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Protein sorting in the synaptic vesicle life cycle

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Abstract

At early stages of differentiation neurons already contain many of the components necessary for synaptic transmission. However, in order to establish fully functional synapses, both the pre- and postsynaptic partners must undergo a process of maturation. At the presynaptic level, synaptic vesicles (SVs) must acquire the highly specialized complement of proteins, which make them competent for efficient neurotransmitter release. Although several of these proteins have been characterized and linked to precise functions in the regulation of the SV life cycle, a systematic and unifying view of the mechanisms underlying selective protein sorting during SV biogenesis remains elusive. Since SV components do not share common sorting motifs, their targeting to SVs likely relies on a complex network of protein–protein and protein–lipid interactions, as well as on post-translational modifications.

Pleiomorphic carriers containing SV proteins travel and recycle along the axon in developing neurons. Nevertheless, SV components appear to eventually undertake separate trafficking routes including recycling through the neuronal endomembrane system and the plasmalemma. Importantly, SV biogenesis does not appear to be limited to a precise stage during neuronal differentiation, but it rather continues throughout the entire neuronal lifespan and within synapses. At nerve terminals, remodeling of the SV membrane results from the use of alternative exocytotic pathways and possible passage through as yet poorly characterized vacuolar/endosomal compartments. As a result of both processes, SVs with heterogeneous molecular make-up, and hence displaying variable competence for exocytosis, may be generated and coexist within the same nerve terminal.

Keywords: Neurotransmission; Exo–endocytosis; Membrane traffic; Secretory pathway; Synaptic vesicle pools; Synaptic-like microvesicles; PC12 cells; Adaptor protein complexes; Phosphorylation

Contents

Abbreviations: AP−, adaptor protein complex−; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; ARF−, ADP ribosylation factor−; AMPA, α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid; APP, amyloid precursor protein; BFA, brefeldin A; PKA, cAMP-dependent protein kinase; DAG, diacylglycerol; ER, endoplasmic reticulum; GAD, glutamic acid decarboxylase; GFP, green fluorescent protein; GAP, GTPase activating protein; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; GTP, guanosine-5′-triphosphate; HRP, horseradish peroxidase; LDCV, large dense core vesicle; MAPK, mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; PI, phosphoinositide; PH, pleckstrin homology; RRP, ready releasable pool; SH3, Src homology 3; SV2, synaptic vesicle protein 2; SV, synaptic vesicle; SLMV, synaptic-like microvesicle; TGN, trans-Golgi network; VAMP2, vesicle associated membrane protein 2; VGLUT, vesicular glutamate transporter; ZnT-3, zinc transport protein-3

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1. Introduction

Synaptic vesicles (SVs) are composed of a specific set of proteins and lipids and carry small hydrophilic molecules, some of which act as neurotransmitters, and ions in their luminal domain. Many SV proteins have been studied in detail using a variety of experimental approaches and linked to at least putative functions in the regulation of SV exo–endocytosis (Südhof, 2004). These studies, together with the physiological description of SV trafficking in the presynaptic terminal, make the SV one of the best characterized organelles of eukaryotic cells. Thus, the SV cycle has served as one of the paradigms for understanding the molecular basis of vesicular traffic in the eukaryotic cell. Three main aspects of the SV life cycle can be distinguished: (a) *de novo* biogenesis of SVs, (b) fusion of SVs with the presynaptic plasma membrane, and (c) recycling of the SV membrane after exocytosis for the re-formation of SVs. While the latter two processes have been extensively studied and are relatively well understood, comparatively little is known about the molecular events underlying the *de novo* biogenesis of SVs.

In the past several years, protein sorting has been approached mainly by means of biochemical methods. Since the large amount of cells required in these experiments limits the use of primary neuronal cultures, neuroendocrine cell lines have become the favored model for addressing the biogenesis of SVs. As a result, almost all the present knowledge about the intracellular traffic routes taken by membrane proteins destined to SVs during the biogenesis of these organelles derives from data collected studying the biogenesis of synaptic-like microvesicles (SLMVs) of rat pheochromocytoma-derived cell line PC12 (Fig. 1B).

In spite of the overall similarity in molecular composition and size between SLMVs and neuronal SVs, neuroendocrine cells lack several distinctive features of neurons. Neuroendocrine cells show neither molecular/functional polarization of neurites nor synaptic specializations. In addition, there are no evidences for the organization of SLMVs in functional pools, as observed in the case of SVs clustered at synapses, and it is questioned whether SLMVs undergo the Ca^{2+}-dependent exocytosis upon stimulation which is typical of SVs (Faundez et al., 1997, but see Bauerfeind et al., 1995). These fundamental differences between neuroendocrine cells and neurons may have important implications for the membrane traffic events, which regulate the biogenesis of either SLMVs or SVs.

Moreover, it is becoming clear that any general model of protein targeting during SV biogenesis is likely to represent an oversimplification of the real situation and that the trafficking routes undertaken by each component should be addressed individually. This is particularly challenging in the case of neurons, as neuronal membrane proteins are subjected to multiple sorting steps during their trafficking: they are sorted to

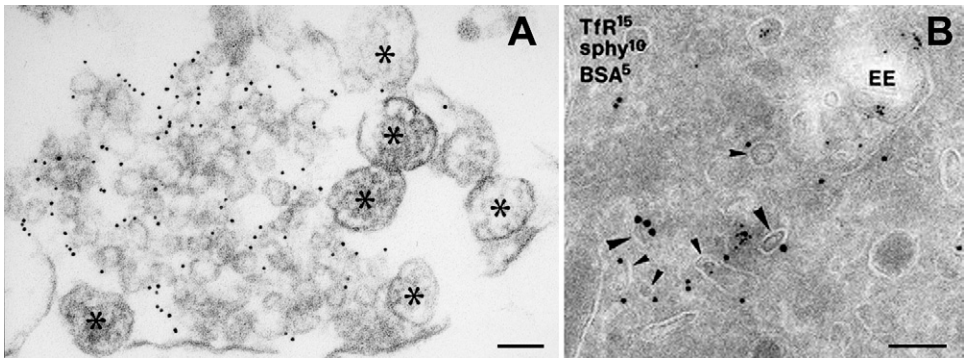

Fig. 1. Selective sorting of SV proteins in neuroendocrine cells. (A) Immunogold labeling for synaptophysin in lysed nerve endings of the neurohypophysis. Synaptophysin is specifically associated with microvesicles while neurosecretory granules (asterisks) are unlabeled. Bar, 100 nm. Reproduced with permission from (Navone et al., 1989) (Copyright 1989, The Rockefeller University Press, NY). (B) SLMV proteins segregate from non-SLMV proteins in early endosomes. Double immunogold labeling for transferrin receptor (TfR, 15-nm gold) and synaptophysin (sphy, 10-nm gold) of ultrathin cryosections of PC12 cells incubated with 5-nm BSA-gold for 40 min at 15 °C to mark early endosomes (EEs) and rewarmed to 37 °C for 5 min to accumulate proteins at the EE exits. Most EE-associated tubules and vesicles contained either transferrin receptor (large arrowheads) or synaptophysin (small arrowheads). Bars, 100 nm. Reproduced with permission from (de Wit et al., 1999) (Copyright 1999, The American Society for Cell Biology, Bethesda, MD).

the axonal or somatodendritic compartment and within the same compartment they may be incorporated into different organelles. Nevertheless, the advent of green fluorescent protein (GFP)-based technology and new optical methods has boosted the field of protein trafficking allowing real-time monitoring of the sorting and transport of individual membrane components in living cells. The impact of the new approaches on the study of SV biogenesis has been terrific: previous models derived from the study of PC12 cells are now corroborated in neurons and new concepts are coming forward.

2. Molecular aspects of synaptic vesicle biogenesis

2.1. Formation of synaptic-like microvesicles from early endosomes

The endocytotic origin of SLMVs has been known for some time. SLMVs in PC12 cells take up the exogenous phase marker horseradish peroxidase (HRP) and, following surface labeling and subsequent warming, labeled proteins become incorporated into SLMVs (Clift-O'Grady et al., 1990; Bauerfeind et al., 1993). Nevertheless, synaptophysin, a major membrane protein of SVs (Wiedenmann and Franke, 1985; Jahn et al., 1985) and SLMVs (Fig. 1A; Navone et al., 1986), shows the intrinsic tendency to accumulate in transferrin receptor-containing endosomes in both PC12 cells and transfected fibroblasts (Johnston et al., 1989; Cameron et al., 1991). Thus, SLMVs biogenesis proceeds from either the plasma membrane or recycling endosomes, or both.

It is important to keep in mind that in either case the formation of SLMVs implies a sorting step that segregates the SLMV membrane proteins from the normal residents of the plasma membrane or endosomes (Fig. 1B).

Several lines of evidence point to the involvement of an intracellular compartment, most likely an endosomal intermediate, in the formation of SLMVs. The fluid phase marker HRP is detected in transferrin receptor-positive endosomes after a pulse internalization (5 min) and short chase (7 min), but appears in SLMVs only after a longer chase (3 h; Bauerfeind et al., 1993). This indicates that SLMVs can be generated by budding from endosomal compartments, albeit in a process requiring >7 min.

Pulse-chase labeling was used by Regnier-Vigouroux et al. (1991) to investigate the exit of newly synthesized synaptophysin from the trans-Golgi network (TGN) and its transport to SLMVs. After synthesis in the rough endoplasmic reticulum and passage through the Golgi complex, synaptophysin moves from the TGN to the plasma membrane in constitutive secretory vesicles, subsequently cycles several times between the plasma membrane and endosomes and eventually segregates from resident proteins prior to being incorporated into SLMVs budded from endosomes. It is unclear whether, in addition to synaptophysin, other SLMV components undertake this routing pathway and to what extent this model can be applied to SV biogenesis in neurons. However, this seminal study contributed to build up a framework to envisage the routes of SV protein trafficking.

The usefulness of PC12 cells as a model system was furthered by the development of a cell-free system for SLMV biogenesis (Desnos et al., 1995). In this assay, PC12 cells placed at 15 °C accumulate internalized antibodies against an epitope-tagged form of synaptobrevin II/VAMP2, an integral protein of both SVs and SLMVs. Upon re-warming to 37 °C, the internalized antibodies are delivered to SLMVs, suggesting that an intracellular intermediate functions as SLMV donor compartment. SLMV formation *in vitro* is time and temperature-dependent, does not require Ca^{2+} but needs ATP and GTP hydrolysis (the dependency on GTP hydrolysis was also reported for the generation of endocytotic vesicles from the plasma membrane, Takei et al., 1995). Importantly, cytosolic extracts from brain, but not fibroblasts, allow vesicles of the correct size to form, implicating a neuro-specific factor in the budding of new SLMVs from the 15 °C donor compartment, a process which has been shown to require also the cytosolic proteins ADP ribosylation factor-1 (ARF1) and adaptor protein complex AP3 (Faundez et al., 1997, 1998), but not clathrin

(Faundez et al., 1998; Shi et al., 1998). ARF1 and AP3 mediate budding from an endosomal precursor, which contains internalized transferrin and this process occurs concomitantly with the sorting of SV proteins from other membrane protein constituents of the endosome (Lichtenstein et al., 1998).

Another member of the ARF GTPase family, ARF6, which is highly expressed in the brain and in neuroendocrine cells, and localized at both plasma membrane and endosomes, has also been implicated in SLMV biogenesis. The expression of ARF6 mutants in PC12 cells affects the selective targeting of proteins to SLMVs (Powelka and Buckley, 2001). It remains to be established whether these effects reflect the involvement of ARF6 in vesicle formation or rather the interference of the exogenous proteins with the ARF1-mediated pathway.

A direct morphological evidence for a pathway in which SLMV membrane proteins recycle from the plasma membrane to endosomes before their incorporation into newly formed SLMVs was provided by de Wit et al. (1999). Quantitative immunoelectron microscopy was used to show that transferrin receptor and SLMV proteins, such as synaptophysin and VAMP2, exhibit a high degree of colocalization in incoming endocytic vesicles, yet they are largely separated after transit through early endosomes (i.e., at the level of early endosome-associated tubulovesicles which represent the domains involved in protein recycling; Klumperman et al., 1993) (Fig. 1B). Thus, it appears that after endocytosis from the plasma membrane SLMV proteins are sorted away from non-SLMV proteins at the level of the vacuolar component of early endosomes and become selectively enriched in the tubular extensions which function as donor compartments for the budding of new SLMVs.

A primary endocytic vesicle involved in trafficking of SV proteins has been identified as part of the endocytic recycling system in neurons and PC12 (Provoda et al., 2000). In PC12 cells, this vesicular compartment contains both SV proteins (synaptophysin, synaptotagmin, SV2) and other recycling proteins (transferrin receptor, glucose transporters). Rather than representing a distinct type of regulated secretory organelles, this vesicle population is likely to correspond to the incoming endocytic vesicles visualized by de Wit et al. (1999), in which SLMV-specific antigens and other recycling proteins are not yet segregated (but see Thoidis et al., 1998).

Endosomal sorting has also been implicated in the generation of different secretory vesicles in the nerve terminal of noradrenergic neurons, namely SVs, which contain acetylcholine and large dense-core vesicles, which contain noradrenaline. The constituents of both types of vesicles are internalized and recycled through a common early endosomal compartment after exocytosis (Partoens et al., 1998). This suggests that *in vivo* the post-exocytic trafficking and, in particular, the ability of the cell to separate different pools of membrane proteins is of vital importance. This concept has been further strengthened by the findings obtained by Cutler's group as to the targeting of exogenous P-selectin in PC12 cells (Strasser et al., 1999). P-selectin is a transmembrane protein originally found in the secretory granules of endothelial cells and platelets that functions as a receptor for leukocytes. After secretagogue-stimulation P-selectin associated with dense-core granules appears on the plasma membrane and then passes through transferrin receptor-positive endosomes en route to SLMVs. Secretagogue-triggered transfer between the two classes of organelles is also observed for VAMP2 and synaptotagmin. Passage through an endosomal sorting compartment might be needed in order to effectively separate proteins destined to SLMVs from proteins that are only to be found in dense-core granules and therefore are en route back to the TGN where this class of organelles is generated (Tooze and Stinchcombe, 1992).

2.2. Formation of synaptic-like microvesicles from the plasma membrane

An alternative pathway of SLMV biogenesis which is AP2, dynamin and clathrin dependent was first described by Schmidt et al. (1997), who proposed the participation of a novel compartment distinct from the transferrin receptor-containing endosome and connected to the plasma membrane via narrow membrane continuity. The key observation, which provided the basis for the identification and characterization of the plasmalemma-associated SLMV donor compartment, was that upon cell surface biotinylation at 18 °C, a temperature which blocks the appearance of synaptophysin in SLMVs, all of the biotinylated synaptophysin is present in avidin-protected membrane, the majority of which is accessible to a reducing agent, and hence in continuity with the plasma membrane. The subplasmalemmal tubulo-cisternal compartment implicated in SLMV targeting of synaptophysin does not contain detectable levels of biotinylated transferrin receptor, indicating that the two proteins segregate at the plasma membrane. From the subplasmalemmal compartment, a minor proportion of synaptophysin (10–15%) is directly incorporated into SLMVs, whereas the majority of the protein is delivered to early endosomes, from which is then recycled back to the plasma membrane. At variance, a careful electron microscopy analysis of the compartments devoted to SLMV protein sorting in PC12 cells did not reveal any connection between early endosomes and the plasma membrane (de Wit et al., 1999). Nevertheless, a plasma membrane-derived pathway of SLMV biogenesis in PC12 cells was independently confirmed using an *in vitro* assay based on tracking of a lumenally tagged VAMP2 derivative labeled with a specific antibody at the cell surface (Shi et al., 1998). Unlike the biogenesis of SLMVs from PC12 endosomes, the formation of plasma membrane-derived SLMVs uses the adaptor protein AP2 instead of AP3 and requires clathrin but not GTP hydrolysis by ARF1.

SLMVs formed *in vitro* from the plasma membrane did not contain the endosomal marker transferrin receptor, arguing in favor of two separate pathways of endocytosis, one exclusive for internalization of SLMV proteins and the other that carries both SLMV components and transferrin receptor (Schmidt et al., 1997; Shi et al., 1998). Although these findings contrast with the evidences of extensive colocalization between SLMV proteins and transferrin receptor in primary endocytic vesicles as shown by de Wit et al. (1999), even in the latter study the

colocalization between different SLMV components was slightly higher than the colocalization between SLMV proteins and transferrin receptor, indirectly suggesting that a partial sorting of SLMV proteins from non-SLMV proteins may take place at the plasma membrane.

Interestingly, while targeting of P-selectin to SLMVs is inhibited by brefeldin A (BFA), which inhibits ARF protein function, implying the requirement for an endosomal intermediate, SLMV targeting of synaptophysin is not affected by the drug (Blagoveshchenskaya et al., 1999b), indicating that synaptophysin may be delivered to SLMVs directly from the plasma membrane, in agreement with what reported by Schmidt et al. (1997). Thus, different SLMV proteins may preferentially use one of the two alternative pathways to SLMVs, perhaps depending on the presence of as yet unidentified targeting signals.

2.3. Two alternative pathways for synaptic vesicle biogenesis

Incubation of purified SVs under conditions which favor their coating while preventing their uncoating has revealed that ARF1 and the AP3 complex are the only cytoplasmic components required for SV formation from endosomes (Desnos et al., 1995; Faundez et al., 1998). The AP3 coat complex is composed by four subunits with different isoforms, two of which (β3B and μ3B) are neuro-specific (Newman et al., 1995). Only the neuronal form of AP3 can produce SVs from endosomes *in vitro* (Blumstein et al., 2001). In addition, the neuro-specific subunits of AP3 are selectively phosphorylated by a casein kinase Iα-like isoform, and preventing this phosphorylation impairs coat assembly and inhibits the formation of SLMVs in PC12 cells (Faundez and Kelly, 2000). These data, in addition to the observation that liver and yeast cytosol could not replace brain cytosol in the reconstitution of vesicle budding from endosomes (Faundez et al., 1998), suggest that SV formation from this compartment may be a function exclusive to neuronal AP3.

Proteomic analysis has been recently carried out to determine the composition of AP3-positive organelles purified from PC12 cells (Salazar et al., 2005b). Several classes of proteins, including SV proteins, cytoskeletal and lysosomal proteins were present on AP3 organelles. Interestingly, phosphatidylinositol-4-kinase type IIα (PI4KIIα), previously implicated in vesicle biogenesis and function (Wenk and De Camilli, 2004), was also found to be enriched in AP3-positive organelles. PI4KIIα displays AP3-dependent targeting to this class of organelles and in turn regulates membrane recruitment and function of the AP3 complex.

Despite the relevance of the AP3-mediated pathway in protein sorting to SLMVs, the *mocha* mouse, which lacks functional AP3 in all tissues, shows a normal SV population in hippocampal mossy fiber nerve terminals and only subtle neurological deficits, including balance and hearing problems, hyperactivity, seizures susceptibility and abnormalities in theta rhythms. At variance, the *mocha* mouse exhibits major defects in melanosome, platelet dense granule and lysosome traffic (Noebels and Sidman, 1989; Kantheti et al., 1998). All these alterations are consistently explained by the missorting of various cargo proteins that are normally delivered to intracellular compartments by the AP3 pathway. These results raise the possibility that the AP3 pathway is not essential for SV formation *in vivo*, and may represent a minor pathway of SV biogenesis, which only operates under certain condition. However, an important physiological role of AP3B in the biogenesis of GABA-releasing SVs is demonstrated by the observation that mice deficient for the neuron-specific subunit μ3B exhibit epileptic seizures and defective inhibitory neurotransmission in the hippocampus. At these synapses, AP3B is specifically required for the sorting of the vesicular GABA transporter VGAT while the expression level and distribution of several other SV proteins remain unchanged (Nakatsu et al., 2004).

On the other hand, considerable evidences indicate that SVs can be formed by a pathway that involves AP2, clathrin, and dynamin (Koenig and Ikeda, 1996; Takei et al., 1996; Cremona and De Camilli, 1997; Shupliakov et al., 1997). Elegant electron microscopic studies have shown that the steps mediated by clathrin and dynamin occur at the plasma membrane, although some budding events may take place also from internalized membranes (Fig. 2B; Takei et al., 1996). The α-adaptin-containing AP2 complex is required for the recruitment to the endocytotic sites of the GTPase dynamin. Consistently, SV recycling is blocked in α-adaptin-deficient *Drosophila* embryos (Gonzalez-Gaitan and Jackle, 1997). The absence of SV retrieval following exocytosis causes depletion of SVs from the nerve terminals and the corresponding expansion of the presynaptic plasmalemma.

Therefore, it appears that neurons use two modes of vesicle formation, one that generates SVs from the plasma membrane using clathrin and dynamin, and a second that uses AP3 and ARF1 to generate SVs from endosomes, even though the severe phenotype observed at synapses of the α-adaptin *Drosophila* mutants compared to the relatively mild neuronal phenotype of *mocha* mice implies that the AP2 pathway is more relevant for SV formation than the AP3 pathway. Interestingly, the AP2 and AP3 pathways appear to exert complementary roles in the recycling of SV proteins such as the vesicular glutamate transporter 1 (VGLUT1), which is internalized through the AP2 pathway during synaptic stimulation and through the AP3 pathway at rest. A competition between the two endocytic routes is suggested by the evidence that preventing VGLUT1 from entering the AP2 pathway during stimulation directs the transporter toward the slower AP3 pathway, while inhibition of the latter route restores the fast kinetics of VGLUT1 retrieval (Voglmaier et al., 2006).

