Synaptic Vesicle Endocytosis

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Neurons can sustain high rates of synaptic transmission without exhausting their supply of synaptic vesicles. This property relies on a highly efficient local endocytic recycling of synaptic vesicle membranes, which can be reused for hundreds, possibly thousands, of exo-endocytic cycles. Morphological, physiological, molecular, and genetic studies over the last four decades have provided insight into the membrane traffic reactions that govern this recycling and its regulation. These studies have shown that synaptic vesicle endocytosis capitalizes on fundamental and general endocytic mechanisms but also involves neuron-specific adaptations of such mechanisms. Thus, investigations of these processes have advanced not only the field of synaptic transmission but also, more generally, the field of endocytosis. This article summarizes current information on synaptic vesicle endocytosis with an emphasis on the underlying molecular mechanisms and with a special focus on clathrin-mediated endocytosis, the predominant pathway of synaptic vesicle protein internalization.

At synapses, the number of synaptic vesicles that undergo exocytosis over a given time period can greatly exceed the supply of synaptic vesicle precursors delivered from the cell body. Thus, nerve terminals have developed efficient endocytic mechanisms to recapture and reuse membranes that have fused with the plasma membrane to release neurotransmitters (Fig. 1A). The occurrence of synaptic vesicle recycling had been inferred early on after the first description of nerve terminal ultrastructure and synaptic vesicles by electron microscopy. However, direct evidence for an endocytic origin of synaptic vesicles was provided by the introduction of extracellular endocytic tracers in the early 1970s (Holtzman et al. 1971). Subsequent electrophysiological studies of stimulated frog neuromuscular junctions in combination with such tracers conclusively established the concept of synaptic vesicle recycling (Ceccarelli et al. 1973; Heuser and Reese 1973). In more recent years, powerful methods have been developed to monitor synaptic vesicle endocytosis and recycling in living neurons. These include imaging methods such as the internalization of fluorescent antibodies directed against luminal epitopes of synaptic vesicles (Matteoli et al. 1992; Kraszewski et al. 1995; Martens et al. 2008), the uptake and release of amphipathic styryl dyes such as FM1-43 (Fig. 1B) (Betz and Bewick 1992), antibodies against luminal domains of synaptic vesicle proteins coupled with pH-sensitive cyanine dye derivative CypHer5E (Hua et al. 2011), and the use of a
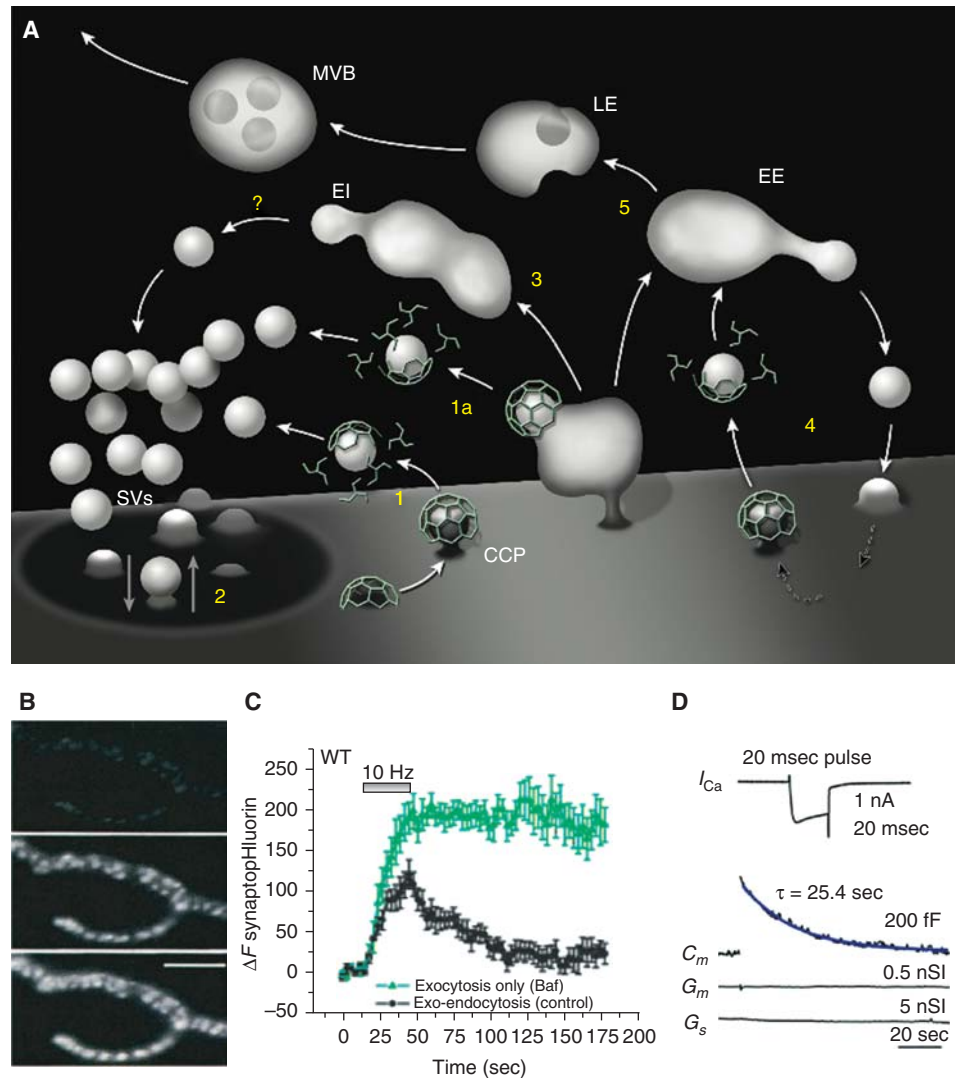
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Figure 1. Pathways of synaptic vesicle recycling and examples of methods to monitor synaptic vesicle endocytosis at living synapses. (*A*) Schematic diagram of membrane traffic in axon terminals illustrating endocytosis of synaptic vesicle (SV) membranes via clathrin-coated pits (CCP) from the plasma membrane and its deep infoldings (1 and 1a), "kiss and run" (2), and bulk endocytosis (3) followed by vesicle formation via unknown mechanisms (?) from endocytic intermediates (EI). This recycling traffic is interconnected with housekeeping membrane recycling (4) involving clathrin-mediated endocytosis and canonical early endosomes (EE) as well as with traffic to the cell body (5) via late endosomes (LE) and multivesicular bodies (MVBs). (*B*) Compensatory endocytosis of synaptic vesicle membranes at the frog neuromuscular junction following stimulation by depolarization in high potassium, visualized by the uptake of the fluorescent styryl dye FM1-43. Partial and full dye loading are shown in the middle and lower panels (Panel *B* is from Betz and Bewick 1992; reprinted, with permission, from *Science* © 1992). (*C*) Time course of exo- and endocytosis in response to a 10-Hz, 30-sec stimulus at synapses of cultured mouse cortical neurons as monitored by the increase and decrease in the fluorescence of synaptopHluorin (spH), a fusion protein of pHluorin to synaptobrevin/VAMP2. Bafilomycin (Baf), a membrane-permeable blocker of the V-type ATPase that is required for vesicle reacidification, was used to trap vesicles at neutral pH after endocytosis, thus allowing spH to remain fluorescent even after endocytosis. Control runs (black) consist of the sum of exo- and endocytosis, and Baf runs (green) monitor all the exocytic events. (*See facing page for legend.*)

pH-sensitive green fluorescent protein (GFP) (pHluorin) fused to luminal domains of synaptic vesicle proteins (Fig. 1C). Given the low pH of the synaptic vesicle lumen and the very rapid reacidification of vesicles after endocytosis, pHluorin's fluorescence monitors the shuttling of vesicle proteins to and from the neutral environment of the extracellular medium (Miesenbock et al. 1998; Sankaranarayanan and Ryan 2000). An additional method that has an exquisite temporal resolution, but can only be reliably applied to large nerve terminals, is the electrophysiological recording of changes in plasma membrane capacitance resulting from exoendocytosis (Fig. 1D) (Neher and Marty 1982; von Gersdorff and Matthews 1994; Sun and Wu 2001; Wu and Wu 2007; Lou et al. 2008). Collectively, these techniques have greatly helped elucidate mechanisms and kinetic parameters of synaptic vesicle endocytosis.

