibit a defect in synaptic vesicle retrieval from the presynaptic plasma membrane (Jorgensen et al 1995).

Depletion of the AP2 adaptor complex from rat brain cytosol strongly impairs the formation of SLMVs from the plasma membrane in the PC12 cell–derived cell-free system (Shi et al 1998). In vivo evidence for an essential role of AP2 in synaptic vesicle formation has come from the analysis of mutations in the $\alpha$-adaptin gene of *Drosophila*. The synapses of mutant embryos were found to be depleted of synaptic vesicles and showed abnormal infoldings of the plasmalemma, which has been attributed to impaired synaptic vesicle membrane retrieval from the presynaptic plasma membrane (González-Gaitán & Jäckle 1997).

How can this evidence for an essential role of AP2, and hence clathrin, in synaptic vesicle formation be reconciled with the concept, described above, that synaptic vesicle re-formation by recycling can occur not only via the clathrin plus dynamin-mediated, budding plus fission type of membrane retrieval, but also via the fission only type mediated by the dynamin machinery without clathrin? The most probable explanation is that, in the study by González-Gaitán & Jäckle, the mutated $\alpha$-adaptin was present throughout development and hence affected not only synaptic vesicle re-formation by recycling, but also formation de novo and formation under renovation, both of which we propose require more than just membrane fission, i.e. protein and lipid sorting and membrane budding. In other words, the $\alpha$-adaptin mutant embryos never develop to a stage at which the fission only type of synaptic vesicle re-formation by recycling becomes effective. A prediction implicit in this interpretation of the González-Gaitán and Jäckle data is that a temperature-sensitive (or rapidly inducible) mutant in the $\alpha$-adaptin gene similar to the *shibire* mutant would show a much less drastic defect in synaptic vesicle membrane recycling than the latter.

**TABLE 1** Cytosolic proteins involved in the recycling of SVs and SLMVs

| | Cell-free | | In vivo |
|---|---|---|---|
| **Cytosolic protein** | **SLMV formation** | **SV recycling** | **SV recycling** |
| Clathrin | Immuno-depletion of clathrin heavy chain from cytosol reduces (9)[a]; addition of anti-clathrin heavy chain antibody inhibits (10) | Clathrin coats are present on vesicular buds forming from presynaptic plasma membrane (4, 5) | Clathrin-coated vesicles are observed at the synapse of the neuromuscular junction (1) |
| AP2 adaptor ($\alpha$-adaptin) | Immuno-depletion of $\alpha$-adaptin from cytosol reduces (9) | $\alpha$-adaptin is present on vesicular buds forming from presynaptic plasma membrane (5) | Mutant form of the homolog of $\alpha$-adaptin in *Drosophila melanogaster* inhibits SV recycling at the synapse (13) |
| AP180 | n.d. | AP180 is present on vesicular buds forming from presynaptic plasma membrane (5) | Mutation that eliminates a homolog of AP180 in *Drosophila melanogaster* impairs the regulation of the size and the recycling of SVs (15) |
| AP3 adaptor | Immuno-depletion of the AP3 complex from cytosol reduces (11); complementation of cytosol immuno-depleted of AP3 with AP3-enriched cytosolic fractions enhances (11) | AP3 adaptor complex is recruited to the membrane of purified SVs (11) | No evidence[b] |
| Dynamin | Depletion of dynamin from cytosol inhibits (10); complementation of cytosol depleted of dynamin with | Dynamin forms spirals on tubular invaginations of presynaptic plasma | *Shibire* thermo-sensitive mutant of the *Drosophila melanogaster* homolog of mammalian dynamin shows |

| | | | |
|---|---|---|---|
| | recombinant dynamin I restores (10); addition of Gst-Dyn-PRD inhibits (9) | membrane (4, 5) | accumulation of “collared pits” at the presynaptic plasma membrane (2, 3) microinjection in the lamprey synapse of a peptide from the PRD of dynamin that corresponds to the putative site of interaction with amphiphysin inhibits SV endocytosis (8) |
| Amphiphysin | Addition of a proline-rich peptide of dynamin corresponding to the binding site of amphiphysin inhibits (14) | Amphiphysin is associated with tubular invaginations of presynaptic plasma membranes (6) | Microinjection in the lamprey synapse of the SH3 domain of amphiphysin inhibits SV endocytosis at the stage of invaginated clathrin-coated pit (8) |
| Synaptojanin | n.d. | Synaptojanin is concentrated at clathrin-coated invaginations of presynaptic plasma membrane (7) | Gene knock-out of synaptojanin shows an accumulation of clathrin-coated vesicles at the synapse, suggesting a role in uncoating (17) |
| SH3P4 | Cytosol depleted of SH3P4 reduces (10, 16); addition of recombinant SH3P4 to cytosol depleted of SH3P4 restores (10, 16) | n.d. | n.d. |
| Epsin | n.d. | Association of epsin with clathrin coats of synaptosomal membranes (12) | n.d. |