It is noteworthy that neurotransmitter release along developing axons is sensitive to inhibition of ARF1 activity by BFA, whereas quantal release from mature nerve terminals is BFA-insensitive (Zakharenko et al., 1999). Thus, the endosomal route of SV biogenesis appears to predominate at either early stages of differentiation or specific sites of the neuronal cells, while direct retrieval of SV components from the plasma membrane by the AP2/clathrin pathway operates

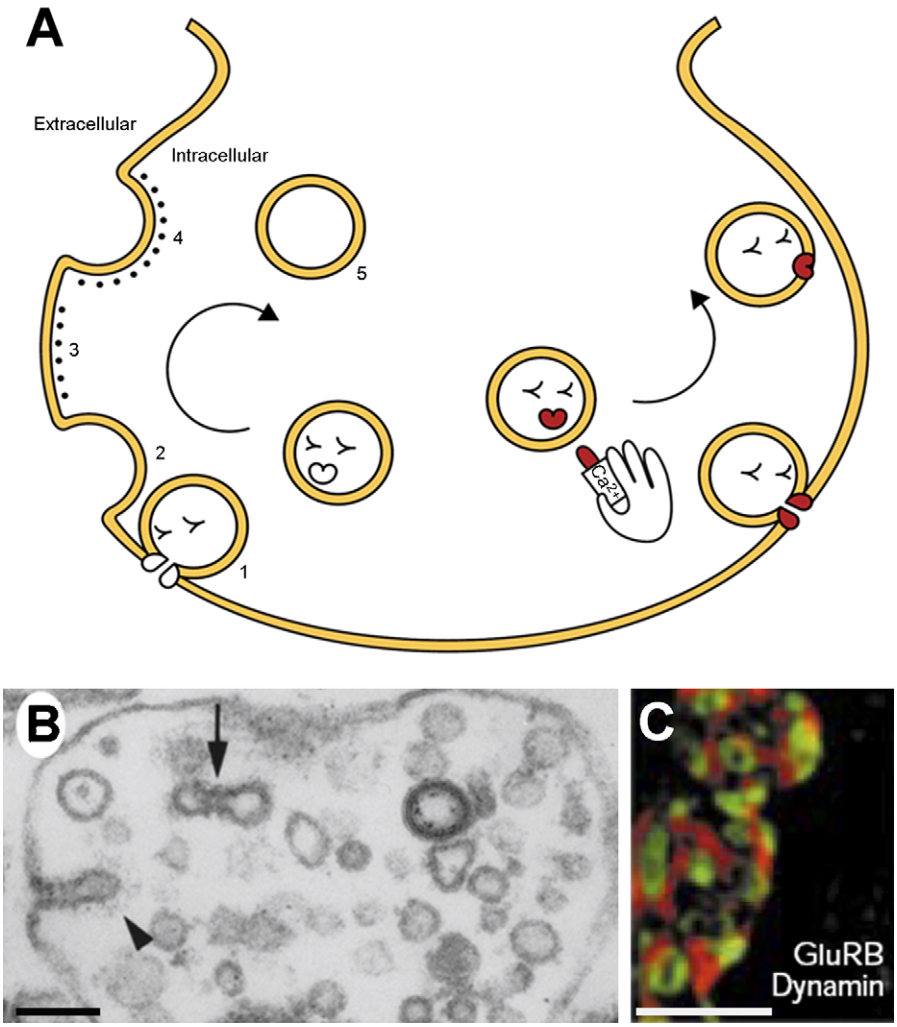

Fig. 2. Modes of SV endocytosis. (A) Neurotransmitter release can occur by two different mechanisms in the nerve terminal. (left) Classical mode. After the opening of a fusion pore, the vesicle undergoes complete fusion with the plasma membrane, accompanied by neurotransmitter release, and is subsequently retrieved through the formation of a coated vesicle. This mechanism takes more than 20 s. (Right) kiss-and-run mode. Opening of a fusion pore is sufficient for discharge of the vesicle content. After closure of the pore vesicles detach from the plasma membrane and are directly reused through a fast (<1 ms) mechanism. Modified from (Fesce and Meldolesi, 1999) (Copyright 1999, Nature Publishing Group, London, UK). (B) Clathrin- and dynamin-dependent budding of SVs from the plasma membrane and endosomal-like structures. Electron micrograph showing the presence of coated buds (arrowhead) originated from either the plasmalemma or internal compartments in nerve terminal membranes incubated with rat brain cytosol, ATP, and GTPγS. Dynamin-like rings (arrow) are located at the neck of a coated bud and around tubular portions of the vacuoles. Bar, 100 nm. Reproduced with permission from (Takei et al., 1996) (Copyright 1996, The Rockefeller University Press, NY). (C) Active zones are surrounded by the machinery for SV endocytosis identified by dynamin staining (red) in resting *Drosophila* neuromuscular junctions. Glutamate receptors (GluRB, green) mark post-synaptic regions juxtaposed to active zones. Bar, 2.5 μm. Reproduced with permission from (Roos and Kelly, 1999) (Copyright 1999, Elsevier, Amsterdam).

during SV recycling at the mature synapse, with only a minor contribution of the AP3/ARF pathway (Murthy and Stevens, 1998). According to this view, the preferential recycling of SLMVs by the AP3 pathway may reflect an intrinsic difference between neurons and neuroendocrine cells, which have not differentiated sufficiently to possess an efficient non-endosomal mechanism.

3. Synaptic vesicle recycling at nerve terminals

3.1. Recycling of synaptic vesicles occurs by endocytosis

SVs are continuously regenerated in the nerve terminals following exocytosis in order to repopulate a pool of vesicles large enough to sustain prolonged synaptic activity. New vesicles need to be endowed with all the molecular components, which make them suited to fulfill the efficient coupling between the arrival of the stimulus and neurotransmitter release.

The existence of a recycling pathway that operates in the nerve terminal was inferred by the evidence that SVs become labeled when their exocytosis is stimulated in the presence of an extracellular tracer (Ceccarelli et al., 1972, 1973; Heuser and Reese, 1973) (Fig. 3). An extended high-frequency stimulation (15 min at 10 Hz) at the frog neuromuscular junction led to the appearance of HRP-filled cisternae/vacuole-like structures which were interpreted as the organelles from which SVs form during a long recovery period (Heuser and Reese, 1973). On the contrary, nerve terminals stimulated extensively at lower

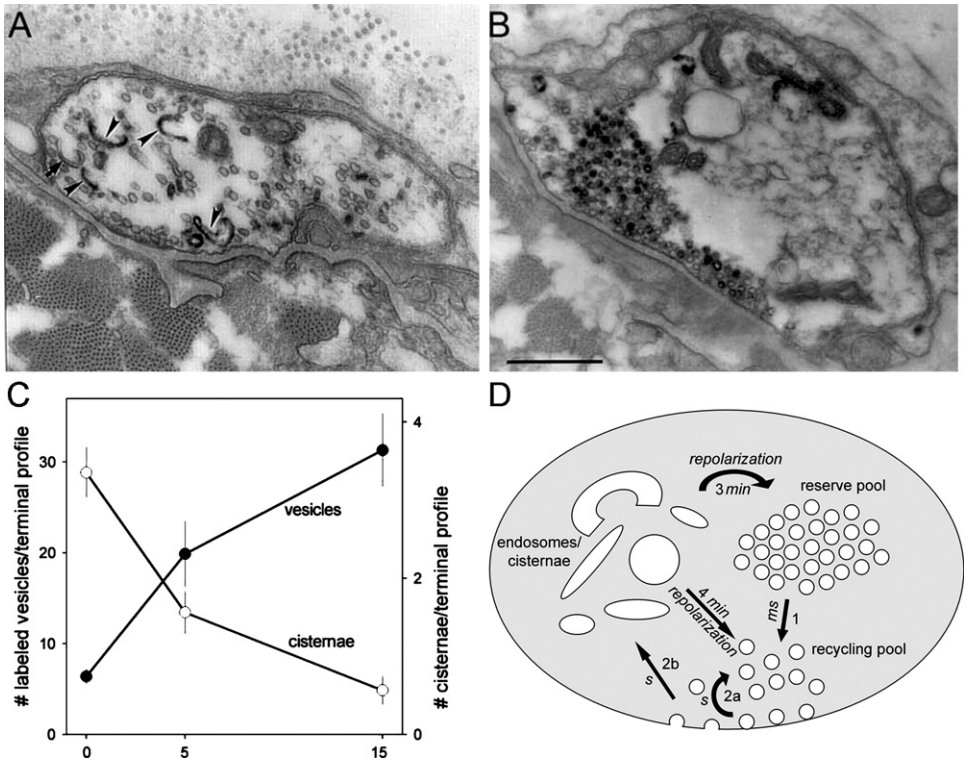

Fig. 3. Pathways of SV recycling. (A–C) New SVs are generated from endosomal-like structures after tetanic stimulation. Frog neuromuscular synapses are stimulated in the presence of FM 1–43 (30 Hz for 1 min) and chased for various times before fixation and dye photoconversion. Immediately after stimulation (A) FM 1–43 is mainly internalized in cisternae and membrane infoldings (arrowheads), whereas 15 min after stimulation (B) cisternae have almost disappeared, and several SVs become labeled. The arrow in A points to an unlabeled cisterna. (C) The appearance of labeled vesicles (filled circles) and disappearance of cisternae (open circles) show comparable time courses. Bar, 0.5 μm. Reproduced with permission from (Richards et al., 2003) (Copyright 2003, Elsevier, Amsterdam). (D) Model of depolarization-induced SV recycling. Short (ms) depolarization activates the transfer of SVs from the reserve to the recycling vesicle pool, and SV docking at the active-zones (1). Fast (s) retrieval of vesicle membrane after prolonged depolarization (s) occurs either directly as SVs (2a), or as larger endosome-like organelles (2b). Following depolarization, SVs are generated from endosomal-like compartments through a slower process (min) (3). Modified from Leenders et al., 2002.

frequencies (4 h at 2 Hz) revealed mainly HRP-filled vesicles even in the absence of a post-stimulation recovery period (Ceccarelli et al., 1973). Vacuolar structures were present in these terminals, although they were not engaged in SV recycling under the stimulation protocol applied.

The endocytic retrieval of SVs can be considered a highly specialized form of a process common to all cells. Indeed, since the machinery necessary to load vesicles with neurotransmitter is present within the nerve terminal, SVs can undergo several cycles of exo–endocytosis without the need of being retrogradely transported to the soma for refilling or sorting of their molecular components (Valtorta et al., 1990). Immunofluorescence and immunoelectron microscopy studies showed that, at the frog neuromuscular junction, SV proteins do not accumulate at the plasma membrane even after high frequency stimulations. In contrast, SVs are permanently incorporated in the axolemma when exocytosis is triggered in the presence of a block of endocytosis (Valtorta et al., 1988, Torri-Tarelli et al., 1990). A similar result was obtained when the rate of exocytosis largely exceeded the rate of endocytosis, resulting in depletion of SVs from the nerve terminal and accumulation of SV proteins in the plasma membrane (Ceccarelli et al., 1972; Torri-Tarelli et al., 1992). However, recent reports show that exogenously expressed SV proteins, such as VAMP2 and synaptotagmin I, accumulate at the cell surface during active SV recycling (Fernandez-Alfonso et al., 2006; Dittman and Kaplan, 2006; Wienisch and Klingauf, 2006).

In order to maintain intact both the vesicle pools and the morphology/composition of the plasma membrane, the rate of SV retrieval needs to be tightly coupled to exocytosis, both spatially and temporally. The molecular bases of this matching have just started to be elucidated, yet current evidences point to Ca^{2+} binding to synaptotagmin I, already recognized as the primary Ca^{2+}-sensor for SV exocytosis, as a critical element of the coupling process (Jarousse and Kelly, 2001; Poskanzer et al., 2003; Poskanzer et al., 2006; see below). In addition, it appears that the fidelity of SV recycling (i.e., the endocytosis of uniformly shaped and sized vesicles) can be mechanistically dissociated from the processes determining the rate of SV retrieval (Poskanzer et al., 2006). Again, different regions of synaptotagmin I take part in both these regulatory steps of endocytosis: Ca^{2+}-coordinating residues are implicated in the control of the endocytic rate, whereas the motif for interaction with the AP2 complex is required for the generation of SVs of correct size and composition (Poskanzer et al., 2006).

3.2. Alternative mechanisms for neurotransmitter release

Strategies exploited by SVs to undergo exocytosis, and hence to release neurotransmitters, have crucial consequences

on the mechanisms underlying their regeneration. It is now widely accepted that neurotransmitter release can occur through two different mechanisms, 'full fusion' and 'kiss-and-run'.

The hypothesis of the 'full fusion' mode of neurotransmitter release has been put forward in the early 1970s by Heuser and Reese. According to this model, SVs release their content after full fusion with the plasma membrane. This implies that, during neurotransmitter release, the SV collapses into the presynaptic membrane and is then retrieved via the formation of a coated vesicle (Heuser and Reese, 1973) (Fig. 2A, left).

Concomitantly, Ceccarelli et al. (1973) proposed an alternative model of SV exo–endocytosis. In this mode, later named 'kiss-and-run' (Fesce et al., 1994), the SV forms a transient pore with the presynaptic plasma membrane through which the neurotransmitter is released and the vesicle recycles quickly by a direct reversal of the exocytic process, without intermixing with the axolemma. Thus, the vesicle maintains its identity throughout the exo–endocytotic cycle and is rapidly returned to the readily releasable pool (RRP) (Fig. 2A, right). As an alternative to the 'kiss-and-run' mode, an even faster recycling mechanism is thought to occur in which the vesicle recycles as the fusion pore opens without undocking from the active zone ('kiss-and-stay'; Südhof, 2004).

The firing pattern of synapses of the central nervous system *in vivo* is often characterized by bursts of high frequency action potentials, with variable interburst periods. Most of these synapses have a limited complement of SVs; thus, in the absence of fast recycling (i.e., kiss-and-run or kiss-and-stay modes), it would be difficult to preserve the ability of terminals to maintain synaptic transmission during high-frequency bursts. In fact, under such conditions most vesicles would be collapsed in the plasma membrane, waiting for endocytosis (Harata et al., 2006a). The proportion of SVs undergoing kiss-and-run is dependent upon the frequency of stimulation and once a vesicle has undergone kiss-and-run it can be quickly and repeatedly reused (Aravanis et al., 2003a,b; Harata et al., 2006b). The possibility of modulating the extent of kiss-and-run and immediate reuse of SVs depending on the physiological firing patterns may have profound effects on the efficiency of neurotransmitter release. The role of kiss-and-run endocytosis by SVs is still fiercely debated. While the idea that separate, differentially regulated mechanisms for SV re-formation via endocytosis coexist at nerve terminals, two for direct SV recycling from the plasma membrane and one for recycling via endosomal intermediates, is supported by several experimental evidences (Valtorta et al., 2001; Harata et al., 2006a), yet recent findings argue against the relevance of the kiss-and-run pathway of SV recycling (Li et al., 2005; Dickman et al., 2005; Wienisch and Klingauf, 2006).

The ultrastructural analysis of the *Drosophila* mutant *shibire*, which harbors a mutation of the dynamin gene that causes a temperature-sensitive block of endocytosis, reveals the presence of two recycling pathways with different ion sensitivity in the same nerve terminal (Koenig and Ikeda, 1996). One recycling pathway emanates from the active zone of exocytosis, displays fast kinetics and sensitivity to low Ca^{2+} levels. It involves small clusters of vesicles that are observed at the active zones. The formation of these vesicles does not include intermediate structures, such as coated pits, coated vesicles, or cisternae, and might be accomplished by a direct pinch-off at the plasma membrane. At variance, the second pathway emanates from sites away from the active zones, has a slower kinetics, is unaffected by reduced Ca^{2+} levels and involves coated collared pits.

Two endocytic pathways displaying different kinetics also operate during the replenishment of distinct functional SV pools at *Drosophila* neuromuscular synapses (Kuromi and Kidokoro, 2002). Refill of the reserve pool of SVs, which is located toward the centre of the bouton and not released by high K^+, occurs by endocytosis of SVs after the cessation of the stimulation and relies on the release of Ca^{2+} from internal stores. At variance, endocytic replenishment of the distally located recycling pool occurs during stimulation and requires external Ca^{2+}.

The localization of the machinery for clathrin-mediated endocytosis, including α-adaptin and dynamin, to areas of the presynaptic plasma membrane that are distinct from the regions predicted to be active zones of exocytosis further indicates that the AP2-mediated SV retrieval operates preferentially in the pathway of endocytic SV re-formation, involving coated vesicles outside the active zones (Gonzalez-Gaitan and Jackle, 1997; Roos and Kelly, 1999) (Fig. 2C). This would imply that SVs are recycled via the kiss-and-run mode or rapid endocytosis (fission only) at the active zone, while a clathrin plus dynamin-mediated type of retrieval (budding plus fission) operates at the non-active zones, in keeping with the 'classic model' of SV recycling.

Further support to the idea of two mechanistically distinct pathways of SV retrieval running at active and periactive zones comes from the study of fly mutants for key components of the endocytic complexes. Mutants for Dap160/intersectin, a synaptic scaffold for endocytic molecules, including dynamin and dynamin-associated proteins, show accumulation of endocytic intermediates, such as omega profiles (i.e., the hallmark of coated pit formation) and collared pits (i.e., the hallmark of dynamin-mediated membrane fission). However, whereas omega profiles are located exclusively at periactive areas, collared pits can be identified both at active and periactive zones (Koh et al., 2004), indicating that molecular complexes coordinated by Dap160 are involved both in clathrin-mediated endocytosis at periactive zones and rapid endocytosis at active zones. Capacitance measurements coupled with the injection of interfering peptides in retinal bipolar cells show that both the fast and the slow modes of endocytosis require the activity of a GTPase, most likely dynamin. However, the slow pathway is clathrin/AP2-dependent, while the fast pathway is clathrin-independent (Jockusch et al., 2005).

Importantly, the composition of the machinery devoted to vesicle fission also appears to vary in the two pathways of SV retrieval, as indicated by the essential requirement for the dynamin-interacting protein amphiphysin in the slow but not in the fast mode of endocytosis (Jockusch et al., 2005). In

addition, flies bearing mutations in the dynamin-associated proteins endophilin and synaptojanin exhibit severe impairment in clathrin-mediated endocytosis at periactive zones, whereas rapid endocytosis at active zones proceeds normally (Verstreken et al., 2002, 2003).

In addition to relying on different pathways of exo–endocytosis, synapses can also adjust the dynamics of SV trafficking according to the demand imposed by chronic changes in network activity. This can be achieved by modulating the rate of either SV mobilization (hippocampal synapses) or SV reuse (neocortical synapses) (Virmani et al., 2006). Although the mechanisms underlying these activity-dependent modifications are unknown, changes in the molecular composition of the recycling machinery may represent a strategy exploited by synapses in order to adapt the rate of SV recycling to physiological needs.

3.3. Clathrin-dependent pathways of synaptic vesicle recycling

The lack of intermixing between SV and plasma membrane components may be due either to the absence of complete SV fusion, according to the 'kiss-and-run' and 'kiss-and-stay' modes of SV exo–endocytosis, or to the rapidity and selectivity of retrieval (Mitchell and Ryan, 2004). When full fusion of SVs with the plasmalemma occurs, some sophisticated sorting events need to take place at the nerve terminal. Indeed, complete fusion is accompanied by diffusion of SV components into the axolemma, with mixing of these components between adjacent synapses (Torri-Tarelli et al., 1990, 1992; Li and Murthy, 2001; Sankaranarayanan and Ryan, 2001).

According to the classical model of exo–endocytosis proposed by Heuser and Reese (1973), collapse of the SV into the plasmalemma is followed by the assembly and endocytosis of clathrin-coated vesicles, which fuse with an endosomal compartment, from which new SVs will bud off. The main function for clathrin-coated vesicles in the brain is to recapture SVs after exocytosis (Maycox et al., 1992). Indeed, a near-complete inventory of the known SV proteins has been detected by tandem mass spectrometry analysis of brain clathrin-coated vesicles, with a lack of abundant presynaptic plasma membrane proteins (Blondeau et al., 2004).

The molecular composition of the machineries for clathrin-dependent endocytosis and membrane fission has been a subject of recent excellent reviews and will not be addressed in detail here (see Edeling et al., 2006; Wenk and De Camilli, 2004; Mousavi et al., 2004; Conner and Schmid, 2003; Slepnev and De Camilli, 2000; Hinshaw, 2000).

The formation of clathrin-coated vesicles in the nerve terminals requires AP2, which initiates the recruitment of the endocytotic machinery. Direct interaction between AP2 and the cytoplasmic C2B domain of synaptotagmin is likely to guarantee the specificity of the endocytotic pathway for SVs re-formation (see below). AP2 acts in concert with the brain specific monomeric adaptor protein AP180 to regulate clathrin-mediated endocytosis of SVs (Ye et al., 1995). Consistently, *Drosophila* mutants lacking the AP180 homologue LAP display severe defects in endocytosis, resulting in the depletion of SVs from the nerve terminals (Zhang et al., 1998). The injection of antibodies against AP180 into the giant presynaptic terminals of the squid leads to block of synaptic transmission and increase in the nerve terminal surface area (Morgan et al., 1999). At variance, the *C. elegans* homologue of AP180, UNC-11, appears dispensable for clathrin-mediated endocytosis but required to maintain the correct size of endocytosed SVs (Nonet et al., 1999).