The time course of endocytosis varies at different synapses and with the intensity of stimulation. Endocytic styryl dyes, applied in a pulse-chase manner after electrical stimulation, revealed that membrane recovery after an exocytic burst (e.g., 20–100 action potentials at 20 Hz at hippocampal synapses in culture or 10 sec at 30 Hz at the frog neuromuscular junction) required less than 1 min to complete and had a time constant in the range of 20–30 sec (Ryan et al. 1996; Wu and Betz 1996), similar to what had been predicted by earlier morphological experiments (Miller and Heuser 1984). Real-time measurements of endocytosis by the use of pHluorin confirmed that the average time constant of endocytosis is in the range of 15–20 sec when the system is not saturated (e.g., 50 action potentials at 10 Hz) and subsequent studies strongly suggested that under these conditions the endocytic process is dependent on

the clathrin coat (Granseth et al. 2006; Balaji and Ryan 2007; Kim and Ryan 2009). At high levels of activity the endocytic rate slows down, indicating that under these conditions the surface accumulation of synaptic vesicle membranes (the exocytic load) exceeds the endocytic capacity (Sankaranarayanan and Ryan 2000; Granseth et al. 2006; Balaji et al. 2008).

Measurements of the endocytic recovery of synaptic vesicle membranes by capacitance recording in frog saccular hair cells, axon terminals of goldfish bipolar neurons, and calyx of Held synapses showed time scales in a similar range and a longer time course after a strong exocytic load (Fig. 1D) (Parsons et al. 1994; von Gersdorff and Matthews 1994; Sun and Wu 2001; Sun et al. 2002; Wu et al. 2005). These studies also revealed the existence of an additional fast kinetic component of endocytosis (~1 sec), which has been proposed to be mediated by a distinct molecular mechanism (von Gersdorff and Matthews 1994; Neves et al. 2001; Hull and von Gersdorff 2004; Jockusch et al. 2005).

It should be noted that most of these studies have investigated time course of endocytosis after a burst of exocytic events. It remains possible that the compensatory endocytic reaction after a single stimulus may be even faster.

PATHWAYS OF ENDOCYTOSIS

The precise pathways taken by synaptic vesicle membranes during their endocytic recycling have been and continue to be an object of debate. This debate has been fueled, in part, by the remarkable plasticity of endocytic traffic in nerve terminals. Although the exo-endocytosis that occurs at rest (the events responsible for miniature synaptic currents), or during modest activity,

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Figure 1. (*Continued*) The difference between the two traces allows estimating total endocytosis occurring during the experiment. (Panel *C* is from Nicholson-Tomishima and Ryan 2004; reprinted, with permission, from *Proc Natl Acad Sci* © 2004.) (*D*) Endocytic recovery of the increase in surface area produced by a secretory burst at a calyx of Held giant nerve terminal as monitored by the measurement of membrane capacitance (C_m, blue trace). Secretion was evoked by a single 20-msec (+10 mV) presynaptic pulse that elicited the Ca^{2+} current shown in the top trace. Membrane conductance (G_m) and series conductance (G_s) did not change during the recording. (Panel *D* is from Lou et al. 2008; reprinted, with permission, from the author.)

does not involve major structural perturbations of nerve terminals, intense activity induces major and reversible structural changes that vary dependent on the type of stimulation (Ceccarelli et al. 1973; Miller and Heuser 1984; Heuser 1989; Holt et al. 2003; Teng et al. 2007; Clayton et al. 2008; Hayashi et al. 2008). A variety of endocytic intermediates have been observed by electron microscopy under these conditions (Heuser and Reese 1973), and it has been difficult to reconcile all these observations into a simple unifying model. Likewise, studies of endocytic membrane recovery using membrane capacitance methods or dynamic fluorescence imaging have suggested multiple modes of endocytosis (Smith et al. 2008). Importantly, it has been difficult to correlate some of the observations obtained by imaging and electrophysiology with results of electron microscopy. Furthermore, although much has been learned about the endocytosis of individual small vesicles by live imaging in nonneuronal cells, for technical reasons this has not been possible yet for nerve terminals, given their very small size. However, some major concepts have emerged.

There is compelling evidence for the importance of clathrin-mediated endocytosis in the internalization of synaptic vesicle proteins and also strong evidence for the hypothesis that synaptic vesicles derive directly from the uncoating of clathrin-coated vesicles (Fig. 1A, and see below). However, clathrin may not be absolutely essential for synaptic vesicle reformation, as a recent study in *Caenorhabditis elegans* has suggested (Sato et al. 2009). Clearly, in response to a very strong exocytic burst, a different endocytic mechanism, called bulk endocytosis, comes into play. Bulk endocytosis leads to the generation of endocytic vacuoles that are subsequently converted into synaptic vesicles by a mechanism that remains elusive (Fig. 1A) (Miller and Heuser 1984; Holt et al. 2003; Paillart et al. 2003; Wu and Wu 2007; Hayashi et al. 2008; Meunier et al. 2010). A third form of endocytosis, the rapid closure of a fusion pore without collapse of the vesicle, termed "kiss and run," remains an attractive model to explain electrophysiological and dynamic imaging data that are difficult to reconcile with other endocytic mechanisms (Fig. 1A)

(Ceccarelli et al. 1973; Fesce et al. 1994; Klyachko and Jackson 2002; Aravanis et al. 2003; Harata et al. 2006; He et al. 2006; Zhang et al. 2009).

These pathways are discussed below with major emphasis on clathrin-dependent synaptic vesicle reformation given the evidence for the importance of this process at synapses and the substantial mechanistic understanding that has been reached for this form of endocytosis.

CLATHRIN-MEDIATED ENDOCYTOSIS

The role of clathrin-mediated endocytosis in the recycling of synaptic vesicles has been supported by many different experimental approaches. (1) Induction of a burst of exocytosis results in a transient accumulation of clathrin-coated endocytic intermediates (Heuser and Reese 1973; Gad et al. 1998). (2) Experimental manipulations of synapses known to block clathrin-mediated endocytosis result in a block of synaptic vesicle recycling (Shupliakov et al. 1997; Zhang et al. 1998; Ringstad et al. 1999; Morgan et al. 2000; Granseth et al. 2006; Ferguson et al. 2007; Heerssen et al. 2008; Kasprowicz et al. 2008; Kim and Ryan 2009; von Kleist et al. 2011). (3) Conversely, many perturbations that block synaptic vesicle recycling result in a major buildup of clathrin-coated intermediates (Shupliakov et al. 1997; Cremona et al. 1999; Ferguson et al. 2007; Hayashi et al. 2008; Milosevic et al. 2011; Raimondi et al. 2011). (4) Synaptic vesicle proteins represent the main cargo of purified brain clathrin-coated vesicles (Maycox et al. 1992; Blondeau et al. 2004). Indeed, the abundance at clathrin-mediated endocytosis at synapses has made brain the tissue of choice for the purification of clathrin-coated vesicles (Kadota and Kadota 1973; Pearse 1975).

CLATHRIN COATS AND THE GENESIS OF A SYNAPTIC VESICLE

Textbook diagrams often show that the fate of presynaptic clathrin-coated vesicles after their uncoating is to fuse with early endosomes, which then function as the source of new synaptic vesicles (Heuser and Reese 1973; Mellman 1996). This model, which fits with the typical

fate of clathrin-coated vesicles in other systems, was first suggested at the time when the coat-dependent synaptic vesicle recycling was first characterized (Heuser and Reese 1973). At the time, coated buds on cisternae not connected to the cell surface in the plane of the section were interpreted as coated vesicles in the process of fusion (Heuser and Reese 1973). However, subsequent studies showed that clathrin-coated vesicles rapidly uncoat after fission (Guan et al. 2010; Milosevic et al. 2011) and that even coated buds located deep in nerve terminals represent budding from plasma membrane invaginations rather than fusion events (Ferguson et al. 2007; Hayashi et al. 2008; Raimondi et al. 2011). Based on these considerations, the direct reformation of synaptic vesicles from the uncoating of clathrin-coated vesicles, as suggested by Figure 1A, was proposed (Takei et al. 1996). According to this second model, at least many of the large and numerous endocytic intermediates observed in nerve terminals after strong stimulation are not stations downstream from clathrin-coated vesicles, but vacuoles that result from the bulk uptake of excess membrane (bulk endocytosis).