[a](1) Heuser & Reese 1973; (2) Kosaka & Ikeda 1983; (3) Koenig & Ikeda 1989; (4) Takei et al 1995; (5) Takei et al 1996; (6) Bauerfeind et al 1997; (7) Haffner et al 1997; (8) Shupliakov et al 1997; (9) Shi et al 1998; (10) Schmidt et al 1998; (11) Faundez et al 1998; (12) Chen et al 1998; (13) Gaitano & Jäckle 1998; (14) A Schmidt & WB Huttner, unpublished observation; (15) P Zhang et al 1998; (16) Schmidt et al 1999; (17) P De Camilli, personal communication.

[b]Mutation in AP3 $\delta$ in the *mocha* mouse results in a dramatic reduction of the SV-associated zinc transporter ZnT-3 but no alteration in the number of SVs (Kantheti et al 1998). Dyn, dynamin; PRD, proline/arginine-rich domain; n.d., not determined.

***AP180*** In contrast to the adaptor complexes AP2 and AP3 (discussed below), AP180, a neuron-specific adaptor protein enriched at synapses, is monomeric (Kohtz & Puszkin 1988, 1989; Su et al 1991). AP180 (previously also referred to as AP3, which is not to be confused with the AP3 adaptor complex) does not exhibit structural similarity to the other adaptor proteins (Hirst & Robinson 1998). AP180 appears to act in concert with AP2 in clathrin coat assembly in vitro (Lindner & Ungewickell 1992) and has been implicated in the generation of particularly small clathrin baskets appropriate to coat synaptic vesicles (Ye & Lafer 1995). Consistent with this, a mutation that eliminates an AP180 homolog in *Drosophila* results in an increased size of synaptic vesicles (Zhang et al 1998).

***EPS15 and Epsin*** Eps15 binds to the $\alpha$-adaptin subunit of the AP2 adaptor complex (Benmerah et al 1996). Eps15, like its recently identified binding partner epsin (Chen et al 1998, McPherson et al 1998), is concentrated in presynaptic nerve terminals (Chen et al 1998). This localization, together with the findings that both Eps15 (Benmerah et al 1998) and epsin (Chen et al 1998) are involved in receptor-mediated endocytosis in non-neuronal cells, suggests that Eps15 and epsin have a role in synaptic vesicle membrane retrieval from the presynaptic plasma membrane. Specifically, the localization of Eps15 at the rim of clathrin-coated pits in non-neuronal cells (Tebart et al 1996) raises the possibility that Eps15 and epsin are organizers of the boundary of the clathrin coat mediating synaptic vesicle budding and the dynamin ring mediating fission. This possibility is consistent with the recent identification of Ese1, a linker protein binding to both epsin and dynamin (Sengar et al 1999).

***AP3 Adaptor Complex*** Using the cell-free system that reconstitutes the formation of SLMVs from early endosomes (i.e. after internalization of the KT3/VAMP-TAg complex at 15°C), Kelly and colleagues have shown that SLMV biogenesis from this donor membrane requires the AP3 adaptor complex (Faúndez et al 1998), previously characterized by two independent groups (Simpson et al 1996, 1997; Dell'Angelica et al 1997). The membrane recruitment of the AP3 adaptor complex involves the small GTPase ARF1 (Faúndez et al 1997) (see below) and is mediated via AP3 interaction with VAMP-2 (Salem et al 1998).

Interestingly, the AP3-dependent cell-free formation of SLMVs from early endosomes is independent of clathrin and dynamin (Faúndez et al 1997). This observation needs to be reconciled with the finding that a certain subunit of the AP3 adaptor complex (the ubiquitous $\beta$3A as well as the neuron-specific $\beta$3B, originally called $\beta$-NAP) (Newman et al 1995) interacts with the N-terminal domain of the clathrin heavy chain (Dell'Angelica et al 1998).

A role of the AP3 adaptor complex in the formation of SLMVs and synaptic vesicles from early endosomes has not been reported for living PC12 cells and neurons, respectively. Nevertheless, a role of the AP3 adaptor complex in neuronal membrane traffic is suggested by the existence of two brain- and neuroendocrine-

specific isoforms: the $\mu$3 chain originally referred to as p47B and subsequently as $\mu$3B (an isoform of the ubiquitous p47A, also referred to as $\mu$3A) (Pevsner et al 1994) and the $\beta$3B chain.