Assembly of the clathrin matrix is a GTP- and ATP-dependent process, whereas the subsequent invagination of the coated vesicle requires exclusively GTP-dependent activities. Fission of the coated vesicle again relies on both ATP- and GTP-dependent processes. The GTPase activity, which mediates the fission step is provided by dynamin, which is required in a variety of endocytotic pathways, including phagocytosis, clathrin/caveolae-mediated endocytosis and some forms of clathrin/caveolae-independent endocytosis (Conner and Schmid, 2003).

The function of dynamin has been elucidated by ultra-structural analysis of the dynamin rings formed around the neck of invaginating coated pits. These transient structures are stabilized by GTPγS, a non-hydrolysable analogue of GTP (Takei et al., 1995) (Fig. 2B). The current model envisioning the action of dynamin in endocytosis postulates that the protein is recruited to coated pits in its GDP-bound state. After GDP/GTP exchange, dynamin assembles at the neck of the coat, forming a helical collar. The hydrolysis of GTP results in a conformational change that precedes fission, followed by dissociation of dynamin from the complex (Conner and Schmid, 2003).

Dynamin appears to be a mechanochemical enzyme directly involved in membrane constriction underlying the fission event (Chen et al., 2004; Danino et al., 2004). Compelling evidence for the mechanical role of dynamin in membrane fission has been recently provided by an *in vitro* assay in which the twisting activity of the protein on lipid tubules was monitored in real time (Roux et al., 2006). This study also indicates that fission requires the coupling of dynamin-mediated constriction with independent mechanisms that generate membrane tension. In addition, binding of the proline/arginine rich domain of dynamin to Src homology 3 (SH3) modules facilitates the recruitment to the fission machinery of accessory molecules that in turn modulate the GTPase activity of dynamin and hence vesicle fission (Conner and Schmid, 2003; Hinshaw, 2000; Schmid et al., 1998).

Two models of clathrin-dependent recycling of SVs have been proposed:

1. The 'classical model' postulates that the coated vesicle undergoes ATP-dependent loss of the clathrin lattice and fuses with the endosomal compartment, from which new vesicles are formed through an independent sorting process (Heuser and Reese, 1973) (Fig. 3).
2. The alternative model proposes that SVs can form directly from clathrin-coated vesicles by loss of the coat, thereby reducing the number of steps needed for the endocytic process (De Camilli and Takei, 1996). According to this

model, endosome-like intermediates in nerve terminals are not preexisting internal structures that act as acceptor membranes of endocytotic vesicles, but originate from deep invaginations of the plasmalemma. These invaginations might either maintain a narrow connection to the plasmalemma or be eventually internalized. In any case, these vacuoles are likely to be molecularly more similar to the plasmalemma rather than to endosomes. Thus, SVs may be directly produced in a single clathrin coat-mediated budding and dynamin-mediated fission step from either the presynaptic plasma membrane, or deep plasma membrane invaginations, or both (Fig. 2B). This would explain the similarity between the molecular composition of clathrin-coats observed on the vacuole membranes and at the nerve terminal surface (Takei et al., 1996; Gad et al., 1998).

It is immediately apparent that the modification of the Heuser-Reese model proposed by De Camilli and colleagues brings the 'classical' and the 'kiss-and run' models closer, yet preserving a central role for clathrin and the participation of vacuolar structures during intense stimulations. Moreover, it provides a conceptual framework to reconcile data indicating that bulk, non-selective endocytosis mediated by large vacuoles (Koenig and Ikeda, 1989) and selective clathrin-mediated endocytosis (Heuser and Reese, 1973) participate in SV recycling.

The molecular identity of intermediate compartments that participate in clathrin-dependent endocytosis and their contribution to the SV life cycle remain uncertain. At snake motor terminals, ultrastructural analysis showed that all of the endocytic SVs, identified by photoconversion of FM dye internalized during a brief, low-frequency stimulation applied at reduced temperature, were clathrin-coated and clustered near active zones. Additional clathrin-coated vesicles budded from either pre-existing cisternae or a small number of macropinosomes that had formed from deep invaginations at periactive zones during stimulation (Teng and Wilkinson, 2000). Membrane retrieval in transient vacuole-like structures that disappeared within several minutes concomitantly with the budding of new labeled SVs was also described at the large Calyx of Held under strong stimulations (de Lange et al., 2003). However, this mechanism is not likely to make a substantial contribution to recycling during low-frequency stimulation. Budding of new SVs from vacuoles during recovery from brief stimulation in high K^+ was also observed in cultured rat cerebellar granules (Marxen et al., 1999). Thus, it appears that new vesicles can be generated from endosomal-like compartments after intense stimulations, which activate bulk endocytosis (Fig. 3). A similar mechanism of activity-dependent bulk endocytosis also operates at synaptic terminals of retinal bipolar cells, characterized by an unusually rapid SV retrieval. At these synapses, brief, low-frequency stimulation leads to the formation of transient, plasmalemma-derived vacuoles, which subsequently bud off SVs (Paillart et al., 2003), whereas strong, sustained depolarization activates bulk membrane uptake that appears to share common features with macropinocytosis described in non-neuronal cells, and might be linked to the regulation of the structural plasticity of the nerve terminal (Holt et al., 2003).

It is unclear whether the internal vacuoles implicated in the clathrin-mediated pathway are distinct from endosomes. In the absence of a detailed molecular characterization of these membrane compartments (see Marxen et al., 1999) the question remains unanswered. If the vacuolar structures described in several studies do not display an endosomal identity, endosomes, which play a major role in SLMV formation in neuroendocrine cells, are dispensable for SV recycling at synapse. Thus, although the presence of endosomes in nerve endings is documented by several studies, their function might be limited to the *de novo* assembly of newly synthesized proteins into mature SVs.

3.4. Do specialized endosomes operate in synaptic vesicle recycling/biogenesis?

It was hypothesized that *de novo* formation of SVs may occur from a specialized class of endosomes (Kelly, 1993). However, the morphological characterization of the endocytic pathway in PC12 cells revealed that the endosomal compartments in this cell line are very similar to those of non-neuroendocrine cells (de Wit et al., 1999). Neither the morphology of the distinct endocytic intermediates nor the comparison between the distribution patterns of SLMV and non-SLMV proteins provides any clues for the existence of a specialized endosome dedicated to SLMV biogenesis/recycling. In agreement with earlier studies (Cameron et al., 1991; Linstedt and Kelly, 1991a; Grote et al., 1995; Grote and Kelly, 1996) synaptophysin and VAMP2 were mainly found on early endosomes as opposed to late endosomes and lysosomes, and especially on the tubular extensions of early endosomes which represent preferential sites for SLMV budding (de Wit et al., 1999).

In neurons, electron microscopy studies have shown the presence of extensive networks of tubular endosomes in dendrites and cell bodies, whereas in the axon early endosomes were found exclusively in presynaptic terminals and varicosities (Parton et al., 1992). Consistent with these observations, the axon shaft of mature neurons is largely devoid of endocytic activity and internalization only occurs at the nerve terminals. In contrast, the entire dendritic plasma membrane of mature neurons shows high endocytic activity. Thus, the axonal and somatodendritic domains of polarized hippocampal neurons seem to possess distinct endocytic circuits (Parton et al., 1992), although they share common components of the endocytic machinery, such as the small GTPase Rab5 (de Hoop et al., 1994) and the SNARE protein syntaxin 13 (Prekeris et al., 1999). Endosomes found in axons and nerve terminals of primary hippocampal neurons (Mundigl et al., 1993) and in neurites of differentiated PC12 cells (Bonzelius et al., 1994) notably lack the transferrin receptor, which is associated with the soma and dendrites.

Brefeldin A has been useful in characterizing functionally specialized endosomes in polarized cells. BFA induces a massive tubulation of transferrin receptor-containing endo-

somes in the somatodentritic region, whereas no obvious morphological changes are produced in axons (Mundigl et al., 1993). Moreover, in cultured hippocampal neurons the transcytotic transfer from dendritic endosomes to the axons is sensitive to BFA (de Hoop et al., 1994). Differences in the sensitivity to BFA action indicate that the molecular composition of the two classes of endosomes may be distinct.

Another molecular difference between the endosomal compartments of the axonal and somatodendritic domains of neurons is the exclusive association of EEA1 with Rab5-positive endosomes of the somatodendritic domain of polarized hippocampal neurons (Wilson et al., 2000). EEA1 is one of the best characterized early endosome marker and vital effector of Rab5 (Christoforidis et al., 1999). In the light of the more widespread distribution of Rab5 relative to EEA1, the nature of the Rab5 effector associated with EEA1-negative presynaptic endosomes remains to be investigated, although the huntingtin-associated protein HAP40 might be a candidate (Pal et al., 2006).

While the role of early endosomes in the control of SV recycling is well supported, the function of tubulovesicular recycling endosomes containing syntaxin 13 associated with the axonal domain of polarized hippocampal neurons is less clear (Prekeris et al., 1999). These endosomes are excluded from synaptic contacts and do not colocalize with SV antigens in either mature or immature neurons, suggesting that they are distinct from the axonal Rab5-positive endosomes. Syntaxin 13, that in non-neuronal cells controls transferrin receptor recycling (Prekeris et al., 1998), might be the SNARE involved in homotypic endosome fusion.

Together, these results have led to the hypothesis that somatodendritic endosomes (transferrin receptor- and EEA1-positive, BFA-sensitive) play a 'housekeeping' role, whereas presynaptic endosomes (transferrin receptor- and EEA1-negative, BFA-insensitive) play a unique role in generating SVs (Parton and Dotti, 1993; de Hoop et al., 1994). However, this picture contrasts with the reported BFA-sensitivity of SLMV biogenesis in PC12 cells (Faundez et al., 1997) and SV recycling in developing axons of frog motor neurons and immature mammalian neuromuscular junctions (Zakharenko et al., 1999; Polo-Parada et al., 2001). This discrepancy is only partially explained by assuming that the BFA-sensitive pathway, implicated in SV biogenesis in PC12 and immature neurons, represents an elementary process that is replaced during nerve terminal maturation with a more efficient and unique neuronal process. Indeed, at variance with aforementioned studies, BFA does not appear to have any effect on recycling of SVs in immature hippocampal neurons, as demonstrated by the uptake of an antibody directed against the luminal portion of synaptotagmin I (Matteoli et al., 1992; Mundigl et al., 1993), possibly reflecting a different timing in the maturation of the neurosecretory apparatus at peripheral and central synapses.

Although the precise contribution of axonal endosomes to SV formation remains to be established, the two small GTPases Rab5 and Rab4, which in non-neuronal cells regulate transport to and from early endosome, respectively (Bucci et al., 1992; van der Sluijs et al., 1992), appear to govern SV recycling/formation.

Rab4 is associated with the early endosomal precursors of SLMVs in PC12 cells, and regulates budding of SLMVs from these compartments. In addition, Rab4 regulates the exit of constitutive recycling proteins from early endosomes in PC12 cells. However, Rab4 does not appear to affect the sorting of VAMP2 from transferrin receptor, suggesting that it acts distally to the primary sorting process of the two transmembrane proteins (de Wit et al., 2001).

The existence of a Rab5-dependent pathway involved in the trafficking of several SV proteins to the nerve terminal has been confirmed by expression of Rab5 mutants in cultured hippocampal neurons. The inhibition of Rab5 activity severely impaired the axonal transport of SV proteins (Kanaani et al., 2004). Axonal Rab5-positive endosomes have been also implicated in the differential sorting of amyloid precursor protein (APP) and SV components, which are internalized together via the same clathrin-coated vesicle at nerve terminals and then directed to either the retrograde transport route or the recycling SV pool, respectively (Marquez-Sterling et al., 1997).

Rab5 was found on synaptophysin-containing vesicles immunoisolated from SV preparations (de Hoop et al., 1994; Fischer von Mollard et al., 1994). Together with the description of Rab5-positive compartments (de Hoop et al., 1994) in both axon and dendrites of cultured hippocampal neurons, these results first argued for the view that early endosomes participate in the biogenesis of SVs. Monitoring of the endosomal compartments in *Drosophila* neuromuscular synapses revealed that the Rab5-positive endosomes are required to sustain SV exo–endocytosis during intense synaptic activity. Altering Rab5 function disrupts the integrity of the presynaptic endosomal network, causing accumulation of endocytic intermediates, and influences synaptic efficacy. Importantly, changes in synaptic performance are due to a change in the release probability of SVs rather than in the size of the recycling vesicle pool (Wucherpfennig et al., 2003). An attractive explanation as to how membrane exchange between vesicles and the endosome could affect the release probability of SVs is to hypothesize that endosome function is required to control the protein and lipid composition of SVs. At synapses, Rab5 has also been implicated in the maintenance of the uniform size of SVs by prevention of homotypic vesicle fusion (Shimizu et al., 2003).

3.5. Phosphoinositide regulation of the synaptic vesicle cycle

Evidence has accumulated that supports the regulatory role of lipids in SV traffic. In particular, several steps of the SV life cycle have been linked to the cycle of synthesis and degradation of phosphoinositides (PIs), which takes place at nerve terminals (Cremona and De Camilli, 2001; Wenk and De Camilli, 2004; Osborne et al., 2006). Although PIs generally constitute $<10\%$ of the total cell phospholipids, yet membrane traffic events involving the sorting of SV proteins depend on the generation of distinct PI species, which are recognized by specific binding modules of target proteins. These protein modules include pleckstrin homology (PH), C2, Phox, Fyve, Dix, and ENTH

domains, as well as short basic amino acid-rich sequences (Lemmon, 2003).

Synthesis and degradation of PIs depend on the activity of specific kinases and phosphatases on the 3′-OH, 4′-OH or 5′-OH positions of the inositol ring. PI(4)P and PI(4,5)P_2 are the most abundant PI species. PI(4)P is primarily localized on the Golgi membranes and Golgi-derived vesicles, including SVs. A phosphatidylinositol 4-kinase activity is associated with SVs and has been attributed to PI4KIIα (Guo et al., 2003). PI(4,5)P_2 is mainly associated with the plasma membrane. In the brain, PI(4,5)P_2 turnover is controlled by the antagonistic activity of synaptically enriched PI(4)P 5-kinase (PIPK1γ) and the PI(4,5)P_2 5-phosphatase synaptojanin 1. Similarly to several other endocytic factors, both enzymes are activated by stimulation-dependent dephosphorylation (McPherson et al., 1996; Wenk et al., 2001; Slepnev and De Camilli, 2000). In addition, synaptojanin 1 is inhibited by Cdk5-operated phosphorylation, which is counteracted by depolarization-induced dephosphorylation by calcineurin (Lee et al., 2004). Phosphorylation of synaptojanin 1 inhibits its binding to endophilin, a component of the synaptic endocytic machinery that activates synaptojanin and binds dynamin (Lee et al., 2004; Ringstad et al., 1997). It appears therefore that during stimulation endophilin synchronizes the action of dynamin and synaptojanin 1 to promote PI(4,5)P2 dephosphorylation on endocytic membranes.

It has long been known that PI(4,5)P_2 modulates neurotransmission through the signaling activity of its metabolites inositol trisphosphate, diacylglycerol (DAG) and arachidonic acid (Berridge, 1993; Tanaka and Nishizuka, 1994). However, early studies also implicated PI(4,5)P_2 itself in the control of secretory granule exocytosis, independently of the generation of metabolites (Eberhard et al., 1990). In cultured neurons, postsynaptically generated nitric oxide retrogradely increases PI(4,5)P_2 production in the presynaptic compartment through a cGMP-dependent mechanism and accelerates SV recycling mainly by impinging on SV endocytosis (Micheva et al., 2003).

The manipulation of PIP(4,5)P_2 levels by the genetic inactivation of either PIPK1γ or synaptojanin 1 has uncovered a major role for PI(4,5)P_2, and more generally for PI metabolism, in SV recycling. PIPK1γ and synaptojanin 1 antagonize each other in the assembly of clathrin coats (Wenk et al., 2001). Loss of the integrity of the phosphoinositide cycle in synaptojanin 1-deficient neurons leads to abnormal accumulation of endocytic clathrin-coated vesicles at synapses and a severe impairment in the progress of recently endocytosed SVs to a fusion-competent state (Cremona et al., 1999; Kim et al., 2002). Defective SV endocytosis probably underlies the enhanced synaptic depression observed in synaptojanin 1-knock out mice (Cremona et al., 1999). Decreased PIP(4,5)P_2 levels in PIPK1 knock-out mice lead to a severe impairment in synaptic transmission linked to defects in clathrin-dependent endocytosis, with reduced number of coated SVs and accumulation of endosome-like structures in nerve terminals (Di Paolo et al., 2004).

The molecular explanation for the critical role of PI(4,5)P_2 in the nucleation and stabilization of clathrin coats during SV endocytosis is suggested by the presence of PI(4,5)P_2-binding modules in key components of the endocytic machinery. These include the clathrin adaptors AP2 and AP180, dynamin and accessory factors for clathrin-mediated endocytosis such as epsin, Hip1/Hip1R, ARH/Dab (Slepnev and De Camilli, 2000; Wenk and De Camilli, 2004). PI(4,5)P_2-binding is responsible for membrane localization of AP2 (Gaidarov and Keen, 1999) and AP180 (Ford et al., 2001). PI(4,5)P_2 is indispensable for the formation of coated buds on the surface of lipid monolayers in the presence of AP180, AP2 and clathrin (Ford et al., 2001).

PI(4,5)P_2-binding and interaction with cargo proteins occur sequentially in a cooperative manner during AP2 recruitment to the plasma membrane. Recognition of both tyrosine-based and acidic dileucine motifs by AP2 only occurs when the cargo is presented in a membrane containing PI(4,5)P_2 (Honing et al., 2005). Interestingly, the small GTPase ARF6, which regulates clathrin-dependent (Altschuler et al., 1999) and clathrin-independent (Brown et al., 2001) endocytic pathways, controls clathrin/AP2 recruitment to synaptic membranes through a direct interaction with PIP1γ that stimulates kinase activity and results in PI(4,5)P_2 production (Krauss et al., 2003). These observations led to the proposal of a dual-key recognition strategy for directing vesicular trafficking, based on a coincident detection mechanism which requires the simultaneous interaction of adaptor proteins with protein cargoes and PIs present in the same membrane to trigger productive clathrin-mediated endocytosis (Wenk et al., 2001; Wenk and De Camilli, 2004). This model ensures efficient compensatory SV endocytosis only after exocytosis and explains how adaptor proteins, which recognize the same signal of cargo proteins are targeted to distinct intracellular locations.

Impaired PI metabolism also alters the exocytic limb of SV recycling. In PIPK1γ−/− mice exocytic defects such as reduced frequency of miniature currents, smaller RRP and enhanced rapid depression (Di Paolo et al., 2004) are suggestive of the involvement of PIs in the regulation of SV priming (i.e., the process that confers competence for Ca^{2+}-triggered fusion to docked vesicles). Importantly, PI transfer protein and PI(4) 5-kinase are essential for vesicle priming in PC12 cells (Hay and Martin, 1993; Hay et al., 1995) and inactivation of ARF6, the upstream regulator of PIPK1γ (Honda et al., 1999; Krauss et al., 2003), leads to defects in Ca^{2+}-dependent LDCV exocytosis correlated with the redistribution of PI(4,5)P_2 to endosomal membranes (Aikawa and Martin, 2003). In a separate study, manipulation of plasma membrane PI(4,5)P_2 levels was associated with changes in the size and refilling rate of the primed vesicle pool in chromaffin cells (Milosevic et al., 2005).

It is also of note that the PI(4,5)P_2 metabolite DAG regulates neurotransmitter release by binding to Munc13, a critical component of the vesicle priming machinery (Rhee et al., 2002). In addition, PI(3)Ps, first implicated in clathrin coat assembly in vitro (Rapoport et al., 1997), also control vesicle exocytosis. The PI 3-kinase isoform PI3K-C2α has a major role in the ATP-dependent priming of LDCV in PC12 cells (Meunier et al., 2005). At variance, pharmacological inhibition of PI 3-kinase at the neuromuscular junction leads to defects in SV recycling consistent with the impairment of endocytic

replenishment of the reserve pool of SVs, with little effect on the RRP (Richards et al., 2004). These results might also originate from the modulatory effects of PI 3-kinases on actin dynamics impinging on vesicle recycling and synapse structure (Holt et al., 2003).

A number of nerve terminal proteins implicated in the control of SV exocytosis bind $PI(4,5)P_2$ via C2 or PH domains. These include proteins of the vesicle membrane, such as synaptotagmin (Schiavo et al., 1996; Tucker et al., 2004) and rabphilin 3A (Chung et al., 1998), the active zone proteins Piccolo and Rim (Gerber et al., 2001; Wang et al., 1997) and cytoplasmic regulators of exocytosis, such as Munc13 (Brose et al., 1995), calcium-activated protein for secretion (CAPS) (Grishanin et al., 2004), and Mint (Okamoto and Sudhof, 1997). Binding of these proteins to $PI(4,5)P_2$ may contribute to the specificity of vesicle exocytosis as well as to membrane fusion. In particular, the Ca^{2+}-dependent binding of the syaptotagmin C2 domains to $PI(4,5)P_2$ and the t-SNAREs syntaxin and SNAP25 is critical for the control of exocytosis (Tucker et al., 2003). These observations support the idea that binding to $PI(4,5)P_2$ increases the affinity of synaptotagmin for Ca^{2+} and enhances its Ca^{2+}-dependent binding to t-SNAREs, which underlies SNARE complex assembly and function (Littleton et al., 2001; Tucker et al., 2003). In addition, the SH3-mediated interaction of the regulatory subunit of PI 3-kinase with synapsin I (Onofri et al., 2000) appears to be required for the stimulation-induced mobilization of SVs from the reserve pool to the RRP (Cousin et al., 2003).