This model is attractive for several reasons. First, studies of vesicle formation in a variety of organisms and at different stations of the exo- and endocytic pathways have established that "coat"-mediated budding is a general mechanism for the production of vesicles with a precise molecular composition because of the property of coats to act as molecular sorters (Rothman 1994; Bednarek et al. 1996). Components of the endocytic clathrin coat, along with their accessory factors, are the major coat proteins concentrated in nerve terminals, strongly implicating them in the formation of synaptic vesicles (Maycox et al. 1992; Ferguson et al. 2007). Second, the model eliminates the requirement for two distinct budding steps in the recycling of synaptic vesicles—a first clathrin-mediated budding from the plasma membrane followed by a second budding reaction from endosomes—and thus is more compatible with the fast time course of this process. Third, clathrin-coated pits of nerve terminals have a remarkably small and homogeneous size, in

the same range of the size of synaptic vesicles (Shupliakov et al. 1997; Raimondi et al. 2011). This characteristic, which contrasts with the heterogeneous size of clathrin-coated buds observed in other systems and even in neurons away from presynaptic sites, is consistent with a direct formation of synaptic vesicles from clathrin-coated vesicles that have shed their coats. Fourth, a scenario in which clathrin-coated vesicles are directly upstream of synaptic vesicles is supported by quantal analysis studies of the uptake and release of styryl dyes (Ryan et al. 1997; Murthy and Stevens 1998).

Some general principles apply to clathrin-mediated endocytosis in all cellular contexts (Conner and Schmid 2003; Jung and Haucke 2007; Dittman and Ryan 2009; Kirchhausen 2009; McMahon and Boucrot 2011; Taylor et al. 2011). The nucleation of a clathrin-coated pit starts with the interaction of clathrin adaptors and/or a subset of their accessory factors with the lipid bilayer and with membrane proteins. In this nucleation stage, obligatory intrinsic components of the vesicle membrane (Koo et al. 2011; Miller et al. 2011), such as those that direct vesicle traffic, are likely to play a dominant role. Subsequently, interactions of the adaptors with each other, with other accessory factors, with cargo proteins, and with clathrin lead to the rapid growth of the coat in a feed-forward, cooperative fashion. Eventually, a deeply invaginated clathrin-coated bud with a narrow neck is formed. Fission of this neck in a reaction that requires the GTPase dynamin (Koenig and Ikeda 1989; Conner and Schmid 2003; Ferguson and De Camilli 2012) leads to a free vesicle that rapidly uncoats.

However, in axon terminals, clathrin-mediated endocytosis has some unique properties. These include, besides the highly homogeneous small size of the vesicles, specificity of the cargo and speed of the process. Because synaptic vesicles have a very precise function, mechanisms must exist to mediate the inclusion into the nascent vesicle of all needed membrane proteins and in the appropriate stoichiometry relative to each other. For example, although some proteins (such as synaptobrevin/VAMP) are present in tens of copies, others are present in few, or

even single copies (e.g., neurotransmitter transporters and the vacuolar ATPase) (Takamori et al. 2006), suggesting the existence of a checkpoint to ensure that all critical components are on board. Little is known about these mechanisms. Given the special nature of clathrin-mediated endocytosis at synapses, several endocytic factors that play general roles in endocytosis are present at synapses as neuron-specific isoforms. An overview of clathrin-dependent endocytosis in presynaptic nerve terminals is presented below.

CORE COMPONENTS AND ACCESSORY FACTORS OF THE CLATHRIN COAT

Like other coats, the clathrin coat is composed of two layers: an inner layer of adaptors and an outer clathrin layer (Table 1). The clathrin adaptors typically comprise a membrane-binding folded module and flexible arms, which may terminate in an additional small folded module (ear) (Edeling et al. 2006). The folded module binds cytoplasmically exposed domains or endocytic "motifs" of vesicle membrane proteins as well as the head group of $PI(4,5)P_2$, a phosphoinositide concentrated in the plasma membrane (Beck and Keen 1991; Traub 2003; Owen et al. 2004; Zoncu et al. 2009; Jackson et al. 2010). The arms bind clathrin heavy chain as well as other adaptors and endocytic factors (Edeling et al. 2006; Schmid and McMahon 2007). Their elongated flexible unfolded structure is optimally suited to capture these proteins in the cytosol and to help concentrate them at growing coated buds.

The most abundant adaptor is AP-2, a member of a heterotetrameric family of adaptors that interacts with tyrosine-based endocytic motifs (Bonifacino and Traub 2003), such as the one present in the synaptic vesicle protein SV2 (Haucke and De Camilli 1999), and with dileucine-based motifs, such as those in vesicular neurotransmitter transporters (Bonifacino and Traub 2003; Fei et al. 2008). The other major synaptic endocytic clathrin adaptor is the ANTH domain-containing protein AP180 (Morgan et al. 2000; Ford et al. 2001), which like its nonsynaptic homolog CALM (clathrin assembly

lymphoid myeloid leukemia) binds the V-SNARE synaptobrevin/VAMP (Nonet et al. 1999; Dittman and Kaplan 2006; Burston et al. 2009; Koo et al. 2011; Miller et al. 2011). Yet another adaptor is stonin 2, that along with AP-2 plays a role in the internalization of synaptotagmin, the Ca^{2+} sensor of the synaptic vesicle (Zhang et al. 1994; Walther et al. 2001, 2004; Diril et al. 2006). Epsin is a clathrin adaptor that binds ubiquitinated cargo and may have additional function in endocytosis (Ford et al. 2002; Boucrot et al. 2012), but the importance of its function in synaptic vesicle endocytosis remains unclear (De Camilli et al. 2002; Ford et al. 2002). FCHo, a protein that comprises an F-BAR domain (see below) and a domain homologous to a cargo-binding subunit of AP-2, may also have adaptor functions for a yet to be determined cargo. Several other proteins with endocytic adaptor properties (also called CLASP for clathrin-coat-associated sorting proteins) (Mishra et al. 2005) have been identified; for example, Dab (Morris and Cooper 2001), ARH (He et al. 2002), β-arrestin (Lin et al. 1997), etc. However, so far these proteins have not been shown to play a major role in synaptic vesicle recycling.

The clathrin lattice is represented by a chicken-wire-like structure that results from the assembly of clathrin triskelia (trimers of clathrin heavy chains with three bound light chains) (Kirchhausen 2000a). Clathrin, which can assemble with various geometries and forms basketlike structures in the absence of a membrane template, had been assumed to be the primary determinant of membrane curvature. However, current models posit that clathrin adapts to, and stabilizes, membrane curvature induced by other factors (the adaptors and its accessory factors), although it may cooperate in propagating curvature once a bud has been nucleated (Ford et al. 2002; Itoh and De Camilli 2006) (see also below). Clathrin also functions as a scaffold for the clustering of the adaptors and thus of membrane cargo to be internalized.