Although recent findings implicate AP3 in the biosynthetic pathway, several lines of evidence are consistent with a role of AP3 in endocytic traffic. Localization studies have shown that $\beta$3B ($\beta$-NAP) is not only localized to the TGN but also to peripheral endocytic structures (Simpson et al 1996). In addition, another subunit of the AP3 complex, $\sigma$3, was found to colocalize with the transferrin receptor in HeLa cells (Dell'Angelica et al 1997). These results are consistent with an involvement of the AP3 adaptor complex in two pathways, one originating from the TGN and the other from endosomes.

Observations made in the *Drosophila garnet* mutant, which is defective in the $\delta$ subunit of AP3 and exhibits a reduced number of pigment granules in various tissues (Simpson et al 1996), are consistent with the possibility that AP3 is primarily involved in Golgi-endosomal membrane traffic because pigment granules are currently viewed as modified lysosomes (Burkhardt et al 1993, Simpson et al 1996). In addition, one of the two color mutant mice strains having defects in both forms of AP3 (Kantheti et al 1998) shows strong neurological symptoms (Noebels & Sidman 1989). The phenotype cannot be attributable to lysosomal dysfunctions because the other strain, defective only in non-neuronal AP3, do not have those symptoms (Seymour et al 1997). Kelly and colleagues have therefore proposed that the neuronal defects may be caused by alterations in synaptic vesicle membrane traffic through endosomes, implicating AP3 in this process (Faúndez et al 1998).

## Dynamin, Dynamin-Interacting Proteins, and Other GTP-Binding Proteins

A role for GTP hydrolysis in the process of vesicle budding and fission was first described at the level of the TGN (Tooze et al 1990). Subsequently, two lines of related investigation, which eventually merged, led to the demonstration that a GTPase is required for the fission of recycling synaptic vesicles from the presynaptic plasma membrane and that this GTPase is dynamin. One line of investigation showed that GTP is required for vesicle formation not only in the secretory pathway (Rexach & Schekman 1991, Balch 1992, Melancon 1993) but also in endocytic membrane traffic (Carter et al 1993), including SLMV formation (Desnos et al 1995a, Shi et al 1998, Schmidt & Huttner 1998) and synaptic vesicle recycling (Hess et al 1993, Takei et al 1995). The other line of investigation emerged from the realization that not only the small, ras-related GTP-binding proteins (Bourne 1988, Balch 1990, Goud & McCaffrey 1991, Pfeffer 1992) have key roles in membrane traffic, but also the larger GTPases, i.e. conventional (Barr et al 1992, Bomsel & Mostov 1992, Leyte et al 1993) and unconventional (Kehlenbach et al 1994) heterotrimeric G proteins and dynamin (Warnock & Schmid 1996).

***Dynamin*** Dynamin is the most intensely studied GTPase involved in the recycling of synaptic vesicles (for recent reviews, see Vallee & Okamoto 1995, Warnock & Schmid 1996, McNiven 1998, Schmid et al 1998, van der Bliek 1999). In mammals, three different genes give rise to three principle dynamin isoforms, each of which exists in several variants because of alternative splicing (for a review, see Schmid et al 1998). Dynamin I is expressed predominantly in neurons, dynamin II is ubiquitous, and dynamin III is found predominantly in testis. The general structure of dynamin is characterized by an N-terminal GTPase domain, a pleckstrin homology domain (PH domain), a GTPase effector domain (GED), and a C-terminal proline/arginine-rich domain (Warnock & Schmid 1996, Schmid et al 1998). One function of the PH domain is to participate in the binding of dynamin to the lipid bilayer, a process in which phosphoinositides are thought to be important (Salim et al 1996, Tuma et al 1993, Lin et al 1997). The latter binding results in stimulation of dynamin's GTPase activity (Lin et al 1997). The proline/arginine-rich domain mediates dynamin's interaction with its SH3 domain–containing partners, in particular amphiphysin and the SH3P4/P8/P13 family (see below).

On the basis of in vitro data, dynamin was originally thought to act as a mechanochemical enzyme in microtubule organization (Shpetner & Vallee 1989). However, molecular cloning of mammalian dynamin (Obar et al 1990) and the *shibire* gene product (van der Bliek & Meyerowitz 1991, Chen et al 1991) revealed that the two proteins are orthologs, suggesting that dynamin's physiological function is in endocytosis, including synaptic vesicle recycling. Moreover, the location of the *shibire* mutation in or near the GTPase domain (van der Bliek & Meyerowitz 1991, Chen et al 1991) suggests a role of this domain in dynamin's function in endocytosis.

Evidence that dynamin is involved in receptor-mediated endocytosis and that the GTP-binding domain is required for dynamin's activity was obtained by transfecting non-neuronal cells with mutated forms of dynamin in which critical lysine residues required for nucleotide binding were substituted (van der Bliek et al 1993, Herskovits et al 1993). Subsequent detailed morphological analysis following expression of a dominant-negative mutant of the neuronal isoform of dynamin, dynamin I, showed that neither recruitment of receptors into coated pits nor coated pit assembly and invagination are affected, but, rather, coated pits fail to constrict (Damke et al 1994). These observations pointed to the involvement of dynamin in late events of coated vesicle formation, i.e. constriction of the vesicle's neck and membrane fission.