As stated above, lipid heterogeneity in the presynaptic plasma membrane might define hotspots of vesicle exo/endocytosis. Remarkably, $PI(4,5)P_2$ microdomains have been described on the plasma membrane of neurons and neuroendocrine cells (Laux et al., 2000; Aoyagi et al., 2005; Milosevic et al., 2005). Part of the $PI(4,5)P_2$ microdomains colocalize with the classical lipid raft marker Thy-1 (Laux et al., 2000; Aoyagi et al., 2005), while other $PI(4,5)P_2$ microdomains are Thy-1-negative and accumulate at syntaxin clusters with docked LDCVs (Aoyagi et al., 2005). Interestingly, the number of $PI(4,5)P_2$-syntaxin-LDCV complexes correlates with the efficiency of Ca^{2+}-dependent LDCV exocytosis. The shaping of $PI(4,5)P_2$ clusters is likely to be governed by the regulated spatial and temporal distribution of the enzymes that control in its turnover. Of note, ARF6 might orchestrate the recruitment of PI(4) 5-kinase at release sites via interaction with mSec7, a guanine exchange factor for ARF6 that binds to Munc13 and possess a PH domain for PI binding (Neeb et al., 1999).

4. Sorting and assembly of synaptic vesicle components

SVs purified from the brain have a distinct and fairly simple protein composition (Huttner et al., 1983). The observation that SVs contain a restricted set of membrane proteins implies the existence of a sorting process ensuring that all nascent vesicles contain the complement of proteins needed for exo–endocytosis.

The vesicle pool in a nerve terminal can completely recycle in the order of one minute (Ryan et al., 1993) and during endocytosis SVs retrieve at least some components from large surface reservoirs (Fernandez-Alfonso et al., 2006). In addition, stimuli known to deplete the RRP of SVs also exhaust a 'readily retrievable pool' of surface-associated proteins that actively participate in SV recycling (Wienisch and Klingauf, 2006). These findings emphasize the need of sorting processes, which generate highly organized endocytic structures at the synaptic plasma membrane. In the absence of such quality control system even a low error frequency would rapidly generate a pool of inert SVs with a composition similar to the plasma membrane.

The sorting of SV proteins is defined as their segregation from non-SV proteins during either the formation of a vesicle from a donor membrane or the formation of distinct microdomains in the plane of the donor membrane (Hannah et al., 1999). In analogy to the raft concept (Simons and Ikonen, 1997), the enrichment of specific lipids within membrane microdomains might allow, by means of distinct protein–protein and protein–lipid interactions, to segregate SV components from resident proteins of the donor membranes. If SV proteins are recruited with the correct stoichiometry in the specialized microdomain, a single budding event might be sufficient for generating new SVs (Fig. 4, upper panel).

Alternatively, the formation of mature SVs, enriched in all the components needed for efficient neurotransmitter release, might rely on a stepwise process requiring multiple cycles of SV exo–endocytosis undertaken either along the axon or at the presynaptic terminals. Indeed, extrapolating from the study of SLMV biogenesis (Regnier-Vigouroux et al., 1991), several passages through the plasma membrane and endosomal compartments might precede the incorporation of SV proteins into mature vesicles (Fig. 4, lower panel).

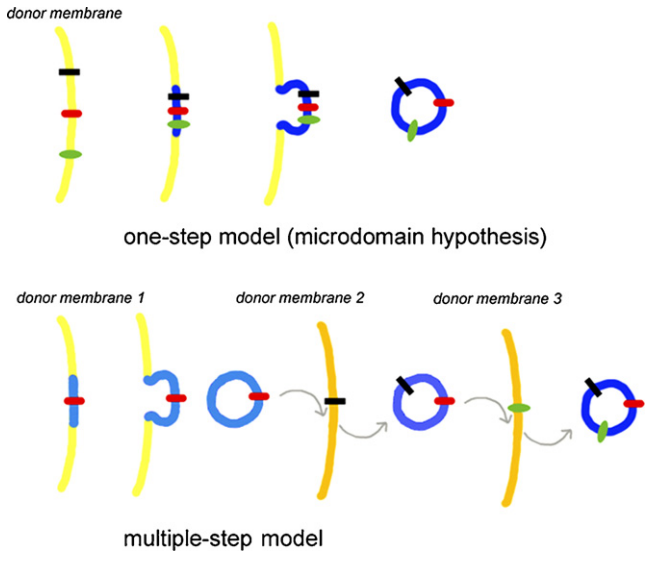

Fig. 4. Models for the assembly of SV components. (Upper panel) one-step model. The lateral organization of microdomains with distinct lipid composition allows SV components to be enriched in these specialized membrane elements and eventually sorted into the same vesicle in a single budding step from the donor membrane. (Lower panel) multiple-step model. Multiple cycles of fusion and budding through various donor compartments, either the TGN, the endosomes, or the plasma membrane, are needed in order for the maturing SV to be endowed with a complete complement of membrane proteins.

The possibility that removal of vesicle components, rather than insertion of new proteins, drives the maturation of the organelles is suggested by the study of granules involved in peptide hormone secretion in a pituitary cell line (Eaton et al., 2000). An ARF-mediated sorting pathway is responsible for functional remodelling of the granule membrane. Several trafficking proteins, including VAMP4 and synaptotagmin IV, are sorted away during the transition from immature granules, which exhibit unregulated secretion after budding from the TGN, to mature, regulated exocytotic carriers. The removal of synaptotagmin IV, which inhibits calcium-triggered exocytosis (Littleton et al., 1999) provides a crucial switch between unregulated and regulated secretion during granule maturation.

Although direct evidence is lacking, the two general mechanisms of protein sorting to SVs described above are not mutually exclusive. Rather, they are likely to work in concert during the SV life cycle, at both plasma membrane and endosomal compartments.

4.1. Microdomain-based sorting of synaptic vesicle proteins

Evidence for the formation of specialized domains in the plasma membrane comes from the study of the role of cholesterol in SV formation and exocytosis.

Limited cholesterol depletion, which has only a weak effect on total endocytic activity, markedly affects both the size of the steady-state SLMV pool and SLMV biogenesis from the plasma membrane in PC12 cells (Thiele et al., 2000). Cholesterol is also essential for the formation of both regulated and constitutive secretory vesicles from the TGN in a neuroendocrine cell line (Wang et al., 2000).

The lipid composition of rat synaptosomal plasma membranes shows similarity with that of purified SVs, but with some striking differences (Vincendon et al., 1972; Breckenridge et al., 1973; Pfrieger, 2003). Gangliosides, which are present at high levels in the synaptosome plasma membrane, are almost completely absent in SVs. In contrast, SVs are enriched in acidic phospholipids and contain unusually high levels of cholesterol. Indeed, the neuronal plasma membranes are themselves enriched in cholesterol (molar ratio of about 0.44), yet SVs show an extra-enrichment (molar ratio about 0.58). In addition, the accumulation of cholesterol in SVs is paralleled by depletion, relative to the plasmalemma, in sphingolipids, with which cholesterol normally interacts. Therefore, SVs are generated by endocytosis of a cholesterol-rich domain of the plasma membrane, at least conceptually analogous to 'lipid rafts', detergent-resistant microdomains enriched in cholesterol and implicated in numerous signal transduction and membrane traffic pathways (Simons and Ikonen, 1997; Ikonen, 2001).

This evidence prompted the investigation of whether one of the protein components of the SV is responsible for cholesterol binding and accumulation. The use of a radiolabeled photoactivatable analogue of cholesterol was exploited by Thiele et al. (2000) to search for cholesterol binding proteins associated with SLMVs of PC12 cells. This approach led to the identification of synaptophysin I as the major cholesterol-binding protein in SLMVs and brain SVs. This finding is of particular interest since synaptophysin I is engaged in a detergent-resistant multimeric complex together with other SV proteins, namely VAMP2, SV2, synaptotagmin and the 39-kDa subunit of the vesicular proton pump (Bennett et al., 1992). Within this large complex, synaptophysin I can interact directly with both VAMP2 and the subunit of the proton pump (see below for references). Consistently, quantitative proteomic analysis of detergent-resistant lipid microdomains isolated from synaptic membranes reveals the presence of several SV proteins, including synaptophysin, in cholesterol-dependent complexes (Jia et al., 2006).

The hypothesis that synaptophysin I organizes lateral membrane domains in which SV proteins are selectively recruited while other membrane proteins are excluded is attractive. The establishment of a network of protein–protein interactions, together with binding of synaptophysin I to cholesterol, might facilitate the formation of membrane domains enriched in SV constituents from which budding of new vesicles may occur. The presence of synaptotagmin I, which binds the clathrin adaptor AP2 (see below), should ensure efficient endocytosis of such preassembled modules.

Interestingly, at the plasma membrane and TGN, cholesterol depletion blocks the late steps of the vesicle budding process, characterized by the generation of negative curvature in the luminal membrane leaflet (Subtil et al., 1999; Wang et al., 2000). Cone-shaped lipids, such as cholesterol, favor negative membrane curvature. This is critical in the case of small organelles such as SVs, whose high curvature would not be normally permitted by the phospholipids present in their membranes. In addition, association of cholesterol with oligomeric proteins, such as synaptophysin, might further contribute to the formation of the highly curved SV membrane (discussed in Huttner and Schmidt, 2000).

Cholesterol-rich domains of the plasma membrane are also implicated in the formation of t-SNARE clusters that might correspond to hotspots of SV exocytosis, although the functional role of such clusters is still controversial (Chamberlain et al., 2001; Lang et al., 2001; Salaun et al., 2005).

4.2. Protein sorting at the TGN

Spatial segregation of cargoes is emerging as a crucial mechanism of polarized post-Golgi sorting. Indeed, apical and basolateral cargoes segregate into large domains in the Golgi/TGN network structures even in non-polarized cells and exit in separate transport containers. Since these transport carriers do not intersect with endocytic structures, lateral segregation in the TGN appears to be the primary sorting event (Keller et al., 2001; Paladino et al., 2006).

In epithelial cells certain apical proteins form detergent-insoluble sphingolipid-cholesterol rafts that are thought to function as platforms for apical delivery. These complexes are formed during protein processing in the Golgi apparatus and act in the TGN as sorting platforms for inclusion of protein cargoes

destined to the apical membrane, which is the non-neuronal counterpart of the axonal membrane (Simons and van Meer, 1988; Dotti and Simons, 1990; Keller and Simons, 1998; Ledesma et al., 1998; Ledesma et al., 1999).

It is less clear whether this mechanism also operates in the sorting/transport of proteins destined to the same subcellular district (apical-axonal or basolateral-dendritic) but to different compartments (e.g., SV versus endosomes or plasma membrane). A post-Golgi segregation step has been reported for different apical proteins transported to the plasma membrane in distinct vesicular carriers (Jacob and Naim, 2001), while axonal proteins belonging to both SVs or plasma membrane have been shown to travel along the axon using the same (Ahmari et al., 2000) or distinct (Zhai et al., 2001) membrane carriers.

It is possible that, similarly to what has been observed for the sorting of apical and basolateral proteins in polarized epithelial cells, axonal or, more specifically, SV membrane constituents are pre-assembled into microdomains with specific lipid composition at the level of the TGN (Hannah et al., 1999). Although SVs are not generated by direct budding from the TGN, it has been suggested that selective association of palmitoylated GAD65 with specialized, cholesterol-rich, membrane microdomains in the TGN is a pre-requisite for GAD65 to enter the axonal trafficking route to presynaptic terminals, shared with other SV proteins (Kanaani et al., 2002; Kanaani et al., 2004).

4.3. Determinants of synaptic vesicle protein trafficking

Signal sequences are thought to mediate the sorting of many proteins to cellular compartments. At present, two major groups of sorting signals have been identified. The first group comprises tyrosine-based signals, which usually conform to the consensus sequences YXXØ (where X is any amino acid, and Ø is a strong hydrophobic amino acid) or FXNPXY. The second group of sorting signals contains di-leucine/di-hydrophobic signals, in which one of the leucines can be substituted by isoleucine, methionine, or valine (Bonifacino and Traub, 2003).

Several lines of evidence indicate that different proteins use distinct pathways to be targeted to the SV. This suggests that each SV protein contains multiple signals that control its trafficking along the various pathways. These signals may mediate different sorting steps at the TGN, plasma membrane or endosomes. The situation is further complicated by the partial overlap between signals responsible for the polarized traffic of SV proteins and signals responsible for their targeting to SVs.

This complex picture is exemplified by the distribution of fluorescent chimeras of various SV proteins expressed in cultured neurons (Pennuto et al., 2003). All exogenous proteins tested were largely confined to the axon, indicating that they contain axonal targeting information. However, whereas synaptophysin I was selectively confined to SVs, the other SV proteins, namely synaptotagmin I, VAMP2 and VAMP1 were not exclusively localized to synaptic sites. Rather, they are diffused throughout the axonal plasma membrane, albeit some enrichment at the level of synaptic puncta was observed. Thus, although these proteins bear the information for axonal targeting, they require additional signals to be sorted to SVs.

Several studies have identified domains and motifs necessary for the correct localization of SV proteins. However, no common motif that could serve as universal targeting signal has been described. The absence of a common sorting signal element in SV proteins contrasts with the targeting to other organelles, where many distinct proteins share the same sorting signals.

At least two different mechanisms of SV protein sorting could account for the lack of a universal SV targeting signal. First, distinct components of SVs may be sorted independently by separate processes. In keeping with this possibility, al least some SV proteins seem to be transported down the axon in different carriers (Okada et al., 1995) and mutations affecting the sorting of a SV protein leave the other components unaffected (Nonet et al., 1999). Second, the sorting information might be present in a few proteins, with other components being secondarily targeted to SVs by interaction with these proteins (Bennett et al., 1992). More likely, a combination of these two mechanisms is used in SV proteins targeting.

Here, we provide an overview of the variety of determinants exploited by individual SV components to be delivered onto maturing vesicles. Besides relying on motifs in their primary structure, SV proteins also make use of post-translational modifications, protein–protein and protein–lipid interactions to reach their final destination.

4.3.1. Synaptobrevin II/VAMP2

In the past 10 years, a number of different laboratories have contributed to a comprehensive study of the sorting determinants involved in the targeting of VAMP2 to SVs. VAMP2 does not possess a signal sequence to be directed to a translocation apparatus of the endoplasmic reticulum (ER) membrane (Kutay et al., 1993). Insertion of VAMP2 into the ER membranes occurs postranslationally and is mediated by the interaction of a conserved bipartite carboxy-terminal motif with an as yet unidentified ER protein(s) (Kutay et al., 1995; Kim et al., 1999). In PC12 cells, after insertion into the ER membrane, VAMP2 is transported via the Golgi apparatus to SLMVs (Kutay et al., 1995).

A GFP-tagged chimera of VAMP2 has been exploited to visualize the post-Golgi traffic of the protein in polarized hippocampal neurons in culture (Sampo et al., 2003). Exogenous VAMP2 was delivered equally to the surface of both axons and dendrites, but was preferentially endocytosed from the dendritic plasma membrane. Thus, selective endocytosis rather than selective delivery appears to be responsible for the axonal polarization of VAMP2. These results have been corroborated by the use of a point mutant of VAMP2, which remains blocked at the plasma membrane in association with t-SNARE proteins due to the inhibition of SNARE complex dissociation (Martinez-Arca et al., 2004). Expression of such mutant showed that, after leaving the Golgi, VAMP2 is directly targeted to the plasma membrane of both axons and dendrites.

A role for endocytosis from the plasma membrane in the sorting of VAMP2 to SVs has been uncovered by genetic ablation of the neuro-specific clathrin-adaptor protein AP180 in *Drosophila* (*lap*) and *C. elegans* (*unc-11*). In both mutants, VAMP2 is mislocalized to the neuronal plasmalemma, and no longer present exclusively on SVs. In the *unc-11* mutant this phenotype appears to be specific for VAMP2 (Nonet et al., 1999), while in mutant flies several other synaptic proteins mislocalized to extrasynaptic regions of the axonal plasma membrane (Bao et al., 2005). Mislocalization of SV proteins may not simply result from the severe defect in SV recycling and depletion of SV pools observed in *lap* mutants (Zhang et al., 1998). Indeed, in *Drosophila* mutants lacking key components of the endocytic machinery, such as endophilin (Verstreken et al., 2002, 2003) or DAP160 (Koh et al., 2004), SV proteins remain localized at nerve terminals despite the severe depletion of SVs. Therefore, either a direct or an indirect interaction of AP180 with VAMP2 and possibly other SV proteins is responsible for the recruitment of VAMP2 to nascent SVs. Whether targeting of VAMP2 to SVs occurs in a single step via UNC-11-mediated endocytosis or whether it requires a passage through endosomal compartments (Salem et al., 1998) is unclear. The trafficking route responsible for VAMP2 targeting to SVs has been further dissected by expressing in hippocampal neurons a chimera consisting of the cytoplasmic domain of VAMP2 (aa 1–93) fused to the amino-terminus of the complete transferrin receptor (West et al., 1997). In striking contrast to the normal distribution of the transferrin receptor, which is localized in the somatodendritic region and excluded from the axon, the chimera was directed to the axon, where it primarily accumulated at presynaptic sites, consistent with the presence of a synapse-targeting signal in the cytoplasmic domain of VAMP2. However, in nerve terminals the VAMP2-transferrin receptor chimera was associated with endosomal compartments rather than SVs. A further sorting decision, mediated by a separate, inhibitory signal within the cytoplasmic domain of VAMP2 (aa 61–70) is required in order for VAMP2 to be targeted from axonal endosomes to SVs. Consistently, deletion of this sequence resulted in shifting of the chimera to SVs. The same inhibitory signal was identified by Grote et al. (Grote et al., 1995; Grote and Kelly, 1996) during a detailed analysis of SLMV targeting and endocytosis efficiencies exhibited by a panel of deletion and point mutants of an epitope-tagged VAMP2 (VAMP2-TAg) expressed in PC12 cells. These studies allowed identifying a sequence (aa 41–50) within the predicted α-helix1 of the cytoplasmic domain responsible for VAMP2 targeting to SLMVs. A point mutation (M46A) within this region severely impaired SLMV targeting. In contrast, amino acid substitutions, which increase the hydrophobicity of the helix (e.g., N49A) enhanced the targeting (Grote et al., 1995). Remarkably, while the positive signals (aa 50–41, Met46) required for VAMP2 targeting to SLMV are also indispensable for endocytosis, the negative signals (aa 61–70, Asn49, Asp44) are dispensable, since their mutations, which enhance targeting to SLMVs, did not affect the endocytosis rate. Importantly, none of these elements conforms to previously identified endocytosis signals (Grote and Kelly, 1996). These results provide evidence that endocytosed VAMP2 is not directly sorted to SLMVs from the plasma membrane: positive sorting signals are required in order for VAMP2 to be endocytosed and sorted to endosomal compartments prior to the targeting to SLMVs, which presumably requires concealing of the negative sorting signals.

Extrapolation of the results obtained in PC12 cells to living neurons as to the role of targeting signals should nevertheless be considered cautiously. Indeed, significant differences have been reported between PC12 cells and neurons from intact animals. Both the M46A and N49A VAMP2 targeting mutants rescued the lethal phenotype of VAMP2-deficient *C. elegans*, and the mutant proteins localized indistinguishably from wild type VAMP2 (Zhao and Nonet, 2001).

Collectively these studies are consistent with the view that VAMP2 undertakes a tortuous trafficking route in order to reach its final destination, regulated by multiple SV targeting and exclusion signals within its primary sequence. It remains to be established whether a precise pattern of protein-protein interactions mediates the activation or the concealing of the various targeting signals of VAMP2 at different stages during its intracellular transport.

4.3.2. Synaptotagmin I

Targeting of synaptotagmin I to presynaptic sites is mediated by palmitoylation of amino-terminal cysteines present in the junction between the transmembrane and cytoplasmic regions of the protein (Kang et al., 2004). Interestingly, the palmitoylated amino-terminus of synaptotagmin VII is not sufficient for synaptic targeting, implying that specific motif(s) may be present only in a subset of the synaptotagmin family members. In particular, presynaptic localization of palmitoylated synaptotagmin I correlates with its internalization efficiency, suggesting the involvement of endocytosis for synaptotagmin targeting to SVs.

Synaptotagmin I internalization relies at least on two distinct signals. A strong, unconventional, internalization motif is present in the carboxy-terminal domain of the protein and appears to be regulated by the C2B domain (Jarousse and Kelly, 2001). In addition, the neuro-specific intralumenal *N*-glycosylation of synaptotagmin I acts in concert with the cytoplasmic domain to direct the protein from the plasma membrane to SVs (Han et al., 2004).