In addition to the core components of the coat, i.e., the adaptors and clathrin, several factors that assist and/or regulate the nucleation, loading, and dynamics of endocytic clathrin-coated pits were identified through biochemical

Table 1. Major endoytic proteins implicated in synaptic vesicle recycling

Protein	Major binding partners	Function	Genetic evidence
Core clathrin coat components			
Clathrin (heavy and light chain)	AP-2, AP180/CALM, Epsin, Amphiphysin, Synaptojanin, Hsc70, Auxilin	Clathrin lattice	+
AP-2 (heterotetramer of $\alpha,\beta,\gamma,\delta$ subunits)	Clathrin, Epsin, AP180/CALM, Stonin, Amphiphysin, Synaptojanin, PIPK1γ, Intersectin, Eps15, Auxilin, Synaptotagmin, SV2, PI(4,5)P$_2$	Clathrin adaptor for several proteins of vesicle membranes	+
AP180/CALM	AP-2, Clathrin, VAMP/Synaptobrevin, PI(4,5)P$_2$	Specific clathrin adaptor for VAMP/Synaptobrevin	+
Epsin	AP-2, Clathrin, Intersectin, Eps15, EHD, ubiquitin, PI(4,5)P$_2$	Clathrin adaptor, specific adaptor for ubiquitinated membrane proteins, role in bilayer deformation and fission	+
Stonin	AP-2, Intersectin, Eps15, Synaptotagmin, PI(4,5)P$_2$	Specific clathrin adaptor for synaptotagmin	+
BAR domain-containing proteins (generate/sense bilayer curvature)			
Endophilin	Dynamin, Synaptojanin	Coordinates curvature acquisition with fission and uncoating of clathrin-coated vesicles	+
Amphiphysin	AP-2, Clathrin, Dynamin, Synaptojanin, Endophilin, N-WASP	Coordinates curvature acquisition with fission?	+
FCHo	Intersectin, Eps15, Dynamin	Key nucleator of endocytic clathrin-coated pits, cargo binding?	a
Syndapin	Dynamin, Synaptojanin, EHD, N-WASP	Coordinates endocytosis with actin nucleation	+
Nervous wreck	Dynamin, Intersectin, N-WASP	Implicated in actin assembly	+
Toca/Fbp17/CIP4	Dynamin, N-WASP	Implicated in actin cytoskeleton nucleation and organization	a
Phosphoinositide metabolism			
Synaptojanin 1 (145 kDa isoform)	AP-2, Clathrin, Endophilin, Amphiphysin, Syndapin, Intersectin	PI(4,5)P$_2$ phosphatase that couples fission of clathrin-coated vesicles to the shedding of the adaptors during uncoating	+
PIPK1γ	AP-2, Talin 2	Major PI(4,5)P$_2$ synthesizing enzyme at the presynaptic plasma membrane	+
Scaffolding			
Intersectin	AP-2, Epsin, Stonin, FCHo, Eps15, Dynamin, Synaptojanin, N-WASP, Cdc42 (GEF for Cdc42)	Scaffolding proteins that coordinate the recruitment of a multiplicity of other factors	+
Eps15	AP-2, Intersectin, Stonin, Epsin		+
Tuba/DNMBP	Dynamin, N-WASP, Ena/VASP, WAVE complex, Cdc42 (GEF for Cdc42)		a

Continued

Table 1. *Continued*

Protein	Major binding partners	Function	Genetic evidence
Membrane fission			
Dynamin	Endophilin, Amphiphysin, Tuba/DNMBP, Syndapin, Nervous wreck, Toca/Fbp17/CIP4, Intersectin, Cortactin, F-actin	GTPase required for endocytic membrane fission	+
Clathrin lattice disassembly			
Hsc70	Auxilin, Clathrin	ATPase that triggers disassembly of clathrin cages	+
Auxilin	Clathrin, AP-2, Dynamin	Cofactor of Hsc70 that recruits Hsc70 to clathrin-coated vesicles	+

[a]Likely play a role in synaptic vesicle recycling but genetic evidence is still missing.

and genetic screens (Slepnev and De Camilli 2000; Toret and Drubin 2006; Dittman and Ryan 2009; McPherson 2010). Some of these proteins, collectively referred to as clathrin accessory factors (Slepnev and De Camilli 2000), may in fact be considered core components of the coat (Table 1). Typically they contain protein–protein or protein–lipid interacting modules as well as extended regions harboring short amino acid sequence motifs that mediate additional intra- or intermolecular interactions. Thus, they are optimally suited to assemble protein networks. Modules often present in such factors include SH3 domains, BAR domains, ENTH/ANTH and PH domains, whereas short amino acid motifs include proline-containing motifs (for SH3 domain binding), NPF motifs (for EH domain binding), clathrin boxes (for clathrin binding), and several motifs that recognize the appendage domains of the clathrin adaptor AP-2 (DPF/DPW, FXDXF, WXXF) (Slepnev and De Camilli 2000; Dittman and Ryan 2009; McPherson 2010). Some of these accessory factors contain enzymatic modules such as protein kinases (for example, Ack and AAK1 [Conner and Schmid 2002; Shen et al. 2010], phosphoinositide-metabolizing enzymes [McPherson et al. 1996; Thieman et al. 2009; Kahlfeldt et al. 2010], and DENN domains, i.e., modules that function as guanylnucleotide exchange factors for Rab proteins [Marat et al. 2010; Wu et al. 2011]). Ubiquitin and ubiquitin-binding motifs are also present in some accessory factors (Slepnev and De Camilli 2000; De Camilli et al. 2002; Polo et al. 2003; Itoh and De Camilli 2006; Dittman and Ryan 2009). Overall, the relatively low affinity involved in the interactions mediated by these modules and motifs (low micromolar range) in clathrin accessory factors is ideally suited to allow the rapid formation and disassembly of molecular networks. The functional characterization of these proteins has generated many insights into the formation and maturation of clathrin-coated vesicles. Some themes that have emerged from the studies of these proteins are discussed below.

CURVATURE GENERATION AND CURVATURE SENSING

Several proteins associated with coats were assigned functions in curvature acquisition and/or curvature sensing (Table 1). The formation of a high-curvature bud requires the existence of mechanisms that counteract the spontaneous tendency of a phospholipid bilayer to adopt a flat shape. Curvature can be induced by the generation of asymmetries between the two leaflets of the bilayer, by curved protein scaffolds that directly or indirectly bind the bilayer, or by cytoskeletal elements that act on the membrane (Farsad and De Camilli 2003). All these sets of

factors may come into play in the formation of clathrin-coated vesicles. (1) Some endocytic proteins contain amphipathic helices that partially penetrate the cytosolic leaflet of the bilayer, thus generating bilayer asymmetry (Farsad et al. 2001; Ford et al. 2002; Gallop et al. 2006; Masuda et al. 2006; Antonny 2011). (2) Other endocytic factors contain curved lipid-binding domains of the BAR superfamily (narrow curvature in the classical BAR domains, shallow curvature in the F-BAR domains) (Peter et al. 2004; Weissenhorn 2005; Shimada et al. 2007; Frost et al. 2008). BAR domains form homo or heterodimers whose concave, positively charged surface (in the case of BAR domain-containing proteins implicated in endocytosis) is optimally adapted to interact with a curved, negatively charged bilayer surface (Frost et al. 2009). (3) Still other factors are actin regulatory proteins that can produce curvature indirectly, via the force applied to membranes by the actin cytoskeleton (Kaksonen et al. 2006; Ferguson et al. 2009), as further discussed below.

Importantly, protein modules that can induce curvature can also sense curvature and bind more efficiently to a precurved bilayer (Antonny 2006; Roux et al. 2010), so that curvature can be rapidly propagated by these modules (Takei et al. 1999; Farsad et al. 2001; Itoh et al. 2005; Tsujita et al. 2006; Frost et al. 2008). Furthermore, curvature sensing by proteins that contain other functional domains represents a powerful mechanism to coordinate progressive acquisition of membrane curvature with changes that must occur at various stages of maturation of the coated pit (Dawson et al. 2006; Itoh and De Camilli 2006).