Evidence complementing the observation that the block in synaptic vesicle endocytosis in *shibire* flies is caused by a mutation in dynamin has been obtained for both synaptic vesicle recycling and SLMV formation from the plasma membrane. With respect to synaptic vesicle recycling, a synthetic peptide corresponding to part of the proline/arginine-rich domain of dynamin I, which blocks the binding of dynamin I to amphiphysin, was found to prevent the reformation of synaptic vesicles from the presynaptic plasma membrane upon microinjection into the lamprey giant reticulospinal synapse (Shupliakov et al 1997). Using the perforated

PC12 cell system that reconstitutes the formation of SLMVs from the plasma membrane and its invaginations, and the observation that SLMV formation in this system requires GTP hydrolysis, evidence for a role of dynamin has been obtained (Schmidt et al 1997a, Schmidt & Huttner 1998). First, depletion of dynamin from rat brain cytosol using the SH3 domain of either Grb2 or amphiphysin (see below) markedly reduces SLMV formation. Second, addition of recombinant dynamin I to dynamin-depleted cytosol restores SLMV formation. Third, a dynamin I mutant carrying a *shibire*-like mutation and defective in GTP binding and hydrolysis not only fails to restore SLMV formation when added to dynamin-depeleted cytosol but inhibits the residual SLMV formation observed with such cytosol (Schmidt & Huttner 1998). Fourth, addition of a synthetic peptide corresponding to part of the proline/arginine-rich domain of dynamin I blocks SLMV formation (A Schmidt & WB Huttner, unpublished observation). Consistent with these observations, Kelly and colleagues have reported that SLMV formation is impaired by addition of the proline/arginine-rich region of dynamin to the PC12 cell-free system that reconstitutes SLMV formation from the plasma membrane (Shi et al 1998).

Insight into the mechanism of action of dynamin in endocytosis has been triggered by studies on the in vitro assembly properties of purified recombinant dynamin (Hinshaw & Schmid 1995, Muhlberg et al 1997) and on the morphological appearance of dynamin recruited to the membrane during synaptic vesicle retrieval from the presynaptic plasmalemma (Takei et al 1995, 1996). Remarkably, purified recombinant dynamin molecules self-assemble into rings (Hinshaw & Schmid 1995). This in vitro assembly property of dynamin appears to reflect the in vivo behavior of the protein because dynamin, when recruited to membranes in a frozen GTP state, is organized in spiral-like rings and appears to induce the formation of narrow necks and tubules via which (clathrin-coated) membrane buds are connected with the presynaptic plasmalemma (Takei et al 1995, 1996). These structures are reminiscent of the collars observed at the necks of invaginated pits accumulating at synapses in *shibire* flies.

That dynamin is indeed sufficient to deform the lipid bilayer into narrow tubules and even to vesiculate these tubules has been demonstrated in recent studies using protein-free liposomes rather than biological membranes (Sweitzer & Hinshaw 1998, Takei et al 1998). Specifically, purified recombinant dynamin assembles in helical spirals on liposomes, leading to the formation of elongated tubules (Sweitzer & Hinshaw 1998, Takei et al 1998). Subsequent addition of GTP, but not GTP$\gamma$S, results in the breakdown of the tubules into vesicles (Sweitzer & Hinshaw 1998). The tubulation and vesiculation capacity of dynamin appears to vary depending on the composition of the liposomes used, with liposomes containing a high proportion of the acidic phospholipid phosphatidic acid favoring tubulation (Takei et al 1998) and liposomes consisting of phosphatidylserine (di-oleoyl) allowing vesiculation (Sweitzer & Hinshaw 1998).

On the basis of these data, it was proposed that a conformational change in dynamin induced by GTP hydrolysis, resulting in the constriction of the dynamin

ring, leads to membrane fission (for reviews see, De Camilli et al 1995, Kelly 1995, Warnock & Schmid 1996, Kirchhausen 1998, McNiven 1998). This pinchase model of dynamin action, however, has been a matter of debate (Roos & Kelly 1997) and has very recently been questioned by Schmid and colleagues (Sever et al 1999). Instead of being a force-generating GTPase, dynamin is proposed to act as a molecular switch that recruits, in a manner regulated by the cycle of GTP binding and hydrolysis, an additional essential component of the pinchase machinery to the neck of the nascent endocytic vesicle (Sever et al 1999, Kirchhausen 1999). Below we propose that, in the case of the recycling synaptic vesicle, this component is the lipid modifying enzyme SH3P4.