The pathway followed by synaptotagmin I to reach its final SV destination also includes the passage through an endosomal compartment. The carboxy-terminal domain of synaptotagmin I contains a di-hydrophobic motif (ML) which, although dispensable for synaptotagmin I internalization, yet supports part (20%) of its trafficking to SLMVs in PC12 cells. All the traffic of synaptotagmin I to SLMVs mediated by the ML motif is BFA-sensitive, strongly suggesting the involvement of an AP3-dependent route through an endosomal compartment (Blagoveshchenskaya et al., 1999a).

The ability of synaptotagmin I to bind AP2 and direct the nucleation of clathrin-coated pits is suggestive of its involvement in quality control during SV formation from the plasma membrane (Zhang et al., 1994; Haucke and De Camilli, 1999).

Synaptotagmin I displays a high affinity, Ca^{2+}-independent interaction of one of its Ca^{2+} binding domains, C2B, with a region of the μ chain of the AP2 complex (Zhang et al., 1994; Haucke et al., 2000). This binding property appears to be highly conserved in the synaptotagmin family (Li et al., 1995a) and dependent on multimerization of the AP2 binding site (Grass et al., 2004). The interaction among cargo proteins, AP2, and synaptotagmin I appears to be cooperative, since the presence of a peptide containing a tyrosine-based internalization signal enhanced AP2 binding to synaptotagmin I *in vitro* (Haucke and De Camilli, 1999).

Accurate mutagenesis studies revealed that the AP2 binding site is not needed for synaptotagmin internalization. A latent internalization signal located within the carboxy-terminal of synaptotagmin I, which appears to be normally concealed by the C2B domain, is unmasked via a neuro-specific mechanism (Jarousse and Kelly, 2001). The cell-type specificity of this mechanism is demonstrated by prominent surface localization of exogenous synaptotagmin I expressed in fibroblasts (Feany et al., 1993). It has been proposed that, in neurons and neuroendocrine cells, the C2B domain acts as a sensor of intracellular changes in Ca^{2+} levels following vesicle exocytosis and couples these changes to endocytosis by relieving the inhibition on the internalization signal (discussed in Jarousse and Kelly, 2001). Several AP2 binding proteins, such as AP180, endophilin, syndapin, dynamin, intersectin and stonin 2 (see below), might mediate the release of this inhibition upon Ca^{2+} binding to the C2B domain. Two immediate corollaries to this model are the tight coupling between SV exocytosis and endocytosis and the generation of SV containing a crucial component of the fusion machinery such as synaptotagmin I.

The observation that synaptic terminals of synaptotagmin mutants of *C. elegans* are depleted of SVs in the absence of transport defects first implicated this protein in SV re-formation after exocytosis (Jorgensen et al., 1995). More recently, a series of elegant experiments based on live imaging of synapto-pHluorin, a pH-sensitive fluorescent VAMP2 derivative, provided a quantitative analysis of the kinetics of SV endocytosis in both synaptotagmin I *Drosophila* mutants (Poskanzer et al., 2003) and cultured hippocampal neurons from synaptotagmin I knock-out mice (Nicholson-Tomishima and Ryan, 2004). These approaches allowed to dissect the exo–endocytic process into separate components during synaptic activity. In particular, using fluorescence-assisted light inactivation after normal, synaptotagmin I-mediated, exocytosis, Poskanzer et al. (2003) inactivated synaptotagmin I only during the endocytotic process. The picture emerging from these studies shows that, in addition to regulating exocytosis, synaptotagmin I plays an essential role during compensatory endocytosis at the nerve terminal, thus participating in the maintenance of the SV pool. As discussed above, this model provides a means to link the extent of SV exocytosis and endocytosis.

At the synapse, AP2 function is regulated by stonin 2, the human homologue of *Drosophila* stoned B, a presynaptic protein implicated in SV recycling (Martina et al., 2001). Binding to synaptotagmin I allows stonin 2 to be recruited to sites of SV recycling, where it regulates the synaptotagmin I-AP2 interaction during SV endocytosis (Fergestad and Broadie, 2001; Walther et al., 2004).

Because of its ability to function as an AP2 dependent sorting adaptor for SV recycling and to interact with a variety of endocytic proteins, stonin 2 was initially thought to participate in the general machinery that controls SV re-formation from the plasma membrane. In particular, *in vitro* studies (Walther et al., 2004) suggest that the association with stonin 2 renders AP2 incompetent to sort tyrosine motif-containing cargo proteins. This finding led to the proposal that stonin 2, by preventing the constitutive internalization of tyrosine motif-containing plasmalemmal receptors by AP2, may contribute to the specificity of protein sorting to SVs. Nevertheless, the role of stonin 2 in endocytosis appears to be less general than previously hypothesized. Indeed, recent findings identify stonin 2 as the first endocytic adaptor specific for internalization and sorting of a SV protein, namely synaptotagmin (Diril et al., 2006).

Stonin 2 is recruited to the plasma membrane only by some members of the synaptotagmin family (I, II, IX) and increases their endocytic rate by associating with AP2. At variance, endocytosis of the synaptotagmin family members unable to recruit stonin 2 to membranes, as well as of other clathrin/AP2-dependent cargoes is not facilitated by stonin 2. Importantly, stonin 2 selectively enhances the sorting of synaptotagmin I to SLMVs rather than to LDCVs in PC12 cells, and to recycling SVs in neurons. The search for cargo-specific regulators of clathrin-mediated endocytosis has been revealing a challenging, yet extremely promising task (Traub, 2003; Tosoni et al., 2005). Stonin 2 might be the first member of an entirely new group of adaptors dedicated to endocytosis and sorting of specific SV proteins.

Since stonin 2 does not appear to alter the kinetics of synaptotagmin I endocytosis during electrical stimulation at synapse (Diril et al., 2006), it remains to be established whether it operates exclusively in the constitutive pathway of synaptotagmin I internalization rather than during activity-dependent SV retrieval. Alternatively, and more attractively, during stimulation stonin 2/AP2 might function in a regulatory step of synaptotagmin I internalization (i.e., SV endocytosis), which is dissociated from a rate-limiting step of the process.

Compelling support to this possibility comes from a recent study from the Davis's group (Poskanzer et al., 2006), which complements previous reports implicating synaptotagmin I in SV endocytosis (Poskanzer et al., 2003; Nicholson-Tomishima and Ryan, 2004). The Authors tested the influence of different regions of synaptotagmin I on either the rate of stimulation-induced SV retrieval or the ultrastructure of endocytosed SVs by introducing several variants of the protein in synaptotagmin-null flies expressing synapto-pHluorin. Through this approach they managed to show that the fidelity of SV re-formation and the rate of endocytosis can be mechanistically separated and were able to assign the control of the two processes to distinct regions of synaptotagmin I. Ca^{2+}-binding to the C2B, but not C2A, domain influences the rate, but not the fidelity of SV endocytosis. At variance, mutation of a poly-lysine motif in the C2B domain, that is the site of interaction with AP2, leads to

endocytosis of abnormally large SVs without altering the rate of SV retrieval. This finding is in keeping with the involvement of stonin 2/AP2-mediated synaptotagmin I endocytosis in the extent, but not in the rate, of endocytosis (Diril et al., 2006). A possible explanation might be the existence of a rate-limiting step influenced by Ca^{2+}-binding to synaptotagmin I, which controls the speed of SV endocytosis, and keeps the pace with exocytosis, followed by a stonin 2-mediated recruitment of AP2 which controls the extent, but not the speed, of SV retrieval.

The overall resemblance of the phenotype of SV protein mislocalization at extra-synaptic sites reported in the *lap* mutant (Bao et al., 2005) with the mislocalization of synaptotagmin I and cysteine-string protein reported in *stoned* mutant flies (Fergestad and Broadie, 2001) points to a crucial role for adaptor complexes in maintaining the fidelity of protein sorting during the SV life cycle.

4.3.3. Glutamic acid decarboxylase 65

Similarly to synaptotagmin I and VAMP2, the targeting of the smaller isoform of the GABA-synthesizing enzyme glutamate decarboxylase (GAD), GAD65, to SV presynaptic clusters is controlled by multiple signals (Kanaani et al., 2002). GAD65 is synthesized as a soluble hydrophilic molecule but undergoes amino-terminal post-translational modification, resulting in targeting to the Golgi compartment en route to presynaptic sites. Three separate signals are required for this process. Two separate sequences in the amino-terminus of GAD65 are required for targeting of the cytosolic protein to Golgi membranes. In addition, palmitoylation of two cysteines is critical for trafficking of GAD65 from Golgi to presynaptic SV clusters via a pathway, which involves Rab5-positive endosomes and is shared with several other SV proteins (Kanaani et al., 2002, 2004). The evidence that reduction in cellular cholesterol levels impairs targeting of GAD65 to presynaptic clusters led to propose that palmitoylation of GAD65 mediates its attachment to specialized membrane microdomains in the TGN, resulting in lateral segregation from non-palmitoylated proteins before the formation of axonal transport carriers (Kanaani et al., 2002, 2004; see below).

4.3.4. Synaptogyrin I

Deletion and mutation analysis of synaptogyrin I, an abundant tetraspan SV protein distantly related to synaptophysin, has defined two sequence motifs responsible for its targeting to SVs (Zhao and Nonet, 2001). A single arginine in one of the cytoplasmic loops and a 38 amino acid sequence in the carboxy-terminal domain seem to be involved in the sorting of synaptogyrin I to SV precursors at the TGN and in endocytosis of the protein from the plasma membrane, respectively. Deletion of three tyrosines in the carboxy-terminal region is sufficient to cause accumulation of synaptogyrin at the plasma membrane, thus implicating tyrosine-mediated interaction with AP2 complex in synaptogyrin endocytosis. Although the signal sequences identified in synaptogyrin I do not share homology with other SV protein-sorting sequences, it is interesting to note that also the long, cytoplasmic carboxy-terminal domain of synaptophysin I appears to contain a signal for endocytosis (Linstedt and Kelly, 1991b).

4.3.5. Vesicular neurotransmitter transporters

Although belonging to the same family of transporters, the vesicular monoamine transporters (VMATs) and the vesicular acetylcholine transporter (VAChT) localize to distinct populations of secretory vesicles. The neuron-specific VMAT2 is preferentially targeted to large dense core vesicles (LDCVs) rather than SVs (Nirenberg et al., 1995). Heterologous expression of VMAT2 in PC12 cells shows VMAT2 primarily localized to LDCVs as the endogenous VMAT1. At variance, VAChT localizes predominantly to SVs in neurons and to SLMVs in PC12 cells (Weihe et al., 1996; Liu and Edwards, 1997; Tao-Cheng and Eiden, 1998). It is important to remember that LDCVs, at variance with SVs, are directly budded from the TGN and undergo a maturation process that involves the removal of proteins destined for other compartments (Tooze and Stinchcombe, 1992).

Sorting signals that interact with cytosolic sorting machineries are responsible for the selective targeting of VMATs and VAChT to different classes of vesicles. A dileucine motif located in the carboxy-terminal domain of both VMAT2 and VAChT is required for endocytosis of the transporters (Tan et al., 1998), while amino acid residues proximal to the dileucine motif contribute to their differential sorting in PC12 cells (Krantz et al., 2000). A carboxy-terminal dileucine motif is also important for the endocytosis of the vesicular glutamate transporter VGLUT1 (Voglmaier et al., 2006). In some cases, dileucine motifs involved in endocytosis require the presence of acidic residues at position-4 and -5 relative to the two leucines, and VMATs contain highly conserved glutamates at both positions. Interestingly, substitution of these residues with alanines does not prevent VMAT endocytosis (Tan et al., 1998) but reduces its targeting to LDCVs (Krantz et al., 2000), indicating that the dileucine motif functions at multiple trafficking steps. In contrast, VAChT contains a serine in position-5 relative to the dileucine motif. PKC-operated phosphorylation at this site introduces a negative charge resembling the glutamate present at the same position in VMATs, and enhances VAChT targeting to LDCVs, whereas prevention of phosphorylation enhances targeting to SLMVs (Krantz et al., 2000).

Recently, GST pull-down assays from rat brain extracts coupled to deletion and site-specific mutagenesis studies have shown that the dileucine motif in the VAChT carboxy-terminal domain is a signal for interaction with the AP1 complex, whereas a non-classical tyrosine-based signal interacts with the AP2 complex (Kim and Hersh, 2004). The two motifs act in concert to permit efficient internalization of the transporter. In addition, the carboxy-terminal tail of VAChT contains a classical tyrosine-based sorting motif that also contributes to the sorting of the transporter to SVs (Kim and Hersh, 2004).

The role of the dileucine motif in AP1 binding is at odds with its requirement for VAChT endocytosis. Indeed, AP1 complexes mediate protein transport from the TGN to either endosomes/lysosomes or the plasma membrane and from

endosomes to TGN, and no evidence supports a role for AP1 in endocytosis. It has been proposed that VAChT is directly sorted from the TGN into immature LDCVs by interaction with AP1, and then moves to endosomes before targeting to SVs (Kim and Hersh, 2004; Varoqui and Erickson, 1998). Thus, preventing the interaction with AP1 might cause missorting of VAChT from regulated to constitutive secretory pathways at the TGN, causing surface accumulation of the transporter. It remains to be tested whether the phosphorylation/dileucine-containing motif in VAChT, besides being involved in AP1 binding, also promotes the interaction of the tyrosine-based motif with AP2, thus enhancing endocytosis of the transporter.

A role for the dileucine motif in VAChT endocytosis has also been uncovered by monitoring the trafficking of a GFP-tagged version of VAChT in the cholinergic cells SN56 (Santos et al., 2001). At variance with the wild type protein, which was targeted to endocytic organelles in the soma and varicosities, a mutant transporter lacking the dileucine motif accumulated at the plasma membrane in the cell body, and exhibited reduced targeting to neurites and varicosities. Therefore, VAChT is first delivered to the plasma membrane in the cell body before undergoing endocytosis and recycling through endosomal compartments en route to SVs. It has also been shown that cAMP-dependent protein kinase (PKA) activity is required for VMAT2 targeting to LDCVs while it is dispensable for VAChT trafficking (Yao et al., 2004), further indicating that distinct mechanisms operate during sorting of these vesicular transporters.

4.3.6. P-selectin

Even though P-selectin does not belong to the endogenous complement of SV proteins, its heterologous expression in PC12 cells has been instrumental in defining the signals required for protein sorting to SLMVs. Targeting of P-selectin has been extensively investigated by Cutler and collaborators by expressing an HRP-tagged version of the protein in PC12 cells and following HRP activity through subcellular fractionation. In PC12 cells, P-selectin is efficiently targeted to SLMVs via a BFA-sensitive route, which relies on distinct internalization and vesicle-targeting signals. Mutagenesis studies show that a tyrosine-based motif within the carboxy-terminal cytoplasmic tail is critical for trafficking of P-selectin to both SLMVs (at the level of early endosomes) and dense-core granules (at the level of the TGN), whereas additional determinants are responsible for the progression of P-selectin through endosomal intermediates en route to SLMVs (Norcott et al., 1996; Blagoveshchenskaya et al., 1999b). These intermediate donor compartments correspond to both early and late endosomes. Interestingly, partially overlapping sequences located in the cytoplasmic domain of P-selectin are responsible for its BFA-sensitive sorting to SLMVs from different sites along the endocytic pathway, namely late or early endosomal compartments. Analysis of mutants bearing deletions in both sorting sequences suggests that P-selectin travels to both SLMVs and lysosomes via the same two subsequent endosomal intermediates (Blagoveshchenskaya and Cutler, 2000).

4.3.7. Synaptophysin I

Synaptophysin I, a major and specific component of SVs, is a tetraspan membrane protein with both the amino- and the carboxy-termini on the cytosolic side of the SV membrane (see Valtorta et al., 2004 for a review). In neuroendocrine cells, synaptophysin I enters a constitutive transport pathway at the TGN, undergoes recycling at the plasma membrane and is eventually sorted onto SLMVs (Regnier-Vigouroux et al., 1991). The trafficking of synaptophysin I in PC12 cells has served as a paradigm for the protein targeting to SVs, yet signals responsible for its sorting remain elusive. *N*-Glycosylation of the first intravesicular loop of synaptophysin I is not required for its targeting to SLMVs (Leube et al., 1989).

Noteworthy, when expressed in cultured neurons, a GFP-tagged derivative of synaptophysin I targets to SVs more efficiently than fluorescent chimeras of other SV proteins (Pennuto et al., 2003). The reasons for this high selectivity are unknown, even though interactions with proteins or lipid components of the SV membrane might be relevant. Interestingly, the overexpression of either synaptogyrin I or its non-neuronal paralog cellugyrin, distantly related to synaptophysin I, promotes SLMV biogenesis and dramatically increases the targeting of synaptophysin I to SLMVs in PC12 cells (Belfort et al., 2005; Belfort and Kandror, 2003). In addition, synaptophysin I binds to cholesterol (Thiele et al., 2000) and this interaction may underlie the preferential targeting of the protein to SVs, which typically contain high levels of cholesterol.

Homo- and hetero-complexes orchestrated by synaptophysin I might also contribute to the targeting of other SV components. Synaptophysin I forms homo-oligomers, from dimers to hexamers, *in vitro* (Jahn et al., 1985; Rehm et al., 1986; Thomas et al., 1988; Johnston and Südhof, 1990) as well as *in vivo* (Pennuto et al., 2002), and the dissociation of these complexes parallels the complete fusion of SVs with the plasma membrane (Pennuto et al., 2002). The assembly of homo-oligomers may facilitate cholesterol accumulation in the plane of the plasma membrane (or possibly endosomal membranes), thus driving synaptophysin I onto the nascent vesicle and perhaps contributing to the recruitment of other SV proteins localized within the cholesterol-rich microdomain. Indeed, synaptophysin I forms hetero-oligomers with various nerve terminal proteins, including VAMP2 (Calakos and Scheller, 1994; Washbourne et al., 1995; Edelmann et al., 1995; Pennuto et al., 2002), the vesicular proton pump V-ATPase (Thomas and Betz, 1990; Galli et al., 1996; Carrion-Vazquez et al., 1998), myosin V (Prekeris and Terrian, 1997), dynamin I (Daly et al., 2000; Daly and Ziff, 2002), and the adaptor AP1 (Horikawa et al., 2002).

The interaction of synaptophysin I with dynamin and cholesterol may underlie the participation of synaptophysin I in both clathrin-independent and clathrin-dependent mechanisms of endocytosis (Daly et al., 2000; Daly and Ziff, 2002). Synaptic vesicle re-formation by endocytosis might also be favored by the presence of an unconventional internalization signal in the long carboxy-terminal tail of synaptophysin I (Linstedt and Kelly, 1991b). The participation of synaptophysin

I in SV biogenesis has also been suggested by the heterologous expression of the protein in epithelial cells (Leube et al., 1989, 1994). In this heterotypic context, synaptophysin I appeared in a class of vesicles, morphologically indistinguishable from SLMVs of neuroendocrine cells. In addition, in retinal rod photoreceptors from synaptophysin I knock-out mice, which lack other members of the synaptophysin family, a reduction in the number of SVs accompanied by an increase in SV size and increase in clathrin-coated vesicles was reported (Spiwoks-Becker et al., 2001). However, it is unlikely that synatophysin I plays an essential role in SV life cycle given the lack of profound alterations in nervous system development and SV recycling in synaptophysin I knock-out mice (Eshkind and Leube, 1995; McMahon et al., 1996) as well as in a recently generated *C. elegans* mutant deficient in all tetraspan SV proteins (Abraham et al., 2006).

Although synaptophysin I might not play a general role in SV assembly, yet it has been implicated in the selective regulation of VAMP2 sorting to SVs (Pennuto et al., 2003). Stimulation-induced dissociation of the synaptophysin I-VAMP2 complex precedes SV exocytosis and might be a prerequisite to make SVs competent for exocytosis (Pennuto et al., 2002; Reisinger et al., 2004; Bonanomi et al., 2005a). Indeed, only after release from synaptophysin I, VAMP2 becomes available for participating in the assembly of fusogenic SNARE complexes (Edelmann et al., 1995). In keeping with this model, the enhancement of neurotransmitter release that follows pharmacological blockade of postsynaptic receptors is accompanied by dissociation of synaptophysin I-VAMP2 complexes (Bacci et al., 2001).

The formation of hetero-complexes also underlies the ability of synaptophysin I to govern the targeting of VAMP2 to SVs (Pennuto et al., 2003). When expressed in cultured hippocampal neurons exogenous VAMP2 is missorted to the axonal plasma membrane, whereas the co-expression of comparable doses of synaptophysin I enhances the recruitment of VAMP2 to SVs. This effect is selective for VAMP2 and requires hetero-complex formation. Indeed, other recombinant SV proteins, which also display the intrinsic tendency to accumulate to the axonal surface but which do not interact with synaptophysin I, are not recruited to SVs by co-expression with synaptophysin I. At present it is unknown whether synaptophysin I redirects newly synthesized VAMP2 to SV precursors already at the level of the TGN or directly in the nerve terminal, at either the plasma membrane or endosomes.

4.3.8. Proteins reversibly associated with the SV membrane

Sorting and targeting of extrinsic SV proteins to their final SV destination could be accomplished either by the presence of specific targeting signals (primary targeting) that direct them towards nerve terminals and/or by specific interactions with primarily targeted SV components (secondary targeting). The major proteins associated reversibly with the SV membrane are the synapsins, Rab3A-C and rabphilin. They all lack transmembrane regions that can mediate their incorporation into the membrane bilayer and their membrane association is obtained by reversible interaction with either SV phospholipids or SV proteins or both. Interestingly, all these proteins cycle between SV-associated and cytosolic forms and, upon dissociation from SVs, disperse into neighbouring preterminal axonal domains during synaptic activity (Fischer von Mollard et al., 1991, 1994; Chi et al., 2001, 2003; Bonanomi et al., 2005b; Star et al., 2005).