Although there is evidence that these various mechanisms play an important role in curvature acquisition and sensing in clathrin-mediated endocytosis, the precise identity and relative importance of the specific factors implicated in shape changes of presynaptic clathrin-coated pits remain poorly understood. Based on in vitro studies, epsin, which shares properties of adaptors, was proposed to function as a critical determinant of endocytic clathrin-coated pit curvature via its amino-terminal amphipathic helix that folds uon $PI(4,5)P_2$ binding

(Ford et al. 2002). A role of epsin in clathrin-dependent synaptic vesicle recycling is supported by antibody-microinjection studies in the lamprey giant axon (Jakobsson et al. 2008), and indirectly by the reported role in this process of its EH domain-containing binding partners Eps15, intersectin, and EHD (Salcini et al. 2001; Marie et al. 2004; Montesinos et al. 2005; Rose et al. 2007; Ko et al. 2010; Pechstein et al. 2010a,b; Jakobsson et al. 2011), but not yet by genetic studies in model organisms (Bao et al. 2008; Chen et al. 2009). It remains possible that a function of epsin in curvature generation, or even in membrane fission, as recently proposed (Boucrot et al. 2012) may be nonessential and redundant with that of other endocytic factors. Endophilin, which contains both a BAR domain and amphipathic helices, is thought to coordinate curvature acquisition with both fission and uncoating of clathrin-coated vesicles via the interaction of its carboxy-terminal SH3 domain with dynamin and the $PI(4,5)P_2$ phosphatase synaptojanin (Ringstad et al. 2001). Endophilin was proposed to start acting at early stages of clathrin-mediated budding, based on antibody-microinjection experiments in giant lamprey axons (Ringstad et al. 1999). However, more recent imaging studies (Perera et al. 2006; Ferguson et al. 2009; Taylor et al. 2011) and genetic studies in *Drosophila*, *C. elegans*, and mice, favor late actions (Schuske et al. 2003; Verstreken et al. 2003; Dickman et al. 2005; Milosevic et al. 2011), primarily in uncoating via endophilin's property to recruit synaptojanin. However, a study in *C. elegans* has suggested dynamin- and synaptojanin-independent action of endophilin (Bai et al. 2010). Because vesicles can be generated, albeit inefficiently, in the absence of endophilin (Schuske et al. 2003; Verstreken et al. 2003; Milosevic et al. 2011), some functions of endophilin in curvature generation/sensing may also be fulfilled by other BAR domain-containing proteins, e.g., amphiphysin, SNX9/SNX18, and tuba (Itoh and De Camilli 2006). Lack of amphiphysin produces only mild defects in synaptic vesicle endocytosis, and its precise site of action remains to be defined (Di Paolo et al. 2002b; Pant et al. 2009).

Based on studies of nonneuronal cells, FCHo, which shares properties of adaptors (see above), was reported to function as the "nucleator" of clathrin-coated pits and as a first inducer of their curvature through its shallow F-BAR domain (Henne et al. 2010). This proposal is supported by its early presence at sites of clathrin-coated pit nucleation along with intersectin and Eps15. A role of an FCHo family member at synapses, however, has not been explored yet. The precise site of action and the role of other F-BAR proteins, such as members of the Toca/Fbp17/CIP4 family, syndapin, as well of nervous wreck in flies, remains to be established, despite their abundance in nerve terminals and of evidence that they participate in endocytosis (Kessels and Qualmann 2004; Dawson et al. 2006; Itoh and De Camilli 2006; Andersson et al. 2008; Rodal et al. 2008; Bu et al. 2010; Wu et al. 2010; Koch et al. 2011).

PHOSPHOINOSITIDE METABOLISM

Some proteins that directly or indirectly interact with core components of endocytic clathrin-coated pits are phosphoinositide-metabolizing enzymes, thus (1) lending support to the physiological importance of the interaction of clathrin adaptors with $PI(4,5)P_2$ and (2) suggesting that $PI(4,5)P_2$ metabolism is tightly coupled to endocytic clathrin coat dynamics (Table 1). Synaptojanin 1, a $PI(4,5)P_2$ phosphatase that can dephosphorylate both the 4 and 5 position of the inositol ring, interacts with endophilin and other BAR proteins (McPherson et al. 1996; Guo et al. 1999; Ringstad et al. 2001). It is therefore recruited (primarily by endophilin) at the necks of clathrin-coated pits just before fission and one of its main functions is to couple the fission reaction of endocytosis to the shedding of the adaptors during uncoating (Cremona and De Camilli 2001; Milosevic et al. 2011).

PIP kinase type 1γ (PIPK1γ), the major $PI(4,5)P_2$ synthesizing enzyme at synapses (Wenk et al. 2001), interacts with talin 2, a protein concentrated at synaptic junctions (Di Paolo et al. 2002a; Ling et al. 2002), and with the clathrin adaptor AP-2 (Krauss et al. 2006; Thieman et al. 2009), thus providing a mecha-

nism to ensure efficient synthesis of $PI(4,5)P_2$ at the nerve terminal plasma membrane and in proximity to endocytic clathrin-coated pits in particular (Di Paolo et al. 2004). These findings have suggested a model according to which a cycle of $PI(4,5)P_2$ synthesis and hydrolysis is nested within the synaptic vesicle cycle (Fig. 2), with $PI(4,5)P_2$ being restricted to the plasma membrane (Di Paolo and De Camilli 2006). Because synaptojanin 1 can dephosphorylate $PI(4,5)P_2$ to PI, PI 4-kinases are also thought to be involved in this cycle. A PI 4-kinase activity was reported on synaptic vesicles (Guo et al. 2003), but PI4P is also synthesized at the plasma membrane (Balla et al. 2008). The source of PI4P implicated in the vesicle cycle and the PI 4-kinase(s) involved remain a matter of debate (Di Paolo and De Camilli 2006).

Other phosphoinositide metabolizing enzymes, such as the inositol 5-phosphatases SHIP2 (Nakatsu et al. 2010) and OCRL (Erdmann et al. 2007; Mao et al. 2009) and the type II PI 3-kinases, also interact with clathrin coat components (Domin et al. 2000; Nakatsu et al. 2010). However, although such interactions further support the critical role of $PI(4,5)P_2$ in clathrin-coated pit dynamics, no direct evidence that they play an important role in synaptic vesicle recycling has been obtained so far. *Drosophila* studies showed a role of Tweek in synaptic vesicle recycling via a functional link to $PI(4,5)P_2$, but the mechanism of action of this protein is unknown (Verstreken et al. 2009).

SCAFFOLDING

The modular structure of some accessory proteins of the clathrin coat is specially designed to provide scaffolding functions for the assembly of other factors. In two large proteins, intersectin and tuba/DNMBP, this function is particularly evident (Table 1). Intersectin contains in sequence (i) amino-terminal EH domains that bind other endocytic factors such as epsin, stonin 2, FCHo and syndapin, (ii) a coiled-coil region involved in homodimerization or heterodimerization with Eps15 (a smaller scaffold protein containing EH domains and AP-2 binding sites), and (iii) SH3 domains that
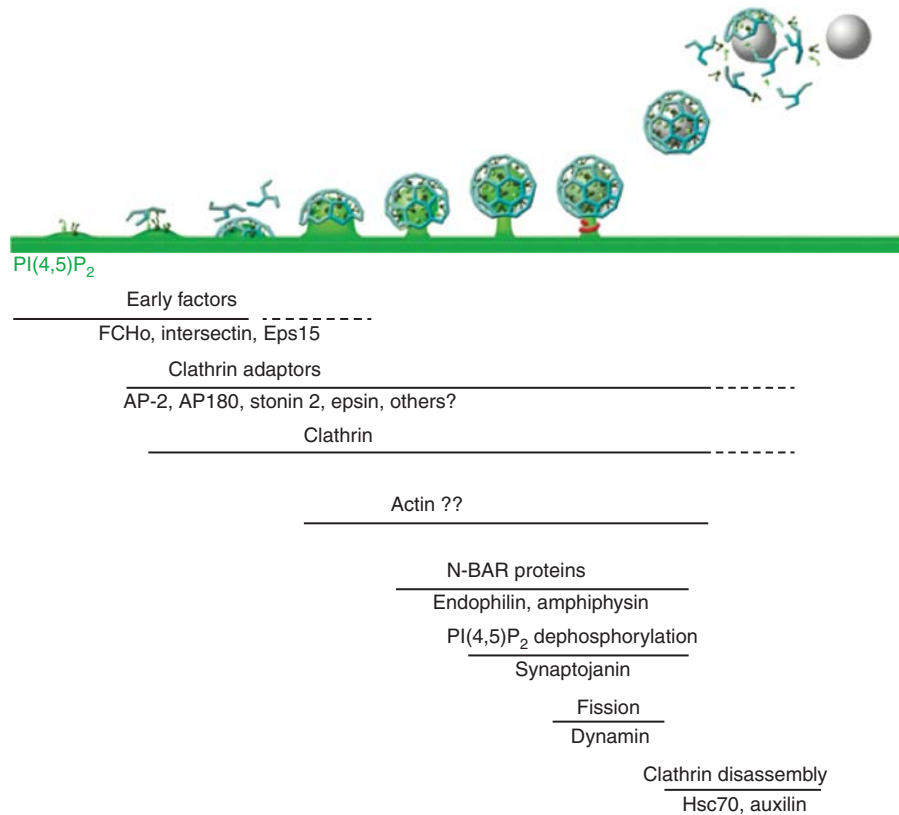
PI(4,5)P$_2$

Early factors
FCHo, intersectin, Eps15

Clathrin adaptors
AP-2, AP180, stonin 2, epsin, others?