***Dynamin-Interacting Proteins*** We confine our discussion to the major proteins that bind to the proline/arginine-rich domain of dynamin, amphiphysin and the SH3P4/P8/P13 family. Because SH3P4 has very recently been found to possess lysophospatidic acid acyl transferase activity, the latter protein family will be discussed under Lipid Modifying Enzymes.

*Amphiphysin* Amphiphysin I, the first member of the amphiphysin family of proteins to be described, was identified as a brain-specific protein partially associated with synaptic vesicles (Lichte et al 1992). The second member of the family, amphiphysin II, was cloned more recently (Sparks et al 1996, Butler et al 1997, Leprince et al 1997, Tsutsui et al 1997, Wigge et al 1997a, Ramjaun et al 1997), shares a similar distribution to that of amphiphysin I, and is highly concentrated in nerve terminals (Wigge et al 1997a, Ramjaun et al 1997). Both isoforms are expressed as ubiquitous splicing variants (Wigge & McMahon 1998). The interaction between the SH3 domain of amphiphysin and the proline/arginine-rich domain of dynamin has been primarily characterized in vitro (David et al 1996), and the consensus sequence within the proline/arginine-rich domain of dynamin responsible for the binding of amphiphysin's SH3 domain has been identified (Grabs et al 1997). Amphiphysin has been implicated in the membrane recruitment of dynamin to clathrin-coated pits (Grabs et al 1997, Wigge et al 1997a, Wigge & McMahon 1998).

Perturbation of the interaction between dynamin and amphiphysin has demonstrated the involvement of the dynamin/amphiphysin complex in clathrin-mediated endocytosis. Microinjection into the lamprey giant reticulospinal synapse of either the SH3 domain of amphiphysin or a peptide containing the amphiphysin-binding site of dynamin blocks endocytosis of synaptic vesicles, which results in the massive accumulation of clathrin-coated pits at the presynaptic plasma membrane (Shupliakov et al 1997). Similarily, overexpression in COS cells of the SH3 domain, specifically of amphiphysin, blocks clathrin-mediated endocytosis (Wigge et al 1997b).

Amphiphysin not only interacts with dynamin but also with the $\alpha$-adaptin subunit of AP2 (David et al 1996; Wigge et al 1997a,b; Leprince et al 1997) and the

lipid modifying enzyme synaptojanin (McPherson et al 1996). Its dual interaction with the AP2 adaptor complex and with dynamin raises the possibility that amphiphysin, like Eps15 and epsin (see above), is involved in the coordination of the interplay of the clathrin coat mediating synaptic vesicle budding and the dynamin ring mediating fission. The interaction of amphiphysin with synaptojanin, an inositol phosphatase, further suggests an important role of phosphoinositides in this interplay.

***Other GTP-Binding Proteins*** Heterotrimeric G proteins are associated with synaptic vesicles (Ahnert-Hilger et al 1994), but the functional significance of this association remains to be established. A potential link between heterotrimeric G proteins and synaptic vesicle formation may be the molecular interaction of $\beta\gamma$ subunits with dynamin, which modulates its GTPase activity (Lin & Gilman 1996, Liu et al 1997). Two types of small GTP-binding proteins are of relevance for synaptic vesicle biogenesis, the ADP ribosylation factor (ARF) and rab proteins.

ARF: SLMV biogenesis from early endosomes (Faúndez et al 1997, 1998), but apparently not that from the plasma membrane (Shi et al 1998), is stimulated by ARF1 and is inhibited by the fungal metabolite brefeldin A, which blocks guanine nucleotide exchange on ARF1 (Donaldson et al 1992, Helms & Rothman 1992). The role of ARF1 appears to be in the membrane recruitment of the AP3 adaptor complex (Faúndez et al 1998). It remains to be investigated whether ARF affects SLMV and synaptic vesicle biogenesis also via activation of phospholipase D and the resulting generation of phosphatidic acid with its consequences on phosphoinositide metabolism—a cascade of reactions thought to be involved in the formation of other types of vesicles (Kahn et al 1993, Ktistakis et al 1996, MA West et al 1997, Chen et al 1997, Tüscher et al 1997).

Rab Proteins: Two rab proteins have been found to be associated with synaptic vesicles, rab3 (Fischer von Mollard et al 1990, 1994a) and rab5 (Fischer von Mollard et al 1994b). While rab3A has been implicated in synaptic vesicle exocytosis (Fisher von Mollard et al 1991, Geppert & Südhof 1998), rab5 may be involved in the endocytosis of the synaptic vesicle membrane. As is the case in non-neuronal cells (Chavrier et al 1990, Bucci et al 1992), rab5 is associated with early endosomes and regulates membrane traffic involving somatodendritic and axonal early endosomes (de Hoop et al 1994). Furthermore, the neuronal growth-associated protein GAP-43 interacts with the rab5 effector rabaptin-5 (Stenmark et al 1995) and participates in endocytosis (Neve et al 1998).