The membrane attachment of the monomeric G protein Rab3 is achieved through the post-translational modification of two COOH-terminal cysteines by geranylgeranylation, although about 30% of prenylated Rab3 remains in a cytosolic store in a complex with Rab-guanine nucleotide dissociation inhibitor (GDI). On SVs, Rab3 is present in the GTP-bound state, in association with SV effectors such as rabphilin and synapsin that bind only or preferentially to GTP-Rab3. During Ca^{2+}-dependent exocytosis, hydrolysis of GTP promotes dissociation of GDP-Rab from SVs and trapping by Rab-GDI. Rab3 eventually reassociates with endocytosed SVs under the GDP-bound state by binding to protein components of SVs that may include synapsin (Chou and Jahn, 2000; Giovedi et al., 2004a). The observation that clathrin-coated SVs are relatively devoid of Rab3 (Maycox et al., 1992, but see Blondeau et al., 2004) indicates that Rab3 dissociates from SVs before endocytosis and reassociates with them soon after uncoating.

At variance with Rab3, both synapsins and rabphilin do not display prenylated residues, and their SV association relies on interactions with SV components. The binding of rabphilin to SV-associated Rab3 is a key determinant of its association with the SV membrane and, consistently, rabphilin follows the Rab3 fate and dissociates from SVs during activity (Geppert et al., 1994a; Stahl et al., 1996; Geppert and Südhof, 1998). In fact, while no mistargeting of Rab3 was observed in rabphilin knockout mice, rabphilin levels were decreased by 70% in Rab3 knockout mice (Geppert et al., 1994a; Schluter et al., 1999). Besides the necessity of Rab3 binding for membrane-specific targeting, an efficient rabphilin association with SVs is also contributed by its COOH-terminal region containing two C2 Ca^{2+}/phospholipid binding domains (McKiernan et al., 1996; Senbonmatsu et al., 1996). Once bound to Rab3 on the SV, rabphilin may stabilize both Rab3 and its binding by inhibiting GAP-activated Rab3 GTPase activity (Kishida et al., 1993; Sakane et al., 2006).

On the other hand, association of synapsins with SVs occurs through interactions with both the phospholipid membrane and SV-associated proteins, including CaM kinase II and c-Src independently of Rab3 (Benfenati et al., 1992; Onofri et al., 1997; Hosaka et al., 1999; Giovedi et al., 2004b). Once on the SV, synapsins accelerate Rab3 cycle by activating Rab3 GTPase activity and facilitating Rab3 stripping from GDI and its reassociation with SVs (Giovedi et al., 2004b).

As far as sorting of peripheral SV protein is concerned, during development both Rab3 and synapsin are first concentrated in cell bodies of developing neurons and then disappear from perykaria and start to concentrate in a polarized fashion in the neuropil of the axon to finally localize exclusively to nerve terminals (Fletcher et al., 1991; Matteoli et al.,1991; Stettler et al., 1994). When synaptic contacts are

formed, synapsin rapidly accumulates at the sites of contacts, which were found by electron microscopy to coincide with presynaptic clusters of synaptic vesicles. This local concentration of synapsin is thought to play an important role in the structural and functional maturation of the evoked release mechanisms and of the nerve terminal ultrastructure (Lu et al., 1992; Valtorta et al., 1995; Fiumara et al., 2001, 2004; Bonanomi et al., 2005b).

The subcellular distribution of synapsin and Rab3 during neuronal development overlaps considerably with that of the integral SV proteins such as synaptophysin or synaptotagmin, except for the fact that, at the Golgi complex, the presence of significant levels of integral SV proteins is not accompanied by detectable levels of peripheral SV proteins (Fletcher et al., 1991; Matteoli et al., 1991). This observation strongly indicates that a considerable fraction of synapsin and Rab3 associates with SV precursors soon after their formation in the cell soma and is transported along the axon even at stages preceding synapse formation. This view was confirmed by experiments aimed at elucidating the anterograde and retrograde transport of integral and peripheral SV proteins along the axon. Synapsins display two different rates of anterograde transport, namely a fast rate typical of membrane components of SVs and a slow rate overlapping with the velocity of a group of cytoskeletal proteins which include a subfraction of actin and spectrin (Baitinger and Willard, 1987). The latter finding suggests that at least a fraction of cytoskeleton-bound synapsin associates with SVs only in nerve terminals. The observation that synapsins and Rab3 travel at a fast rate is consistent with the finding that, after a nerve crush or ligation, both proteins rapidly accumulate in parallel with integral SV proteins proximal to the crush site (Booj et al., 1989; Li et al., 1995b, 1996; Li and Dahlstrom, 1997). Interestingly, at variance with the behavior of integral SV proteins, both synapsin and Rab3 did not substantially accumulate distally to the nerve crush or ligation (Booj et al., 1989; Li et al., 1995b), implying that SV membranes moving retrogradely from the nerve terminal to the cell body do not carry appreciable amounts of peripheral proteins, which are presumably degraded in nerve terminals.

The sorting/targeting determinants within the multiple synapsin domains were investigated in detail. To this aim, either synapsin Ib, its NH_2-terminal region (domains A–C) or its COOH-terminal region (domains D and F) were expressed in the photoreceptor cells of transgenic mice that lack expression of the endogenous protein. The results show that both the full-length and the NH_2-terminal region, but not the COOH-terminal region were efficiently targeted to SVs, indicating that a major targeting determinant is present in the NH_2-terminal domains, which are largely homologous in all synapsin isoforms (Geppert et al., 1994c). More recently, the analysis of the specific nerve terminal targeting of GFP chimeras of single or combined synapsin domains demonstrated that the correct localization of synapsins to SVs requires multiple sites, including domains A–C, that are shared by all isoforms, and domain E restricted to all A-isoforms (Gitler et al., 2004). As domains with a negative influence on targeting, such as domain D, also exist in the synapsin molecule, the B-isoforms lacking the positive influence of domain E can be primarily targeted with less efficiency. However, synapsin isoforms undergo extensive homo- and hetero-oligomerization mediated by domain C (Esser et al., 1998; Hosaka and Südhof, 1999), and therefore the possibility exists that some synapsin isoforms can be indirectly targeted to SVs through heterodimerization with synapsins possessing stronger targeting determinants.

4.4. Regulation of vesicular content by sorting of neurotransmitter transporters

Heterogeneity in the distribution of vesicular zinc content at the single-neuron level suggests that the SV-associated zinc transport protein ZnT3 could be subjected to the control of local mechanisms affecting its transport activity. In fact, the AP3-dependent sorting of either chloride channels or the vesicular glutamate transporter 1 and ZnT3 are reciprocally regulated (Salazar et al., 2004a; Salazar et al., 2005a). These findings indicate that the biogenesis of SVs is likely to be much more elaborate than previously envisioned, pointing to the existence of a sequential process in which multiple, differentially regulated sorting pathways converge on the same vesicle to define its membrane composition and lumenal content. In particular, changes in the amount and relative distribution of vesicular neurotransmitter transporters have the potential to profoundly affect neurotransmission, given that the quantal size is directly linked to the membrane content of vesicular transporters (Fremeau et al., 2004; Wojcik et al., 2004; Song et al., 1997). Appropriate sorting of vesicular transporters is critical for synaptic functions. Heterologous expression of VGLUTs is sufficient to induce glutamate release from inhibitory neurons (Takamori et al., 2000; Takamori et al., 2001), and mistargeting of the vesicular GABA transporter VGAT in μ3B−/− mice lacking functional AP3B impairs inhibitory synaptic transmission and causes an epileptic phenotype (Nakatsu et al., 2004).

The two main isoforms of the VGLUT family, VGLUT1 and VGLUT2, exhibit complementary patterns of expression in the adult brain, with VGLUT1 being present in the cortex and VGLUT2 in the brainstem (Fremeau et al., 2001; Varoqui et al., 2002). During development the two isoforms are transiently coexpressed by the same cells in the cortex but segregate to distinct synaptic sites associated with different release kinetics (Fremeau et al., 2004, but see Wojcik et al., 2004). Importantly, in VGLUT1 knockout mice the vast majority of excitatory synapses which normally express this isoform are silent, probably reflecting the fusion of empty vesicles (Fremeau et al., 2004). Indeed, empty SVs have been shown to recycle at normal rates, both when the vesicular uptake is blocked by drugs (Ceccarelli and Hurlbut, 1975) and when expression of the transporters is prevented by gene ablation (Wojcik et al., 2004; Croft et al., 2005). These findings indicate that the quantal nature of neurotransmitter release depends on the reliability of vesicle filling, ultimately dictated by the control of transporter expression and sorting, rather than on regulatory mechanisms preventing the fusion of unfilled SVs. Remarkably, the sorting of VGLUT1 to SVs is subjected to a tight control by

the circadian clock (Yelamanchili et al., 2006). In spite of the lack of changes in the total amount of VGLUT1 protein in brain homogenate and synaptosomes (i.e., the starting material of the SV preparation) pure SVs prepared at different times during the day exhibit oscillations in the levels of VGLUT1. At variance, SVs prepared from mice lacking period 2, a critical component of the circadian clock, or kept in darkness, show no circadian changes of VGLUT1. Other SV proteins do not display light-dependent oscillations. Thus, at individual terminals a light-dependent, specific sorting mechanism accounts for the regulated targeting of VGLUT1 from a donor compartment, most likely the plasma membrane, to SVs.

A further level of regulation has been suggested by the ultrastructural analysis of excitatory synapses in the hippocampus and cerebellum of VGLUT1 knockout mice (Fremeau et al., 2004). This study revealed a dramatic reduction in the number of SVs at these synapses relative to wild-type littermates, resulting from selective alteration of the reserve SV pool, and accumulation of irregularly shaped SVs and elongated tubulovesicular membranes absent in wild-type synapses. Levels of synapsin I and Rab3, major regulators of SV organization and recycling, are also substantially reduced in the knockout mice, whereas other SV proteins are relatively unaffected. These data first implicate vesicular neurotransmitter transporters in SV biogenesis, arguing for a feedback regulatory mechanism which maintains an intimate coupling between transporter levels and the total number of SVs. Surprisingly, a fundamental difference between SV biogenesis in glutamatergic and dopaminergic neurons emerged from the analysis of VMAT2 knockout mice (Croft et al., 2005), which do not show changes in ultrastructure, distribution and number of SVs in dopamine-releasing terminals, thus indicating that VMAT2 is not required for SV biogenesis.

5. Functional heterogeneity in the synaptic vesicle population

5.1. Organization of synaptic vesicles in functional pools

SVs are organized in functionally distinct pools within the synapse. The classical view of SV pools is based on both morphological parameters and functional properties of SVs. Thus, the small number of SVs (5–10% of the total pool), which resides in the RRP are docked at the active zone and are competent for release elicited by depolarization or hypertonic stimulation. A separate pool of SVs, the 'reserve pool', comes into play during sustained high-frequency stimulations.

At the frog neuromuscular junction the RRP and the reserve pool use different strategies for recovery after depletion. The RRP is exhausted by brief, high-frequency nerve stimulation, and endocytosed SV either repopulate the recycling pool (Richards et al., 2003) or are recruited in the reserve pool and hence are not released by subsequent stimulation (about 30% of recycled vesicles; Rizzoli and Betz, 2004). Nevertheless, the RRP is refilled in 1 min exclusively by direct endocytosis from the plasma membrane instead of mobilization from the reserve pool. At variance, the reserve pool is depleted during prolonged, high-frequency stimulation and recovers slowly (half-time 8 min) through SV endocytosis from cisternae and plasma membrane infoldings (Richards et al., 2003) (Fig. 3). The preferential targeting of endocytosed SV to the pool of origin may arise from their tagging with distinctive markers, which are sorted to the re-forming vesicle at the plasma membrane or endosomal compartments. However, this description does not apply to all synapses. Indeed, in cultured hippocampal neurons neurotransmission during high-frequency tetanic stimulation is maintained by SV recycling (i.e., reuse), whereas mobilization from the reserve pool is exploited at lower-frequency stimulation (Sara et al., 2002). A different report addressing the post-endocytic traffic of SVs in hippocampal neurons expressing synapto-pHluorin underlies the importance of mobilization from the reserve pool rather than vesicle reuse to replace SVs of the RRP that have undergone exocytosis (Li et al., 2005).

Synaptic vesicle pools are classically believed to reflect localization of vesicles at distinct sites within the nerve terminal (Kuromi and Kidokoro, 1998). Recent findings have challenged this view, so that spatial segregation between SVs is no longer considered the sole factor regulating the formation of functional vesicle pools. Indeed, at the frog neuromuscular junction tracking of SVs of the RRP during recycling in the presence of FM 1-43, followed by dye photoconversion and ultrastructural analysis, showed that SV are first endocytosed at sites away from the active zones and within minutes become distributed randomly throughout the SV cluster instead of being concentrated in the proximity of the plasma membrane as previously shown (Schikorski and Stevens, 2001). Subsequent stimulation induces exocytosis of labeled SVs located at the surface of the vesicle cluster (Rizzoli and Betz, 2004).

Photoconversion of newly endocytosed SVs, labeled with FM dyes, reveals the existence of a large 'resting pool' of SVs, which do not undergo recycling even under strong stimulation (Südhof, 2000; Harata et al., 2001). The number of non-recycling SVs varies from 50 to 80% of the total SV pool (on average constituted by 30–45 SVs at hippocampal synapses) depending on the type of synapses analyzed and the experimental conditions (Harata et al., 2001; Li et al., 2005; Darcy et al., 2006). For instance, in hippocampal neurons resting vesicles encompass 50% of the total SV pool at excitatory synapses and 30% at inhibitory synapses, as determined by synapto-pHluorin-based analysis of SV recycling (Li et al., 2005). A large non-recycling pool of SVs comprising 30–40% of the entire vesicle pool has also been described at the *Drosophila* neuromuscular junction (Poskanzer and Davis, 2004).

Whatever the exact number, it is apparent that a substantial amount of SVs are in a 'dormant' state within the nerve terminal. The molecular explanation of such refractoriness to participate in recycling is as yet unclear. 'Dormant' SVs might be characterized by a protein complement that makes them reluctant to recycle and/or might be located in an unprivileged position relative to the presynaptic machinery for exocytosis. In support of the latter possibility, in the *shibire* flies exocytosis of resting vesicles is triggered under conditions of endocytic

blockade, which depletes the recycling SV pool (Poskanzer and Davis, 2004). These results have been interpreted as an indication that SVs of the 'resting pool' are prevented from approaching the release sites by the pool of recycling SVs located in the proximity of the active zones. In addition, this study shows that non-recycling SVs are competent for exocytosis. Nevertheless, though at the frog neuromuscular junction SVs recruited for exocytosis are preferentially located at the margins of the vesicle cluster, spatial constraint imposed by other vesicles does not appear to be sufficient to impede SV mobilization, and proximity to active zones is not a requirement for recycling SVs (Rizzoli and Betz, 2004).

A population of SVs reluctant to undergo exocytosis has also been described within the RRP of glutamatergic synapses, but surprisingly not GABAergic synapses (Moulder and Mennerick, 2005). The key observation of this study was that in excitatory terminals estimates of the size of the RRP obtained by hypertonic stimulation appeared to be several folds greater than estimates derived from action potential trains commonly assumed to deplete the RRP. Reluctant SVs are nevertheless competent for release, since can be forced to recycle by strong depolarization and supra-physiological Ca^{2+} influx. Several mechanisms may underlie vesicle reluctance (see Moulder and Mennerick, 2006), including molecular specification of SVs, their proximity to Ca^{2+} channels or the spatial extent of Ca^{2+} microdomains at individual boutons (Beaumont et al., 2005).

Functional diversity of SVs of the same terminal in the absence of anatomical equivalents is also extended to the preference for different recycling pathways. In dissociated hippocampal neurons, spontaneously recycling SVs display limited cross-talk with SVs that populate the activity-dependent recycling pool (Sara et al., 2005). Spontaneously endocytosed vesicles are more likely to be reused spontaneously, whereas they are only scarcely available for evoked release.

The spontaneously and activity-dependent recycling vesicle pools may rely on distinct priming processes, as inferred by the different sensitivity of the two populations to the diacylglycerol analog PMA (phorbol 12-myristate 13-acetate), which regulates the priming machinery (Virmani et al., 2005; Rhee et al., 2002). PMA triggers mobilization from the reserve pool of SVs belonging to the activity-dependent recycling pool while leaving SVs of the spontaneously recycling pool unaffected. When PMA is applied in the absence of stimulation, SVs that would normally be released under activity are recruited for spontaneous recycling, yet after endocytosis they return to populate the activity-dependent recycling pool (Virmani et al., 2005). Importantly, in VAMP2-deficient neurons activity-dependent and spontaneously recycling vesicle pools mix randomly, so that SVs endocytosed either spontaneously or upon stimulation undergo spontaneous exocytosis at similar rates (Sara et al., 2005). This observation suggests that the molecular complement of SVs may influence their recruitment in different functional pools.

Further support to the idea of a limited intermingling among pools of SVs competent for the different modes of exo–endocytosis comes from the molecular dissection of the endocytic pathways operating in retinal bipolar cells. Selective blockade of the slow clathrin-dependent pathway prevented retrieval of a fraction of SVs that were not substituted by vesicles undergoing endocytosis through the fast clathrin-independent mechanism (Jockusch et al., 2005).

5.2. Molecular variability and competence maturation

Separation of SVs among functional pools does not imply spatial segregation of vesicles but rather may result from the tagging of SVs with a unique molecular repertoire and/or the presence of molecular scaffolds that maintain SVs in distinctive, yet ultrastructurally undistinguishable locations within the SV cluster.

Heterogeneity in the protein complement of SVs may arise from differences in biogenetic origin, trafficking routes, age of the vesicles (see Duncan et al., 2003), and subtle variability in the sorting process as the vesicle cycles through the plasma membrane or intracellular compartments. Molecular diversity within the RRP may provide an explanation for the high levels of variability in the release probability found at central glutamatergic synapses (Hessler et al., 1993; Rosenmund et al., 1993) and may underlie a new form of plasticity based on the use of functionally distinct vesicle pools (Moulder and Mennerick, 2005).

Might the stepwise assembly of SVs provide a source of heterogeneity? The sequential enrichment of proteins during the endo–exocytic cycle of SVs implies that vesicles with different membrane composition can be present at the same time in the nerve terminals. This assumption contrasts with the concept that all SVs from a particular neuron have uniform composition and possess at least one member of each family of SV proteins (Südhof et al., 1993; Takamori et al., 2000). However, it should be considered that although biochemical analysis of isolated SVs argues for uniformity of their protein complement, minor differences might have escaped detection. In addition, it is also likely that vesicles isolated by currently available procedures represent only a subset of the whole population. Of note, purification of the total fraction of SVs from rat synaptosomes revealed that this preparation represents a mixture of at least two individual vesicular populations, which differ in buoyant density and sedimentation. Remarkably, one of the vesicle pools carries some plasma membrane proteins (aminopeptidase, the neuro-specific isoform of the glucose transporter protein GLU3) in addition to specific SV proteins. The presence of these extra-proteins is accompanied by decrease in the specific content of certain non-regulatory components of the SVs, such as the proton pump (Thoidis et al., 1998). It has been put forward that these vesicles may be 'maturing' SVs isolated during their cycling between donor membrane compartments.

Results obtained by the study of *mocha* mice, which lack functional AP3 complexes, highlight the physiological impact of variability in the SV repertoire. At least some of the neurological deficits reported in the *mocha* mouse can be attributed to the apparent loss of vesicular zinc in several brain regions, due to the lack of the SV-associated zinc transport protein ZnT-3 (Kantheti et al., 1998). Remarkably, this

phenotype is due to alteration in a subpopulation of SVs that contain the zinc transporter or in the targeting of a discrete set of SV proteins, rather than to a generalized defect in SV biogenesis. Further reports showed that ZnT3 and synaptophysin are preferentially targeted to distinct brain SV populations due to their differential interaction with the AP3 complex. While targeting of ZnT3 to SVs occurs via the AP3 pathway, synaptophysin seems to be directly sorted to SVs from the plasma membrane, likely via the AP2/clathrin pathway (Salazar et al., 2004b), supporting the exciting possibility that the preferential use of alternative pathways of SV biogenesis underlies the generation of distinct SV populations within the same nerve terminal.

The idea that a certain degree of heterogeneity is present in the SV population has been tested directly in the case of synaptotagmins. Analysis of SVs purified from adult rat brain cortex revealed a partially overlapped pattern of distribution of synaptotagmin I, II and IV in the same population of SVs (Osborne et al., 1999). Synaptotagmin I and II are present on the same SV, and heterodimerize efficiently in a Ca^{2+}-dependent manner, whereas synaptotagmin IV shows incomplete overlap with the other two isoforms and does not exhibit Ca^{2+}-dependent interaction with synaptotagmin I.