Clathrin

Actin ??

N-BAR proteins
Endophilin, amphiphysin

PI(4,5)P$_2$ dephosphorylation
Synaptojanin

Fission
Dynamin

Clathrin disassembly
Hsc70, auxilin

Figure 2. Sequential recruitment of endocytic proteins during clathrin-mediated synaptic vesicle endocytosis. Assembly and maturation of endocytic clathrin-coated pits involves the sequential action of a variety of protein factors. Putative sites of action of various factors are indicated (see text for discussion). PI(4,5)P$_2$ (green), which is generated at the synaptic plasma membrane primarily by PIP kinase type 1γ (PIPK1γ), plays a critical role both in exocytosis and in the recruitment of endocytic factors.

collectively bind dynamin, synaptojanin, N-WASP, and several signaling proteins (Yamabhai et al. 1998; Okamoto et al. 1999; Marie et al. 2004; Kelly and Phillips 2005; Montesinos et al. 2005; Malacombe et al. 2006; Pechstein et al. 2010a). A long intersectin isoform selectively expressed in the nervous system of vertebrates contains an additional carboxy-terminal extension containing a DH-PH module that acts as a guanine nucleotide exchange factor (GEF) for Cdc42 (an actin regulatory protein) and a C2 domain (Hussain et al. 2001; Pechstein et al. 2010a). Tuba/DNMBP comprises a central DH-BAR module that also acts as a Cdc42 activator. This domain is flanked by amino-terminal SH3 domains that bind dynamin, and by car-

boxy-terminal SH3 domains that bind N-WASP and a variety of actin regulatory proteins (Hussain et al. 2001; Salazar et al. 2003; Kelly and Phillips 2005; Pechstein et al. 2010a). Because N-WASP and active Cdc42 act synergistically to stimulate Arp2/3-dependent actin nucleation, an important function of these scaffold proteins is to control actin dynamics at endocytic sites.

The importance of intersectin and of its binding partner Eps15 in coordinating the function of multiple early endocytic factors in endocytosis (Henne et al. 2010), including synaptic vesicle endocytosis, is supported by genetic studies (Salcini et al. 2001; Koh et al. 2004, 2007; Marie et al. 2004; Rose et al. 2007) and by microinjection

experiments in giant lamprey axons (Evergren et al. 2007). Tuba localizes to clathrin-coated pits (Ferguson et al. 2009), but its role in synaptic vesicle endocytosis remains to be shown.

THE ACTIN CYTOSKELETON

Several clathrin accessory factors are directly or indirectly linked to the regulation of the actin cytoskeleton (Table 1). The examples of intersectin and tuba were mentioned above. These proteins activate Cdc42 via their DH domains (Hussain et al. 2001; Salazar et al. 2003). Active Cdc42, in turn, acts in concert with their interactor N-WASP to stimulate Arp2/3-dependent actin nucleation (Miki et al. 1998). PI(4,5)P$_2$, also present at endocytic sites, further activates N-WASP (Miki et al. 1996). Several other BAR/F-BAR domain-containing proteins besides tuba, such as syndapin, nervous wreck, and Toca family members (Toca-1/FBP17/CIP4), bind N-WASP via their SH3 domains (Toca family members also bind active Cdc42) (Itoh and De Camilli 2006; Takenawa and Suetsugu 2007). These findings converge with many studies of clathrin-mediated endocytosis in a variety of systems to suggest a strong link between clathrin-mediated endocytosis and actin.

However, the role of actin in clathrin-mediated endocytosis at synapses has been and continues to be a highly debated issue. Studies of synapses of the giant axon of the lamprey have shown that actin-perturbing agents have a powerful inhibitory action on clathrin-dependent synaptic vesicle recycling (Shupliakov et al. 2002; Bourne et al. 2006). In contrast, pharmacological disruption of actin did not seem to affect clathrin-mediated endocytosis at mammalian CNS synapses in vitro (Sankaranarayanan et al. 2003). Conflicting results on the role of actin in clathrin-mediated endocytosis have also been reported for non-neuronal cells (Merrifield et al. 2005; Ferguson et al. 2009; Saffarian et al. 2009). The emerging consensus is that actin dynamics may help counteract membrane tension, so that its importance varies depending on the tension of the membrane and the geometry of the membrane patch to be internalized (Boulant et al. 2011).

How could actin function in endocytosis? Important lessons have been learned from yeast, in which clathrin is implicated in the endocytosis that occurs at actin patches (Engqvist-Goldstein and Drubin 2003; Kaksonen et al. 2003). Although this process differs in many respects from clathrin-mediated endocytosis in metazoan—for example, yeast endocytic buds have a tubular rather than a spherical shape, the fission reaction does not require dynamin, and the dependence on actin is much stronger—several important features are conserved (Kaksonen et al. 2005; Ferguson et al. 2009). Thus, the many advantages offered by this organism for the functional characterization of proteins with genetic methods have greatly helped define a sequence of action of many endocytic factors and of actin in particular. As in mammalian cells, endocytosis in yeast starts with the assembly of clathrin coat proteins, and many of the subsequent stages are orchestrated by proteins whose homologs are also present at mammalian clathrin-coated pits (Kaksonen et al. 2005; Ferguson et al. 2009; Taylor et al. 2011). Importantly, yeast studies have shown that N-WASP- and Arp2/3-dependent actin polymerization plays a major role in the invagination of the buds and in the events leading to fission (Kaksonen et al. 2005). Analysis of clathrin-mediated endocytosis in mammalian cells, either in living cells or in a cell-free system under conditions in which endocytosis is blocked, has supported this model because they have shown an actin dependence of the elongation/tubulation of the neck of the arrested clathrin-coated pits (Ferguson et al. 2009; Wu et al. 2010).

MEMBRANE FISSION

Although the clathrin coat and some of its interactors can drive progression of an endocytic bud to a deeply invaginated state, the physical separation of the clathrin-coated bud to generate a free vesicle requires the GTPase dynamin, a member of a family of membrane-remodeling GTPases (Table 1) (Praefcke and McMahon 2004; Ferguson and De Camilli 2012). Dynamin oligomerizes into spirals at bud necks and mediates fission in a process involving GTP

hydrolysis (Hinshaw and Schmid 1995; Takei et al. 1995; Roux et al. 2006; Pucadyil and Schmid 2008). Of the three dynamins encoded by the human genome, dynamin 1 and dynamin 3 are expressed almost uniquely in the brain (Cao et al. 1998), where dynamin 1 is present by far at the highest concentration, whereas dynamin 2 is expressed at much lower concentration in all cells (Ferguson et al. 2007, 2009). A striking demonstration of the importance of dynamin in endocytosis is provided by the temperature-sensitive *shibire* mutation in *Drosophila* (Koenig and Ikeda 1989). Within less than a minute following a shift to the restrictive temperature, these mutant flies become paralyzed owing to a depletion of synaptic vesicles. Although their synaptic vesicles continue to fuse with the plasma membrane, their endocytosis is arrested at the stage of "collared" buds (Koenig and Ikeda 1989). A dramatic arrest of clathrin-mediated endocytosis was also observed in a variety of model synapses after perturbation of dynamin function (by dominant-negative interference or pharmacological inhibition) (Shupliakov et al. 1997; Yamashita et al. 2005; Newton et al. 2006), and more recently, at mouse synapses that lack dynamin 1 (Ferguson et al. 2007), or, in a more severe form, at synapses that lack both dynamin 1 and 3 (Raimondi et al. 2011). At such synapses, the arrest of clathrin-coated pit fission leads to a massive accumulation of clathrin-coated pits on deep plasma membrane invaginations that may fill the entire nerve terminal and replace the synaptic vesicle cluster (Ferguson et al. 2007; Hayashi et al. 2008; Raimondi et al. 2011). Surprisingly, neither dynamin 1 nor even dynamin 1 and 3 together, are essential for the occurrence of clathrin-mediated endocytosis at low level of activity. The presence of high levels of dynamin (primarily dynamin 1) at synapses is needed to allow the rate of endocytosis to scale with an increase of the secretory response (Lou et al. 2008). In the absence of dynamin 1 and 3, probability of release is reduced, most likely to prevent synaptic transmission failure owing to vesicle depletion (Lou et al. 2012).