## Actin and Actin-Binding Proteins

Studies in yeast have pointed to a crucial role of the actin cytoskeleton in endocytosis (Kübler & Riezman 1993, Munn et al 1995). In mammals, there is also increasing evidence for the involvement of the actin system in clathrin-mediated

endocytosis in non-neuronal cells (Gottlieb et al 1993, Durrbach et al 1996). Several observations suggest a role of actin/actin-binding proteins with respect to synaptic vesicle recycling. Amphiphysin exhibits homology to the yeast protein Rvs167, which regulates the cortical actin cytoskeleton and is involved in endocytosis (Munn et al 1995). A similar potential link is suggested by the sac1 homology of synaptojanin, which is discussed below (for a discussion on actin cytoskeleton and synaptic vesicle recycling, see (Cremona & De Camilli 1997).

In addition, from studies using the perforated PC12 cell system that reconstitutes SLMV formation from the plasma membrane and invaginations, the potential role of profilins in SLMV biogenesis has been suggested (Schmidt & Huttner 1998). First, the neuron-specific profilin, profilin II, interacts with dynamin (Witke et al 1998). Second, there is increasing evidence for a role of phosphoinositides in synaptic vesicle recycling (De Camilli et al 1996, McPherson et al 1996, Schmidt & Huttner 1998), which raises the possibility that proteins of the actin system known to bind phosphoinositides, such as profilin, are involved in this process. Indeed, recombinant profilin II was found to promote SLMV formation (Schmidt & Huttner 1998).

Obvious proteins to be considered in this context are the synapsins, which are actin-binding, synaptic vesicle-associated proteins (De Camilli et al 1990, Greengard et al 1993). However, as far as we are aware, the evidence available to date indicates their role is in the regulation of the reserve pool of synaptic vesicles rather than in the retrieval of the synaptic vesicle membrane from plasmalemmal and endosomal donor membranes (Ryan et al 1996).

## Lipid-Modifying Enzymes

The dissection of the cytoplasmic machinery involved in the (re-)formation of synaptic vesicles and SLMVs has revealed that two of the major components are enzymes modifying the levels of acidic membrane phospholipids, i.e. phosphatidic acid and phosphoinositides. A role for inositol phospholipids in the process of vesicle budding and fission was first described at the level of the TGN (Ohashi et al 1995). The identification of synaptojanin as an inositol-5-phosphatase then suggested a link between phosphoinositides and synaptic vesicle recycling (McPherson et al 1996, De Camilli et al 1996). The search for binding partners of synaptojanin in turn led to the identification of the previously described SH3P4/P8/P13 family (Sparks et al 1996, Giachino et al 1997) as dynamin- and synaptojanin-binding proteins (Ringstad et al 1997, De Heuvel et al 1997), of which SH3P4 has recently been shown to convert lysophosphatidic acid into phosphatidic acid (Schmidt et al 1999b).

***Synaptojanin*** Synaptojanin, specifically the 145-kDa splice variant of synaptojanin 1, is an amphiphysin-binding protein enriched in nerve terminals (McPherson et al 1994a,b, 1996; David et al 1996). Synaptojanin exhibits inositol-5-phosphatase activity and can use both soluble inositol polyphosphates and membrane

phosphoinositides as substrate (McPherson et al 1996, De Camilli et al 1996). Because of these properties, synaptojanin may affect $PIP_2$ levels in the synaptic vesicle membrane during its retrieval from the presynaptic plasma membrane, and thereby regulate the membrane recruitment of dynamin and other proteins potentially involved in synaptic vesicle recycling such as phosphoinositide-binding proteins of the actin system (De Camilli et al 1996, Cremona & De Camilli 1997). Consistent with the latter possibility, synaptojanin contains an N-terminal domain homologous to the yeast protein sac1 (McPherson et al 1996), which has been implicated in both phospholipid metabolism and the function of the actin-based cytoskeleton (Cleves et al 1989, Novick et al 1989).

Functional data showing that synaptojanin has a role in synaptic vesicle recycling remain to be reported. However, it is interesting to note that yeast contains three synaptojanin-like genes (*SJL1, SJL2,* and *SJL3*) which, though not essential for growth, are involved in receptor-mediated and fluid phase endocytosis. Double mutants lacking *SJL1* and *SJL2* are severely impaired in this process in addition to exhibiting actin and cell polarity defects (Singer-Kruger et al 1998).