These results led to the proposal that the repertoire of synaptotagmin isoforms with different Ca^{2+}-binding features present on a single SV determines its exocytotic properties. This prediction was tested *in vivo* in *Drosophila*, where synaptotagmin IV bears an amino acid substitution, which abolishes its ability to bind membranes in response to Ca^{2+} influx. Synaptotagmin I and IV colocalize on the same population of SVs in *Drosophila* and the formation of hetero-oligomers decreases the ability of synaptotagmin I to penetrate membranes, resulting in downregulation of synaptic transmission owing to less efficient coupling between Ca^{2+} entry and secretion (Littleton et al., 1999). Hence, changes in the relative abundance of distinct synaptotagmin isoforms on the same SV may modulate the Ca^{2+} sensitivity of vesicle fusion. Interestingly, a pool of highly Ca^{2+}-sensitive vesicles which exhibit fast kinetics of release has been uncovered in the calyx of Held (Schneggenburger and Neher, 2000; Bollmann et al., 2000), ribbon synapses (Beaumont et al., 2005; Thoreson et al., 2004) and chromaffin cells (Yang et al., 2002).

Functional heterogeneity of SVs may also be linked to the use of different VAMP family members in the fusion process. Indeed, variability in the distribution of the v-SNARE isoforms VAMP1 and VAMP2 at a single terminal of the same neuron (Morgenthaler et al., 2003) may impinge on the release properties of these synapses. Interestingly, since at least some of the VAMPs (such as VAMP2 and the non-neuronal isoform cellubrevin/VAMP3) appear to be functionally interchangeable (Bhattacharya et al., 2002; Deak et al., 2006), their differential expression patterns and trafficking pathways are crucial for the specificity of vesicle fusion. A developmental switch in VAMP isoform expression might contribute to the insensitivity of SV recycling to tetanus toxin (which cleaves VAMP2; Schiavo et al., 1992) in developing axons and in a subset of mature synapses (Verderio et al., 1999). It is possible that tetanus-toxin insensitive TI-VAMP/VAMP7, which is implicated in neurite outgrowth and localized to organelles that do not contain VAMP2 (Coco et al., 1999; Martinez-Arca et al., 2000), participates in this alternative form of vesicle recycling.

Heterogeneity in the content of vesicular neurotransmitter transporters might have profound effects on synapse physiology. Since recycling of empty or partially filled SVs is not prevented (Wojcik et al., 2004; Croft et al., 2005) it is possible that during cycling through donor compartments SVs incorporate variable amounts of vesicular neurotransmitter transporters, perhaps even generating a pool of empty vesicles which do not contribute to neurotransmission. The fine tuning of VGLUT1 sorting by circadian clock argues for the existence of physiologically relevant mechanisms to increase plasticity of the synaptic response at the same nerve terminals by modulating the levels of SV filling (Yelamanchili et al., 2006).

The concept linking a certain level of variability in the composition of SVs to their exo–endocytotic properties has been exploited to illustrate a process of 'competence maturation' that might determine the location of SVs in the various functional pools and even the recycling route followed by SVs after neurotransmitter release (Valtorta et al., 2001). According to this model, the molecular make-up of SVs, at least in terms of accessory proteins, ultimately dictates whether the vesicle is competent for fast or slow exo–endocytosis, whether it undergoes spontaneous or evoked exocytosis, whether it is kept in a 'dormant' state or participates to recycling. However, 'competence' is also contributed by the interaction of SVs with nerve terminal proteins and by post-translational modifications, which modulate the properties of most SV proteins.

The study of the development of SV pools during hippocampal neuron differentiation in culture points to a matching between the emergence of the RRP and the acquisition of SV competence for effective neurosecretion (Mozhayeva et al., 2002). Synapses lacking the RRP are functionally impaired, since SVs at these terminals can be recycled only in response to strong stimuli. These SVs progressively mature into competent SVs capable of release under physiological stimulation. This competence maturation may involve the availability of active zone components and/or changes in the molecular make-up of the vesicles. Surprisingly, central GABAergic neurons do not show the developmental maturation of the RRP observed at glutamatergic synapses (Mohrmann et al., 2003).

Extension to the concept of different biogenesis as a cause of variability in SV competence for exocytosis comes from the recent discovery that newly endocytosed SVs can be constitutively transported to neighboring boutons and enter the native pool of SVs. Interestingly, although imported SVs undergo exocytosis at the same rate as native vesicles, a larger fraction of these vesicles remains non-releasable, suggesting that a maturation process is needed in order for imported SVs to acquire competence for exocytosis after incorporation at the host synapse (Darcy et al., 2006).

Clues as to the importance of protein composition of SVs in determining the choice between different recycling pathways at the synapse come from genetic studies showing that

spontaneous fusion rate was unchanged in mice knock-out for synaptotagmin I or complexins, proteins critical for evoked synchronous neurotransmitter release (Geppert et al., 1994b; Reim et al., 2001). In contrast, spontaneous fusion rate was significantly reduced after deletion of the VAMP2 gene, or completely abolished after genetic ablation of munc-18 or munc-13 isoforms (Schoch et al., 2001; Varoqueaux et al., 2002; Verhage et al., 2000). In addition, synaptotagmins and VAMP2 play complementary roles in SV endocytosis. Synaptotagmins are associated with the clathrin-dependent slow pathway of endocytosis (von Poser et al., 2000; Diril et al., 2006), whereas VAMP2 has been implicated in the fast pathways for both exocytosis and endocytosis (Schoch et al., 2001; Deak et al., 2004). Consistently, fast Ca^{2+}-triggered SV exocytosis is particularly affected in VAMP2-deficient synapses (Schoch et al., 2001) and mutant synapses display a switch from fast (i.e., kiss-and-run) to slow recycling rather than a general impairment in endocytosis (Deak et al., 2004).

5.3. Functional heterogeneity of synaptic vesicles by differential targeting of peripherally associated components

Of particular importance for the generation of functional heterogeneity in the SV population are the targeting dynamics of proteins peripherally associated with SVs, such as the synapsin and Rab3 families. As both protein families consist of multiple isoforms whose expression and targeting can be differentially regulated, and their association with SVs is dynamically modulated during synaptic activity and by post-translational modifications, they can significantly contribute to constitutive and regulated functional heterogeneity in SV populations.

Heterogeneity can arise from distinct mechanisms. First, the various isoforms can be differentially expressed both within the same neurons during ontogeny and among distinct neuronal populations. In the case of synapsins, whose family includes the products of three genes spliced in multiple isoforms (Hilfiker et al., 1999; Baldelli et al., 2005), synapsin III is expressed early during development in a period that corresponds to active process elongation and its expression declines thereafter, whereas synapsins I and II progressively increase their expression during synaptogenesis and maintain elevated levels in mature neurons (Staple et al., 1997; Ferreira et al., 2000). In the mature brain, the various synapsin gene products and splicing isoforms are differentially expressed among neurons (De Camilli et al., 1983; Südhof et al., 1989; Mandell et al., 1990, 1992; Stone et al., 1994; Staple et al., 1997; Matus-Leibovitch et al., 1997). The majority of nerve terminals contain at least one synapsin isoform, regardless of the neurotransmitter secreted. For example, mossy fiber terminals of hippocampal granule cells contain all four synapsins, Purkinje cell axon terminals do not contain detectable amounts of synapsin IIa and retinogenicolate terminals do not contain either synapsin I or synapsin II (Südhof et al., 1989; Kielland et al., 2006). In addition to a genetically programmed expression, the expression levels of synapsin isoforms can also be differentially regulated by neural activity (Morimoto et al., 1998). The only neuronal cells which do not express any synapsin isoform are cells involved in sensory transduction displaying ribbon-type synapses, which express most pre-synaptic proteins except synapsins and rabphilin (Favre et al., 1986; Mandell et al., 1990; Finger et al., 1990; Von Kriegstein et al., 1999).

Interestingly, at early stages of neuronal differentiation synapsins associate with a subpopulation of the total SV pool undergoing more sustained basal recycling (Bonanomi et al., 2005b). Possibly, synapsins identify a more mature state of these vesicles, maybe dictated by the presence of either a molecular marker, which could act as a receptor for synapsin binding to the vesicle or a distinctive lipid composition. Interestingly, synapsin co-localization with SVs increases in growth cones during development and is strongly enhanced in synaptic varicosities (our unpublished observations), supporting the idea of a molecular change in the protein/lipid composition of SVs which parallels their functional maturation and coincides with an increased affinity for important regulators of SV life cyle.

A second possibility is that the association of distinct protein isoforms with the SV membrane is modulated by post-translational modifications such as phosphorylation or guanine nucleotide binding. Rabphilin is phosphorylated by both protein kinase A and CaM kinase II and its phosphorylation reduces its membrane affinity (Foletti et al., 2001). All synapsin isoforms are targets of cAMP- and Ca^{2+}/calmodulin stimulated kinases, but only synapsin I is also phosphorylated by MAP kinase and CaM kinase II. As synapsin phosphorylation regulates its association with actin and/or the SV membrane and hence the availability of SV for release, a different mosaic of synapsin isoforms may endow a nerve terminal with distinct functional responses to the activation of various signal transduction pathways (Hilfiker et al., 1999; Baldelli et al., 2005).

As various isoforms display distinct functional properties, the developmental or regional heterogeneity in the expression of synapsin isoforms, as well as of other SV proteins, can account for functional heterogeneity among synapses in terms of release probability, kinetics of release and short-term plasticity properties (Knight et al., 2005). Only the A-isoforms of synapsins possess domain E, critical for the assembly of the reserve pool of SVs and for propelling post-docking steps of release (Hilfiker et al., 1998, 2005; Fassio et al., 2006). Synapsins I and II make excitatory nerve terminals more resistant to synaptic depression during sustained high-frequency stimulations by increasing the size of the total recycling SV pool, whereas synapsin III enhances depression and reduces the size of the recycling pool (Rosahl et al., 1995; Li et al., 1995c; Ryan et al., 1996; Feng et al., 2002). The heterogeneous substrate properties of the various synapsin isoforms for protein kinases or specific changes in isoform expression may affect the plastic responses of nerve terminals in releasing neurotransmitter. For example, synapsin I, but not synapsin II, mRNA levels increase in hippocampal granules after kindling (Morimoto et al., 1998) and the expression levels

of synapsin I, but not synapsin II, correlate with the extent of SV cycling in nerve terminals labeled with FM dyes (Staple et al., 1997).

Similarly to what reported for synapsins, Rab3 isoforms are also differentially expressed in various brain region, with Rab 3A and Rab3C being the more abundant and widespread isoforms (Schluter et al., 2002). However, at variance with synapsins, a complete genetic analysis of mutant mice lacking single or multiple Rab3 isoforms revealed that Rab3 isoforms are functionally redundant (Schluter et al., 2004). Nevertheless, as the SV association/dissociation/reassociation of Rab3 is the result of multiple protein interactions with an array of Rab effectors including rabphilin, RIM, synapsin I, GDI, GTPase activating protein (GAP) and guanine nucleotide exchange factor (GEF) that modulate the functional cycle of Rab3, a different constellation of these accessory proteins in distinct nerve terminals is able to affect the functional properties of exocytosis by modulating the Rab3 cycle and its effects in the delivery of SVs to the active zone and the subsequent steps of priming and fusion (Leenders et al., 2001; Geppert et al., 1997; Geppert and Südhof, 1998). In addition, a careful analysis of mice deficient for the four Rab3 isoforms revealed that the activity of this family of GTP-binding proteins is required in order to increase the Ca^{2+} sensitivity of a subset of already primed vesicles, thus generating heterogeneity in the intrinsic release probability among vesicles of the RRP. Whether the interaction of Rab3 with its effectors, including synapsin I, underlies the enhancement of SV priming remains to be investigated (Schluter et al., 2006).

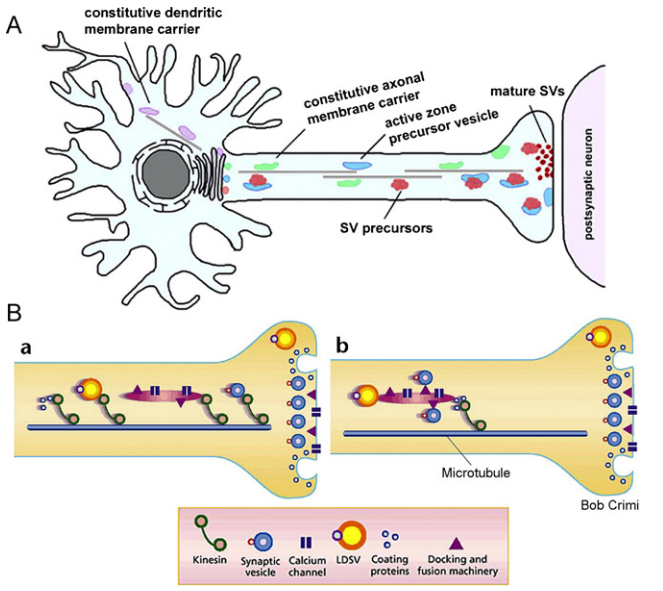

Fig. 5. Trafficking routes of membrane proteins in neurons. (A) After leaving the TGN, membrane proteins are targeted either to carriers mediating constitutive secretion in either axons or dendrites, or to carriers specialized in the transport of active-zone and SV proteins. SV precursors assemble in clusters which travel along the axon in association with active-zone precursor vesicles. At the nerve terminal cycles of exo–endocytosis of the precursor vesicles may underlie the generation of a pool of mature SVs. (B) Transport of prefabricated prototerminals. Past models (a) predicted that the components of the nerve terminal (coating proteins, LDSVs, membranes containing presynaptic membrane proteins and SVs) are transported down the axon in individual packets, with each packet recognized and transported by a different member of the kinesin family. The current model (b) envisages a pre-assembled complex of SV proteins, calcium channels, endocytotic machinery and LDSVs transported as a unit along microtubules to nascent synapses. Reproduced with permission from (Roos and Kelly, 2000) (Copyright 2000, Nature Publishing Group, London, UK).

6. Axonal transport of synaptic vesicle proteins

6.1. How many carriers?

Axonal transport is essential to maintain the function, integrity and viability of neurons (Goldstein, 2003). Components of the various membrane compartments of the axon are transported from the cell body to their destinations in the axon and synapses (Fig. 5A).

The original concept that SVs are generated by direct budding from the TGN (see Zimmermann et al., 1993 for a discussion) was dismissed in the light of the key observation that membrane components accumulating along the axon at the site of an experimentally induced transport block could be classified either as vesiculotubular structures of 50–80 nm with some continuity with the axonal smooth endoplasmic reticulum, moving anterogradely, or as large multivesicular bodies moving in the retrograde direction. Remarkably, no typical 50 nm spherical clear vesicles, i.e. vesicles of the size and shape of SVs, were visible (Tsukita and Ishikawa, 1980). The lack of SVs in the axon implies that their components are transported down the axon on membrane precursors of mature SVs. SVs are then generated by either a single or sequential steps along the endo–exocytic pathway.

It is unclear whether, in analogy to the traffic of newly synthesized SLMV components (Regnier-Vigouroux et al., 1991), SV proteins exploit membrane carriers mediating the constitutive axonal transport in order to be delivered to the site(s) where they are assembled onto the final organelle. The visualization of GFP-tagged SV and plasma membrane proteins in living neurons allowed the identification of tubulovesicular organelles as general carriers for newly synthesized proteins along the axon. Indeed, the dynamics and morphology of SV precursors containing exogenous synaptophysin are largely similar to those displayed by the carriers used by functionally distinct plasma membrane proteins (Nakata et al., 1998). These vesicles move at a fast speed (~1 μm/s), bidirectionally, with frequent changes in direction, and are not labeled by endosomal tracers.

Nakata et al. (1998) defined a form of axonal transport shared by proteins destined to different compartments, leaving however unanswered the question as to whether all membrane proteins are transported by the same tubulovesicular carrier or whether they are sorted into different vesicles that are morphologically or behaviorally indistinguishable. Clues supporting the latter possibility come from the characterization of the motor proteins of the kinesin superfamily, which mediate anterograde transport. The neuro-specific kinesin KIF1A and the widely distributed isoform of the conventional microtubule motor KIF1Bβ, which share largely homologous tail domains,

appear to be specific for the anterograde axonal transport of SV precursors, but not of presynaptic membrane proteins (Okada et al., 1995; Zhao et al., 2001). Consistently, mice lacking either KIF1A or KIF1Bβ exhibit lethal neurological phenotypes associated with a reduction in the density of SVs in the nerve terminals (Yonekawa et al., 1998; Zhao et al., 2001). In contrast, the conventional microtubule motor kinesin-I associates with the plasma membrane protein APP and mediates its axonal transport on an organelle, which also contains the neuronal membrane protein GAP43 but is devoid of SV proteins. As expected, mice lacking functional kinesin-I show a marked decrease in the axonal transport of both APP and GAP43, whereas the transport of SV proteins is normal (Kamal et al., 2000).

These data were corroborated by the use of dual-color video microscopy to track simultaneously the axonal transport of APP and synaptophysin I (Kaether et al., 2000). These two proteins are transported in different structures moving at different speeds. APP is transported in fast-moving (up to 4.5 μm/s) elongated (up to 10 μm in length) tubules, whereas synaptophysin I is sorted to smaller tubulovesicular carriers. Furthermore, using morphological and biochemical approaches, Zhai et al. (2001) provided evidence that the active zone component Piccolo is transported to nascent synapses in ~80 nm dense-core granulated vesicles together with other constituents of the active zone. These granulated vesicles have been proposed to serve as a precursor for the presynaptic active zone assembly. Remarkably, none of the components of SVs is present in the active zone precursor vesicles. These studies argue against the possibility that all synaptic membrane proteins are transported on the same organelle. It appears that membrane proteins destined to the different synaptic domains are already sorted in the cell body to specific carriers associated with distinctive motors.

The complexity of the axonal membrane traffic was further illustrated by live imaging of GFP-tagged VAMP2 during synaptogenesis in hippocampal cultures (Ahmari et al., 2000). Exogenous VAMP2 associates with large motile puncta comprising dense-core and pleiomorphic vesicles as well as tubulovesicular elements, containing SV proteins, a component of the endocytosis machinery (amphiphysin I) and Ca^{2+} channels. Interestingly, nothing resembling a typical clear 50 nm SV was revealed by ultrastructural analysis. These fluorescent puncta, named 'transport packets', are recruited at the site of contact between an axon and a dendrite. Activity-dependent recycling of SVs is rapidly established at new axo-dendritic sites (<1 h; see also Friedman et al., 2000). The dense core granulated vesicles found by Ahmari et al. (2000) in transport packets are likely to correspond to the active zone precursors described by Zhai et al. (2001).

Thus, it appears that membrane carriers of different nature and bearing functionally different cargoes are bundled together in a mobile unit containing many or all of the components required for construction of a presynaptic active zone (Fig. 5B). However, these studies do not clarify whether different SV proteins are delivered in the same precursor vesicle toward the site(s) where SVs are generated (i.e. either the axon or the synapse). Although the available data are somewhat contradictory, the picture emerging is consistent with the existence of multiple carriers involved in SV protein transport. It should also be considered that the situation might be significantly different in the case of mature versus immature neurons. Okada et al. (1995) reported that the axonal membrane carriers associated with KIF1A contain some of the proteins common to SVs, such as synaptotagmin I, synaptophysin I and Rab3A, but do not contain other SV proteins, such as SV2. This finding would imply that the incorporation of these proteins in the same SV, which seems to be mandatory for efficient neurotransmission, occurs at the synapse. In contrast, vesicles associated with the KIF1Bβ motor were found to contain synaptotagmin I, synaptophysin I, and SV2 (Zhao et al., 2001), indicating that different motors are involved in the transport of a heterogeneous population of SV precursors with variable composition.

Interestingly, in immature neurons treated with BFA to induce tubulation of the Golgi apparatus, synaptophysin I redistributed to a tubular perikaryal-dendritic network positive for the transferrin receptor, whereas other SV proteins (SV2, synaptotagmin, synaptogyrin, VAMP2, Rab3a) maintain a vesicular distribution (Mundigl et al., 1993). The heterogeneous distribution of SV proteins reported in this study further suggests that neurons, or at least developing neurons, contain a substantial fraction of SV proteins which are not yet coassembled in SVs or SV precursors in the soma, consistent with a biogenesis of mature SVs distal to the TGN. In support of this concept, three different SV proteins, namely synaptophysin I, SV2 and synaptotagmin I, display a differential intracellular distribution when expressed in fibroblasts: synaptotagmin I was found associated with the plasma membrane, synaptophysin I with early endosomes and SV2 with an as yet unidentified organelle (Feany et al., 1993).

Altogether these findings strengthen the idea that endo–exocytotic cycling of SV precursors, perhaps requiring passages through endosomal compartments, is essential to redistribute SV components to the same vesicle. It is noteworthy, however, that vesicles bearing synaptotagmin I, synaptophysin I or SV2 were associated with synapsin I in axonal growth cones (Bonanomi et al., 2005b). In view of the role of synapsin I in the regulation of SV precursors localization and recycling at early developmental stages (see below), this finding implies that vesicles with heterogeneous composition are maintained in the same pool and might be subjected to similar control mechanisms.