The precise mechanism through which dynamin mediates fission remains unclear, but recent structural studies have provided significant insight. These studies have confirmed that dynamin is a member of a family of GTPases whose catalytic activity is dependent on the GTP-dependent dimerization of the GTPase module (Gasper et al. 2009; Chappie et al. 2010; Ferguson and De Camilli 2012). Importantly, they have shown that such dimerization can only occur between adjacent rungs within the dynamin spiral and that GTP hydrolysis results in a conformational change of neighboring domains to produce a constriction of the dynamin spiral and thus of the underlying tubular bud neck (Chappie et al. 2011; Faelber et al. 2011; Ford et al. 2011). It remains unclear whether fission is the direct result of this constriction or of the disassembly that follows constriction and that exposes an unstable narrow tubular intermediate (Roux et al. 2006; Mears et al. 2007; Bashkirov et al. 2008; Pucadyil and Schmid 2008; Low and Lowe 2010; Chappie et al. 2011; Faelber et al. 2011; Ford et al. 2011).

Dynamin recruitment occurs as pits mature to their deeply invaginated state (Merrifield et al. 2002; Perera et al. 2006; Taylor et al. 2011). The PH domain of dynamin, which binds acidic phospholipids and $PI(4,5)P_2$, and the proline-rich carboxy-terminal region, which functions as a protein–protein interaction domain, are important for dynamin's function and recruitment (Okamoto et al. 1997; Shupliakov et al. 1997; Lee et al. 1999; Vallis et al. 1999; Szaszak et al. 2002). Many of the proteins that bind this region (generally through SH3 domains) are BAR domain-containing proteins and thus proteins that may help coordinate formation of the vesicle neck with the recruitment of dynamin (Itoh and De Camilli 2006). Importantly, BAR proteins that bind dynamin often also bind the $PI(4,5)P_2$ phosphatase synaptojanin and or N-WASP, thus suggesting a functional partnership between these proteins (Itoh and De Camilli 2006; Takenawa and Suetsugu 2007). Indeed, the recruitment of synaptojanin to sites of fission couples separation of the coated bud from the plasma membrane with its uncoating. It has also been proposed that the $PI(4,5)P_2$ phosphatase activity of synaptojanin may have a direct action in fission by creating a lipid-phase

boundary, and thus an interfacial force, between a PI(4,5)P_2-depleted bud and the PI(4,5)P_2-rich plasma membrane (Liu et al. 2009), or by promoting the dissociation of dynamin after constriction of the dynamin collar (Chang-Ileto et al. 2010). However, genetic studies in mice support a primary uncoating function of synaptojanin, although they do not rule out other roles. (Cremona et al. 1999; Hayashi et al. 2008; Milosevic et al. 2011). Concerning N-WASP, its activation in proximity of dynamin (Kessels and Qualmann 2002; Koch et al. 2011) may reflect a role of actin in neck formation/elongation (see above yeast studies) or in fission, by providing tension at bud necks, or in propelling newly formed vesicles away from endocytic sites.

UNCOATING

After fission, newly generated clathrin-coated vesicles rapidly shed their coat proteins and are recaptured in the synaptic vesicle cluster for reuse. Clathrin shedding is ATP-dependent and requires Hsc70 ATPase and its cofactor auxilin (Guan et al. 2010; Xing et al. 2010; Yim et al. 2010), whereas shedding of the adaptors is dependent on PI(4,5)P_2 hydrolysis by the PI(4,5)P_2 phosphatase synaptojanin (Table 1). Genetic disruption of synaptojanin 1, or of endophilin (the protein that plays a critical role in recruiting synaptojanin to endocytic sites), produces a striking accumulation of clathrin-coated vesicles in nerve terminals and delayed recycling kinetics in mice, worms, and flies (Cremona et al. 1999; Schuske et al. 2003; Verstreken et al. 2003; Dickman et al. 2005; Hayashi et al. 2008; Milosevic et al. 2011). It is the combined action of Hsc70 and synaptojanin that makes the uncoating reaction "catastrophic" and irreversible. A critical question is how these two reactions are coordinated. Although auxilin binds clathrin and AP-2, its recruitment to budding coated vesicles also requires its tensin homology domain (a tandem arrangement of a PTEN-like phosphatase domain and a C2 domain). Because the tensin homology domain binds membrane monophosphoinositides (Guan et al. 2010), it has been proposed that the catalytic action of synaptojanin 1 may mediate

such coordination. Conversion of PI(4,5)P_2 to PI4P by synaptojanin would not only remove membrane-binding sites for the clathrin adaptors but also generate binding sites for auxilin (Guan et al. 2010).

SPATIAL SEGREGATION OF EXO- AND ENDOCYTIC SITES

Clathrin-coated pits are typically observed at the outer margins of the active zones of secretion (Heuser and Reese 1973; Heuser 1989; Pieribone et al. 1995; Gad et al. 1998; Bloom et al. 2003). This spatial segregation is likely explained by the presence of a highly specialized membrane anchored matrix at the active zone, which is involved in capturing and docking of synaptic vesicles in preparation for exocytosis (Jin and Garner 2008). Although active mechanisms for the rapid export of components of the newly fused membrane away from sites of exocytosis cannot be excluded, the nonexpandable nature of the active zone, which is in precise register with, and anchored to, the postsynaptic membrane, may be sufficient to account for a rapid passive translocation of newly fused membrane material. Only after massive nerve terminal perturbations is the active zone disrupted (Shupliakov et al. 1997).

The area that surrounds the active zone and that is specialized for endocytosis forms an anatomically and biochemically distinct domain called the endocytic or periactive zone (Wan et al. 2000). This zone, which is particularly obvious at some specialized giant synapses such as those of lamprey reticulospinal axons, is typically marked by the presence of actin (Fig. 3), thus providing important evidence for the strong link between actin and endocytosis (Pieribone et al. 1995; Gad et al. 1998; Bloom et al. 2003). Studies in *Drosophila* have shown that several mutations in endocytic genes (e.g., synaptojanin, intersectin, nervous wreck, and endophilin) also produce defects in bouton size and organization at neuromuscular junctions (Rikhy et al. 2002; Coyle et al. 2004; Koh et al. 2004, 2007; Marie et al. 2004; Dickman et al. 2006). This finding may be explained by the importance of these genes both for endocytosis

and for actin function or by indirect effects of an endocytic impairment on signaling pathways that control synaptic size.

COUPLING BETWEEN EXOCYTOSIS AND ENDOCYTOSIS

In nonneuronal cells, clathrin-mediated endocytosis is predominantly a constitutive process. In contrast, at synapses, clathrin-dependent endocytosis is primarily a compensatory reaction aimed at recapturing excess membrane delivered to the cell surface by exocytosis. Accordingly, clathrin-coated profiles are rarely observed in resting nerve terminals, and their number greatly increases after stimulation (Heuser and Reese 1973; Gad et al. 1998). This implies the occurrence in nerve endings of a large pool of "nonengaged" clathrin coat proteins and of mechanisms to trigger their engagement during and after a stimulus.

A key trigger for the endocytic burst that follows a stimulus is the exocytic delivery of synaptic vesicle membranes to the plasma membrane, where they act as nucleating sites for coated pits. This function is mediated by binding sites for endocytic clathrin adaptors that are present in the cytoplasmic domains of several synaptic vesicle proteins but that need to be presented in the context of a $PI(4,5)P_2$-rich membrane (the plasma membrane) to trigger efficient recruitment of the adaptors. Because of the lack of $PI(4,5)P_2$ on synaptic vesicles, exocytosis represents the switch that makes these sites competent for adaptor recruitment (Fig. 2) (Di Paolo and De Camilli 2006). Interestingly, the presence of $PI(4,5)P_2$ in the plasma membrane also plays an important role in defining this membrane as the target for synaptic vesicle exocytosis and Ca^{2+}-dependent fusion (Osborne et al. 2001; Bai et al. 2004; Gong et al. 2005; Milosevic et al. 2005). $PI(4,5)P_2$, therefore, plays a fundamental role in exo-endocytosis coupling.