***SH3P4*** Originally identified in a search for novel SH3 domain–containing proteins (Sparks et al 1996, Giachino et al 1997) the SH3p4/SH3p8/SH3p13 protein family (also referred to as endophilins 1, 2, and 3, respectively) (Micheva et al 1997, Schmid et al 1998) was suggested to have a role in endocytosis when it was found that these proteins, via their highly conserved C-terminal SH3 domain, interact not only with the proline/arginine-rich domain of synaptojanin but also with that of dynamin (Ringstad et al 1997, De Heuvel et al 1997). Interestingly, the tissue distribution of the three members of this family shows a remarkable match to that of the three mammalian dynamins, with SH3P4 being predominantly expressed in brain, SH3P8 being expressed in all tissues, and SH3P13 being most abundant in testis (Giachino et al 1997, Ringstad et al 1997). The co-expression of the SH3p4/SH3p8/SH3p13 family members and the dynamins points to an intimate functional link and, in the specific case of synaptic vesicle recycling, raises the possibility that SH3P4 cooperates with dynamin I in fission.

Two converging lines of investigation recently demonstrated that SH3P4 functions in endocytosis and provided insight as to its mechanism of action (Schmidt et al 1999b). Using the perforated PC12 cell system that reconstitutes the formation of SLMVs from the plasma membrane and from invaginations, SH3P4 was found to be a rate-limiting component of the cytoplasmic machinery mediating this vesicle budding and fission process (Schmidt & Huttner 1998). Independently, the purification from brain of a lysophosphatidate acyltransferase (LPAT) activity by Söling and colleagues, which originated from the observation that this enzyme activity is increased in response to stimulation of regulated secretion (Söling et al 1987, 1989a,b), revealed its identity as SH3P4.

In collaboration with Söling and colleagues, we have found that the stimulation of SLMV formation by recombinant SH3P4 indeed involves the conversion of lysophosphatidic acid to a phosphatidic acid that bears or contains arachidonate in

position 2 of the glycerol backbone (Schmidt et al 1999b). Remarkably, a deletion mutant of SH3P4 lacking the SH3 domain, though still exhibiting LPAT activity, no longer promotes SLMV formation (Schmidt et al 1999b). Since the SH3 domain mediates the binding of SH3P4 to dynamin, we propose that the LPAT activity of SH3P4 needs to be targeted, via interaction with dynamin, to the relevant membrane site in order to promote fission, i.e. the neck of the nascent synaptic vesicle. This proposition is in line with that of Schmid and colleagues (Sever et al 1999) concerning the role of dynamin as a molecular switch rather than a pinchase on its own and implies that the pinchase function results from the concerted action of dynamin and SH3P4 (Schmidt et al 1999b). The exact mechanism of how the LPAT activity of SH3P4 at the neck of the nascent synaptic vesicle stimulates its pinching-off from the presynaptic plasma membrane remains to be established. An appealing possibility is that the conversion of lysophosphatidate, an inverted cone-shaped lipid, to phosphatidic acid, a cone-shaped lipid, affects the curvature of the cytoplasmic membrane leaflet such that fission is promoted (Schmidt et al 1999b).

***General Considerations on The Roles of Lipids in Synaptic Vesicles and SLMV Biogenesis*** The observations that the cytoplasmic machinery mediating synaptic vesicle and SLMV (re-) formation includes lipid-modifying enzymes (SH3P4, phosphatidylinositol transfer protein, synaptojanin) (Schmidt et al 1998) raises the general question about the roles membrane lipids play in this membrane traffic step. In this context, it is interesting to note that the lipid-modifying enzymes include both enzymes affecting the polar head groups (synaptojanin) and those affecting the hydrophobic backbone (SH3P4) of phospholipids.

As exemplified by the binding of dynamin's PH domain to phosphoinositides, certain membrane lipids can serve as specific sites for the membrane recruitment of cytoplasmic proteins mediating budding and fission. If so, why have lipids, especially the phosphoinositides, evolved as such recruitment sites in addition to the cytoplasmic domains of transmembrane proteins? Compared to the latter, lipids may offer advantages not only sterically, especially at the neck of the nascent synaptic vesicle, but also in terms of lateral mobility and availability. In the case of dynamin, for example, membrane recruitment to a lipid microdomain that is near, but not necessarily at, the future site of fission would presumably allow for the more rapid redistribution and concentration of dynamin to the neck than would be the case if the recruitment site were the cytoplasmic domain of a transmembrane protein. A corollary of the lateral movement of lipid-bound dynamin to the forming neck would be the clustering of the dynamin-bound lipids, which may promote membrane constriction and fission. In addition, in the kiss-and-run, fission only type of membrane retrieval, the very site of bilayer fusion of the synaptic vesicle and the presynaptic plasma membrane may be largely devoid of transmembrane proteins except for the transmembrane domains of the SNAREs, and lipids may be the most available and easily accessible sites for the recruitment of the fission machinery.