6.2. Interplay between constitutive membrane traffic and synaptic vesicle biogenesis

It is unclear at present whether the vesicular tubules associated with nascent synapses represent endosomal membranes or a repository for at least some components of the mature synapse. Evidences have been accumulating which support the existence of a partial overlap between the trafficking route of SV proteins along the axon and constitutive endosomal recycling:

1. Although the bulk of exogenous APP and synaptophysin I are sorted in distinct, highly mobile carriers which are not stained by endosomal tracers, the two proteins are occasionally found to colocalize in slow or immobile short tubulovesicular/rounded structures that internalize either transferrin or dextran (Kaether et al., 2000).
2. Similarly to plasma membrane proteins, most of the GFP-tagged synaptophysin I is transported anterogradely by tubulovesicular carriers (Nakata et al., 1998), supporting the possibility that SV proteins are directly transported from the TGN to the nerve terminal and then recycled rather then being targeted to axonal endosomes and then recycled to the axon. However, exogenous synaptophysin I also associates with globular organelles which are labeled by endocytic tracers and exhibit a preferential retrograde movement. The question arises as to whether synaptophysin I in large globular endosomes is again recruited for the biogenesis of SVs, or is en route to the cell body and destined for degradation.
3. Along the axon syntaxin 13, a marker of recycling endosomes, is associated with two classes of organelles: round-oval, stationary structures, and fast-moving tubulovesicular elements. Membrane dynamics suggestive of fusion and budding events between the two classes of organelles were reported, consistent with the hypothesis that the mobile tubulovesicular endosomes might shuttle cargo to and from the stationary endosomes. Neither types of organelles contain a significant amount of SV proteins, although colocalization is occasionally observed (Prekeris et al., 1999). However, it should be noted that the identification of transporting vesicles by immunocytochemistry of fixed samples might be hampered by the small amount of membrane proteins being transported relative to the fraction that has already reached the target. The use of dual-color videomicroscopy to monitor in real time the axonal trafficking of fluorescent chimeras of both SV and endosomal proteins, in combination with biochemical studies, will be critical to unequivocally estimate the molecular identity of the tubulovesicular structures delivering SV proteins to the synapse.

7. Synaptic vesicle recycling in developing neurons

7.1. Mechanisms of neurotransmitter secretion from developing axons

Neurotransmitter release appears early in the developing embryo and might play a role in morphogenesis, neuronal communication and axonal navigation. Several lines of evidence indicate that neurons are endowed with a functional apparatus for neurotransmitter release prior to the establishment of the synaptic contacts. The rapidity of the onset of regulated secretion after synapse formation (Friedman et al., 2000) suggests that neurons may have already acquired the appropriate machinery of neurotransmitter release before encountering the target cell.

Both spontaneous and evoked release of various neurotransmitters from developing axons has been reported from a number of preparations. Neurotransmitter release is restricted to the distal axon and growth cones, whereas is not detected at the level of the soma. In particular, evoked release is detected only at selected sites along the axon and in the growth cone, which displays the most efficient excitation-secretion coupling (Young and Poo, 1983; Hume et al., 1983). A gradient of secretory activity along the axon, which is shaped by developmentally regulated mechanisms, has been described in *Xenopus* spinal cord neurons (Antonov et al., 1999). At early stages of axonal growth, neurotransmitter release takes place along the axon to the same extent as in the growth cone, whereas at later stages it follows a proximodistal gradient, with the highest level at the growth cone, that parallels the targeting of SVs to the distal domains of the axon (Fletcher et al., 1991). These results point to the presence of regulatory elements underlying the preferential localization of the machinery for neurosecretion at the growth cone.

Whether at these stages SVs are needed for neurotransmitter release is unknown. Neurotransmitters might be stored and released from cellular compartments other than SVs, such as the endoplasmic reticulum or the cytoplasm (Fig. 6A). It was initially hypothesized that the release of neurotransmitters during neuronal development occurs as a consequence of the incorporation of new membrane during neurite extension (Young and Poo, 1983). This conclusion was suggested by the pulsatile nature of both spontaneous and evoked release in developing neurons relative to the kinetics of release measured at synapses. Although this hypothesis has not been completely ruled out, the presence of vesicles bearing SV proteins in young neurons (Fletcher et al., 1991), together with the evidence that SVs are not engaged in neurite extension (Leoni et al., 1999), argue against a role for membrane carriers devoted to axon outgrowth in this immature form of neurotransmitter release.

While release of neurotransmitters might be mediated by reversion of transporters (Gaspary et al., 1998), exchangers (Warr et al., 1999), or a developmentally regulated paracrine mechanism (Demarque et al., 2002), the involvement of vesicles in neurotransmitter secretion along the axons has been inferred by its sensitivity to BFA, which does not affect SV recycling at mature synapses (Zakharenko et al., 1999). Since the effect of BFA on neurotransmitter secretion was observed even in transected axons, it is likely to depend on the inhibition of the ARF-mediated local recycling of vesicles through axonal endosomes rather than of vesicle budding from the TGN. The existence of a vesicular mechanism underlying neurotransmitter secretion along the axon is also supported by the increase in either GABA (Gao and van den Pol, 2000) or acetylcholine (Zakharenko et al., 1999) release by α-latrotoxin, which is known to cause SV exocytosis (Valtorta et al., 1988). In addition, the secretion of acetylcholine can be triggered by a hypertonic solution, suggesting the presence of a pool of fusion-competent vesicles docked at the axolemma (Zakharenko et al., 1999).

Important clues as to the mechanisms underlying the release of neurotransmitters from developing neurons came from the study of quantal neurotransmitter secretion from either non-neuronal cells loaded with neurotransmitter or *Xenopus* spinal

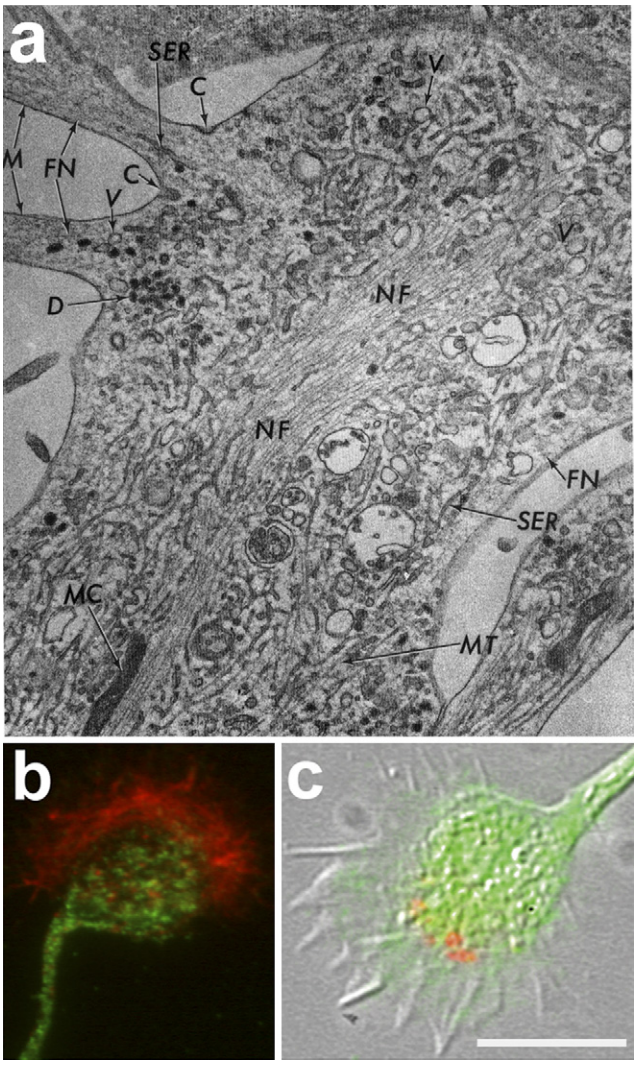

Fig. 6. SV precursors in developing neurons. (a) Electron micrograph of a dorsal root ganglion cell growth cone. Pleiomorphic vesicles (V) and dense-core granules (D) are dispersed in a dense filamentous network (FN) which fills the periphery of the growth cone. Vesicles are excluded from filopodia (M, microspike) where extensions of the smooth endoplasmic reticulum (SER) are visible. Coated vesicles (C); Neurofilaments (NF); microtubules (MT) mitochondrion (MC). Reproduced with permission from (Yamada et al., 1971) (Copyright 1971, The Rockefeller University Press, NY). (b) Immunofluorescence of a growth cone stained for synaptophysin I (green) and F-actin (red). Vesicles bearing synaptophysin I are clustered in the core of the growth cone and excluded from the peripheral domain enriched in F-actin. (c) SV recycling is dissociated from constitutive membrane retrieval in the growth cone. Basal uptake of FM 4–64 (red) in a growth cone incubated with the dye for 1 min and retrospectively stained for synaptophsyin I (green). The overlay between the fluorescence and DIC images is shown. FM 4–64 is internalized in large endocytic compartments but does not label SVs, indicating a negligible rate of SV recycling relative to the high levels of constitutive endocytosis observed in growth cones. Reproduced with permission from (Bonanomi et al., 2005b) (Copyright 2005, Society for Neuroscience, Washington, DC). Bar, 1.6 μm in A, 8 μm in B and 6 μm in C.

neurons manipulated into contact with a muscle cell used as a detector for acetylcholine (Dan and Poo, 1992; Girod et al., 1995). Both spontaneous and evoked release are reported to occur from myocytes and fibroblasts loaded with acetylcholine, which reflect the Ca^{2+}-dependent exocytosis of acetylcholine-filled vesicles (Dan and Poo, 1992; Morimoto et al., 1995). Similarly, neurotransmitter release could be detected in *Xenopus* oocytes loaded with mRNA isolated from rat cerebellum (Alder et al., 1992). Compared with the regulated neurotransmitter secretion from presynaptic nerve terminals of the neuromuscular junction, excitation-secretion coupling in non-neuronal cells is significantly weaker, but surprisingly close to that found at developing neuromuscular synapses in culture (Evers et al., 1989; Girod et al., 1995). The kinetics with which neurotransmitter packets are discharged from either non-neuronal cells or developing neurons is comparable, although the speed of secretion in non-neuronal cells is slower and more irregular.

The striking similarity of the size and distribution of miniature endplate currents typical of neurons with those detected in non-neuronal cells supports the notion that SVs in developing neurons are derived from a similar population of vesicles used for constitutive secretion in all cell types (Girod et al., 1995).

In developing neuromuscular junctions the quantal size varies over a wide range, resulting in a skewed distribution of miniature endplate currents detected in the postsynaptic muscle cell (Evers et al., 1989). A high variability in the amplitudes of quantal transmission has also been found at nascent central synapses (Liu and Tsien, 1995). A skewed amplitude distribution may result from the accumulation of either different concentrations of neurotransmitter in vesicles of similar size or comparable concentrations in vesicles of different size. The presence of pleiomorphic vesicles along isolated axons and nascent synapses (Kraszewski et al., 1995; Ahmari et al., 2000) is consistent with the latter possibility. Thus, neurotransmitter secretion in developing neurons may be sustained by a population of SV precursors of variable size and may originate from a ubiquitous pathway used for constitutive secretion and membrane trafficking in non-neuronal cells, which acquires specific regulatory properties during neuronal differentiation (Fig. 6). Consistent with this picture, isolated neurons exhibit a limited spontaneous quantal neurotransmitter secretion, and marked increase in the frequency of secretion can be induced by specific contact with a target (Xie and Poo, 1986; Sun and Poo, 1987). Thus, it is conceivable that the presence of a neurosecretory apparatus in developing neurons has the direct functional consequence of rapidly establishing synaptic transmission at the nascent synapse (Ahmari et al., 2000). However, in the light of these results neurotransmitter secretion measured in a myocyte manipulated into contact with the axon as a detector (Sun and Poo, 1987; Zakharenko et al., 1999) might reflect the rapid induction of the program of synaptic maturation by contact with the myocyte rather than the genuine properties of neurotransmitter release from isolated processes.

7.2. Properties of synaptic vesicle recycling in growing axons

Expression of SV proteins occurs early during neuronal development. Before synaptogenesis, vesicles containing SV proteins concentrate in the axon and upon contact with the

postsynaptic cell become clustered at synaptic sites (Fletcher et al., 1991). However, the relationship between vesicles recycling along the axon and genuine SVs in the nerve terminal remains to be established (i.e., it is unknown whether SVs in immature neurons differ in their composition from SVs present at the synapse). The absence of classical small SVs in the 'transport packets' indicates that pleiomorphic precursor vesicles are likely to be involved in the trafficking of SV proteins along the axons (Ahmari et al., 2000; Kraszewski et al., 1995; Matteoli et al., 1992).

Although the contribution of *bona fide* SVs to neurotransmitter secretion in developing neurons remains to be determined, vesicles bearing SV antigens have been shown to recycle along the axon. A seminal set of experiments has been performed studying SV recycling by internalization of an antibody directed against the lumenal domain of synaptotagmin I (Matteoli et al., 1992; Kraszewski et al., 1995). Using this technique clusters of ~10–20 SVs were shown to undergo multiple cycles of exo–endocytosis along isolated axons. Since labeled SVs are eventually found at synapses formed days after the uptake of the antibody, it appears that following repetitive exchanges of components with the axolemma SVs are recruited to the presynaptic terminals. As observed at the mature synapse (Betz and Henkel, 1994), SV clusters are maintained through a phosphorylation-dependent mechanism, since the inhibition of phosphatase activity causes their disruption (Kraszewski et al., 1995). SV clusters move in anterograde and retrograde directions in the axon and, upon contact with the target neuron, become immobilized into the newly formed presynaptic terminal (Kraszewski et al., 1995; Ahmari et al., 2000). The mechanisms underlying the recruitment and immobilization of SV packets into the nascent synapse are still unknown, yet actin-binding proteins of the synapsin family, which are implicated in SV clustering at mature nerve terminals, are implicated in this process (Bonanomi et al., 2005b).

Some mismatches are found between SV dynamics during synaptogenesis as monitored through synaptotagmin I recycling and developmental changes in neurotransmitter release kinetics. Recycling of SVs in isolated axons of dissociated hippocampal neurons is Ca^{2+}-dependent and poorly sensitive to depolarization, although high levels of constitutive exo–endocytosis are detected. The formation of synaptic contacts correlates with a downregulation of the basal rate of SV recycling (Kraszewski et al., 1995), accompanied by reduction in spontaneous release along the axons consistent with recruitment of SVs at nerve terminals (Chow and Poo, 1985). Inhibitory mechanisms of spontaneous recycling associated with a Ca^{2+}-regulated enhancement of SV exocytosis are needed to gain an efficient excitation-secretion coupling at the synapse (Popov and Poo, 1993). In addition, *in vivo* imaging of SV precursor clusters combined with the use of FM dyes to visualize activity-dependent endocytotic processes reveals that the mobile clusters in isolated axons do not undergo evoked recycling, whereas SVs located at stable synaptic puncta (Ahmari et al., 2000) or at the tip of growth cone filopodia (Sabo and McAllister, 2003) show effective activity-dependent FM loading and release. At variance, spontaneous neurotransmitter release from isolated axons has been reported to be inhibited in *Xenopus* spinal neurons, and inhibition is reduced upon contact with the post-synaptic cell (Xie and Poo, 1986; Sun and Poo, 1987).

In spite of some inconsistency that might result from either the use of different experimental models or alternative mechanisms of neurotransmitter release, these results indicate that in the axon SVs recycle along a constitutive pathway of secretion which is scarcely sensitive to depolarization, and enter an activity-regulated pathway as recruited at nascent synapses. This view is supported by differences between the machineries involved in SV recycling in developing and mature neurons. Consistently, BFA displays selective effects on neurotransmitter secretion in young but not mature neurons (Zakharenko et al., 1999), indicating that an ARF/AP3-mediated process of vesicle re-formation from endosomes similar to that described in neuroendocrine cells (Faundez et al., 1997, 1998) takes place at early developmental stages. Such process is later substituted by a dynamin/AP2 mechanism of vesicle generation directly from the plasma membrane (De Camilli and Takei, 1996).

The functional maturation of neurotransmitter release apparatus is possibly underlain by the involvement of the BFA-insensitive ARF6 (Aikawa and Martin, 2003; Krauss et al., 2003). In addition, the differential expression of vesicle components might also underlie the presence of distinct regulatory mechanisms of SV exocytosis during neuronal development. Before synapse formation, the exocytic machinery effective in isolated axons utilizes a tetanus-toxin-resistant isoform of VAMP2 which is substituted by a VAMP2-dependent exocytotic pathway as synapses form (Verderio et al., 1999). This change in the composition of the exocytotic machinery correlates with a change in the recycling properties of SVs, as shown by the reduction in the rate of spontaneous exocytosis following the establishment of synaptic contacts.

Thus, during neuronal development changes in the expression of SV components and in the pathway of SV biogenesis are needed to generate a pool of homogeneous clear small vesicles, which are typically associated with the presynaptic terminals, from the pleiomorphic vesicle population found in the isolated axons. It has been proposed that the multiple cycles of exo–endocytosis undertaken by vesicle precursors along the axon might play a role in the morphological and functional maturation of SVs, allowing redistribution of membrane components between the SV and the axolemma (Matteoli et al., 2004).

This hypothesis has been corroborated by the study of axonal membrane trafficking in *Drosophila* neurons lacking sec5, an essential component of the exocyst complex implicated in trafficking to the cell surface. This mutation impairs membrane addition of newly synthesized proteins, whereas SV fusion at the synapse is not affected. Importantly, newly synthesized synaptotagmin I, though transported down the axon, does not enter the pool of mature SVs clustered at synaptic boutons and remains in the axon or is sent retrogradely back to the soma. Therefore, it seems that vesicles bearing SV

antigens which cannot fuse with the plasma membrane in the absence of a functional exocyst complex are not matured into SVs (Murthy et al., 2003).

Accumulating evidences show that in growth cones SV distribution and recycling are already subjected to regulatory mechanisms similar to those operating at the mature synapse. SVs appear to be retained in the central core of the growth cone and are not engaged in rapid constitutive membrane recycling visualized by FM uptake, thus suggesting that a control device might be effective prior to the formation of synaptic contacts to differentiate SV recycling from constitutive secretion pathways (Bonanomi et al., 2005b) (Fig. 6B and C). Constitutive secretion from *Xenopus* myocytes and fibroblasts artificially loaded with acetylcholine is indeed more prolonged and irregular compared to the release from the neuronal growth cone (Girod et al., 1995). The storage of neurotransmitter in a class of vesicles (i.e., SVs or their precursors) different from those involved in constitutive axonal membrane trafficking and the early expression of regulators of the SV life cycle may account for these effects. Consistently, the SV-associated phosphoprotein synapsin I has been recently shown to control the spatial organization and basal rate of recycling of SVs in the growth cones. Phosphorylation of synapsin I at a PKA/CaMKI site in response to intracellular cAMP increase leads to dissociation of synapsin I from the SV membrane paralleled by vesicle relocalization and enhanced recycling in the growth cone. As a consequence, lack of synapsin I expression impairs the ability of the growth cone to retain SVs in the core domain at rest, while changes in SV distribution in response to cAMP and constitutive SV recycling are impaired by expression of mutated synapsin I lacking the PKA phosphorylation site (Bonanomi et al., 2005b). This picture is reminiscent of the mechanisms at the basis of synapsin function in the regulation of neurotransmitter release at the mature synapse (Greengard et al., 1993) and provides a molecular explanation for previous studies showing impaired spontaneous neurotransmitter release from growth cones of *Drosophila* fly memory mutants with constitutively enhanced PKA activity (Yao et al., 2000).

It remains to be established whether neurotransmitter release in developing neurons is regulated at multiple levels by different signaling pathways as it occurs at mature synapses. However, evoked GABA secretion from growth cones is stimulated by protein kinase C activation (Girod et al., 1995; Gao and van den Pol, 2000) and stimulation of axonal AMPA receptors triggers SV exocytosis in the growth cone through a MAPK-dependent pathway which leads to synapsin I phosphorylation (Schenk et al., 2005). Thus, synapsin I might represent a key point of convergence of various signaling cascades effective in the control of SV exocytosis in developing neurons.

Synapsin-modulated SV exocytosis in growth cones might be linked to the release of neurotransmitters, which instruct axon pathfinding during the formation of neuronal networks. Alternatively, synapsins may provide a device to inhibit SV fusion in the resting state and direct the rapid reorganization of the SVs present in the growth cone into the various functional vesicle pools during establishment of the synaptic contact. Indeed, SVs in the growth cone are likely to be used for the formation of the presynaptic pool of vesicles (Chow and Poo, 1985; Ahmari et al., 2000).

8. Concluding remarks

The generation of vesicles of uniform size and endowed with a molecular repertoire, which confers competence for efficient neurotransmitter release is a formidable task. Common motifs for protein sorting to SVs are missing, thus individual components exploit different strategies to reach the target organelle. The contribution of trafficking pathways utilizing either the plasma membrane or endosomal compartments as donor membranes for SV biogenesis varies throughout neuronal development. In addition, diverse adaptor complexes might give cargo-specificity to protein retrieval during endocytic regeneration of SVs. Kinetic tagging of morphologically alike SV pools by electrophysiological analysis, complemented by novel optical approaches, provides evidence for an unpredicted level of functional diversity in the SV population at individual terminals. Thus, although the molecular specification of SV pools is not definitely proven, remodeling of the vesicle membrane through the differential use of various sorting mechanisms might introduce some degree of molecular heterogeneity in the SV make-up, possibly underlying the definition of functionally distinct SV pools. Continuous shaping of the molecular identity of SVs at single boutons would widen plasticity of the nerve terminal allowing adapting neurotransmission to physiological demands. Testing these emerging models is a challenge for future research and will add new dimensions to the physiology of the nerve terminal.

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