As discussed above in the context of the LPAT activity of SH3P4, addition of an acyl chain to a lyso-phospholipid may have profound effects on membrane curvature. Obviously, the same holds true for the reverse reaction, i.e. the conversion of a phospholipid to a lyso-phospholipid by phospholipase A2. Perhaps, the inhibition of neurotransmitter release resulting from the block of synaptic vesicle recycling, which is caused by certain snake neurotoxins exhibiting phospholipase A2 activity (Verheij et al 1981), reflects a shift in the balance between cone-shaped and inverted cone-shaped lipids that is incompatible with budding and/or fission.

It should be noted that the curvature-inducing properties of membrane lipids can be changed not only by modification of the hydrophobic backbone but also by that of the polar head group (Düzgünes 1995). The inositol 5-phosphatase activity of synaptojanin presumably results in too subtle a change in this regard. However, it is conceivable that enzymes such as phospholipase C, which converts $PIP_2$ to diacylglycerol, affect synaptic vesicle recycling not only via production of soluble inositol polyphosphates, which can act as modulators of the clathrin coat budding machinery (Beck & Keen 1991, Ye et al 1995, Hao et al 1997b) and its recruitment to the membrane (Fukuda et al 1995), but also via an effect on membrane curvature.

## Regulation by Protein Phosphorylation-Dephosphorylation

The seminal work of Greengard and colleagues has shown that protein phosphorylation-dephosphorylation is a major mechanism of cellular regulation, in particular at synapses (Greengard et al 1993). In fact, the study on depolarization-induced changes in protein phosphorylation-dephosphorylation in synaptosomes by Krüger et al (1977) constitutes the first description of the protein, originally called dephosphin, that was later isolated and named dynamin (Shpetner & Vallee 1989).

In line with this general notion, most of the proteins involved in synaptic vesicle (re-)formation are regulated by phosphorylation-dephosphorylation. The proteins dephosphorylated in response to nerve terminal depolarization, which results in an increase in both exocytosis and endocytosis (for reviews, see Robinson et al 1994, Wigge & McMahon 1998), include dynamin I (JP Liu et al 1994), amphiphysins I and II (Bauerfeind et al 1997, Wigge et al 1997a, Marks et al 1998), synaptojanin (McPherson et al 1994b), and Epsin and its binding partner Eps-15 (Chen et al 1999).

Dephosphorylation (at least in the case of dynamin) was shown to be mediated by calcineurin, a calcium- and calmodulin-dependent phosphatase enriched at the synapse (JP Liu et al 1994). Upon repolarization of the nerve terminal, these proteins are rephosphorylated in a process that is mediated by protein kinase C (Robinson et al 1993, Wigge et al 1997a). Phosphorylation increases and dephosphorylation decreases the GTPase activity of dynamin, consistent with dynamin-GTP promoting synaptic vesicle endocytosis (Robinson et al 1993).

Moreover, phosphorylation of the proline/arginine-rich domain of dynamin decreases its interaction with amphiphysin and the $\alpha$ and $\beta$ subunits of the AP2 adaptor complex (Slepnev et al 1998).

Epsin and its binding partner Eps-15, which are thought to be involved in the rearrangement of the clathrin coat during the budding/fission process, undergo stimulation-dependent dephosphorylation in nerve terminals, which enhances their interaction with AP2 (Chen et al 1999). Together, these observations indicate that the assembly of the endocytic machinery is favored by the dephosphorylated state of the above proteins, which is induced by nerve terminal depolarization. On the other hand, nerve terminal depolarization also results in the phosphorylation of certain proteins (Robinson & Dunkley 1983). Interestingly, phosphorylation of SH3P4 increases its LPAT activity (Schmidt et al 1999b), consistent with the increase in synaptic vesicle (re-)formation upon nerve terminal stimulation.

## CONCLUSION

Substantial progress has been made toward understanding the trafficking of synaptic vesicle proteins, the formation of synaptic vesicles from their donor membranes at the synapse, and the cytoplasmic machineries involved. This insight raises additional questions: What changes occur in the trafficking of synaptic vesicle proteins during the transition from a growth cone to a synapse, and how are these changes regulated? What is the contribution of secretory granule membrane constituents, both proteins and lipids, to synaptic vesicle biogenesis, and how does this vary between developing and mature neurons and between those that are neuropeptide rich and poor? What is the significance of the specific lipid composition of synaptic vesicles for biogenesis, and are lipid-protein interactions relevant in this regard? What are the exact roles of covalent modification of membrane lipids in synaptic vesicle budding and fission, and do such reactions also operate in the formation of other membrane vesicles of eukaryotic cells? And finally, is the variation in the membrane protein composition of individual synaptic vesicles of a given neuron a means of synaptic plasticity?



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