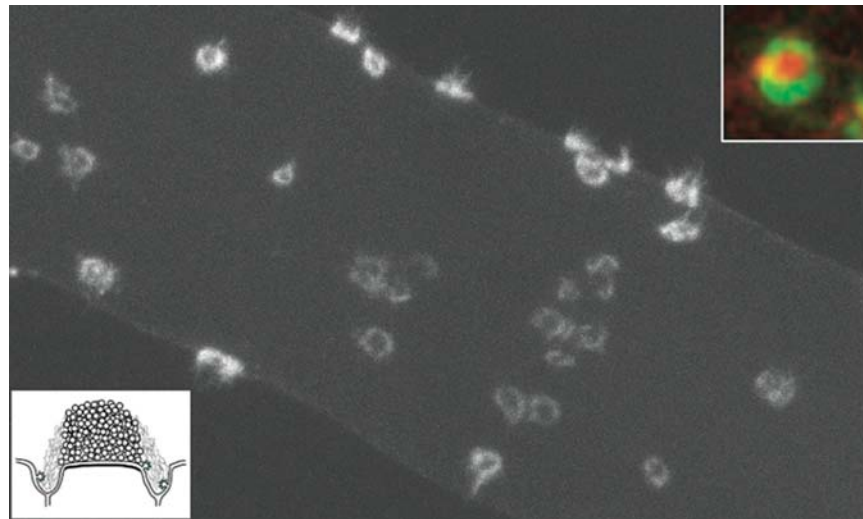An important open question that has implications for the exo-endocytic coupling is the

Figure 3. Presence of actin at periactive zones of synapses of the giant reticulospinal axon of the lamprey. Fluorescence image of a giant axon microinjected with Alexa Fluor 488-phalloidin. Fluorescent rings represent actin at periactive zones surrounding the vesicle cluster. The top inset is from an axon preincubated with an FM dye (red) to produce an endocytic labeling of synaptic vesicles, then fixed and labeled with phalloidin (green) to reveal actin. Note the actin ring nesting the vesicle cluster. The bottom inset is a schematic diagram of the synapses of the giant axon illustrating the vesicle cluster and the periactive zone (J Morgan, V Pieribone, and P De Camilli, unpubl.).

fate of vesicle proteins after the collapse of the vesicle into the plasma membrane. Removal of newly fused membrane material from sites of fusion seems to be required for new vesicles to dock (Hosoi et al. 2009). Whether vesicle proteins retain some association with each other after fusion, or disperse throughout the plasma membrane remains a debated issue. If they disperse and intermix with a small pool of synaptic vesicle proteins already present at the cell surface, the molecules taken up by compensatory endocytosis may not necessarily be the newly delivered ones (Tabares et al. 2007). Supporting this idea, the existence of a rapidly retrievable pool of synaptic vesicle proteins (possibly a preassembled endocytic patch or bud) whose endocytosis is triggered by exocytosis, was recently suggested (Fernandez-Alfonso et al. 2006; Wienisch and Klingauf 2006; Hua et al. 2011). Such a preassembly may explain the stochastic distribution of endocytic events after a single action potential (time constant $\sim$14 sec) with such events occurring as early as 1–2 sec after exocytosis (Balaji and Ryan 2007), i.e., an extremely short delay that seems difficult to reconcile with the formation of a new clathrin-coated pit (Kirchhausen 2009). The coupling between exocytosis and the internalization of preformed buds may be triggered by the decrease in surface tension resulting from expansion of the plasma membrane produced by exocytosis (Dai et al. 1997). Clearly, as shown by the analysis of capacitance in response to a secretory stimulus, the surface of an axon ending is set at a very precise value, and compensatory endocytosis rapidly resets an increase in surface area to the original value (Fig. 1D).

Another debated issue is whether and how the increase in cytosolic Ca^{2+} that has a critical role in exocytosis also plays some role(s) in the regulation of the subsequent endocytic process. Although there is evidence that cytosolic Ca^{2+} is needed for endocytosis, elevations of cytosolic Ca^{2+} above its physiological levels are not essential for this process (Ceccarelli and Hurlbut 1980; Wu and Betz 1996; Gad et al. 1998; Balaji et al. 2008). However, a transient elevation in cytosolic Ca^{2+} triggers various forms of clathrin-independent endocytosis in a variety of

cell types (Artalejo et al. 1995; Neves et al. 2001; Wu et al. 2009; Lariccia et al. 2010), and a stimulus-dependent cytosolic Ca^{2+} increase may also play a role in bulk endocytosis at synapses. Furthermore, a sustained increase in cytosolic Ca^{2+} accelerates endocytosis (Sankaranarayanan and Ryan 2000, 2001; Wu et al. 2005; Balaji et al. 2008). A synaptic vesicle protein, flower, was proposed to oligomerize and to form a Ca^{2+} channel on exocytosis, thus contributing to the coupling between exo- and endocytosis (Yao et al. 2009).

The regulatory effect of Ca^{2+} on endocytosis may be mediated by Ca^{2+} effectors that participate in the endocytic reaction (for example, synaptotagmin, which is thought to function as a Ca^{2+} sensor, acts both in exocytosis and in endocytosis [Yao et al. 2011]), but also by phosphorylation–dephosphorylation reactions. Importantly, several endocytic proteins are constitutively phosphorylated in resting nerve terminals but undergo rapid dephosphorylation by calcineurin (a Ca^{2+}-calmodulin-dependent phosphatase) on stimulation. These proteins, sometimes referred to as dephosphins, include proteins with different functions in endocytosis including dynamin, synaptojanin, PIPKIγ, AP180, epsin, Eps15, and several others (Liu et al. 1994; McPherson et al. 1994; Chen et al. 1999; Cousin and Robinson 2001; Lee et al. 2004, 2005; Nakano-Kobayashi et al. 2007). Typically, their dephosphorylation enhances their interactions with other endocytic factors and promotes their recruitment to endocytic sites (Liu et al. 1994; Slepnev et al. 1998; Anggono et al. 2006). Conversely, their constitutive phosphorylation, mediated at least in part by Cdk5, may serve to keep them inactive yet concentrated in nerve terminals by interactions that are mutually exclusive with those responsible for endocytosis (Tan et al. 2003; Tomizawa et al. 2003; Lee et al. 2005). Thus, phosphorylated endocytic proteins may represent a reservoir from which proteins can be recruited on stimulation. The Ca^{2+}-dependent dephosphorylation of PIPK1γ and synaptojanin 1 is of particular interest, because such dephosphorylation is coupled to their recruitment and activation (Lee et al. 2004, 2005;

Nakano-Kobayashi et al. 2007). This may represent a mechanism to couple the traffic of synaptic proteins through the plasma membrane with a metabolic flux of PI(4,5)P$_2$.

Other changes that are dependent on synaptic vesicle exocytosis, but not necessarily on Ca^{2+}-dependent dephosphorylation, may help link exo- to endocytosis. Some endocytic proteins are bound to the synaptic vesicle cluster and relocate to the periactive zone after stimulation (Evergren et al. 2007). In the case of endophilin, such reversible relocation was proposed to be linked to the exocytosis-dependent shedding of Rab3, a synaptic vesicle-associated protein (Bai et al. 2010).

KISS AND RUN

Clathrin-mediated budding provides a mechanism to generate a vesicle enriched in a specific cargo. The need for a molecular sorting mechanism to select cargo could be bypassed if vesicles did not fully collapse into the plasma membrane after fusion, thus retaining their identity. The potential existence of this endocytic mechanism, subsequently called kiss and run (Fesce et al. 1994), was raised already at the time when synaptic vesicle recycling was first described in studies of the frog neuromuscular junction (Ceccarelli et al. 1973; Heuser and Reese 1973; He and Wu 2007). Although the importance of clathrin-mediated endocytosis was subsequently corroborated by biochemical, biophysical, and genetic studies, evidence for kiss and run of synaptic vesicles remains primarily indirect, based on observations that appear incompatible with clathrin-mediated endocytosis or bulk endocytosis. Such evidence includes incomplete discharge of FM dyes (Aravanis et al. 2003), quenching of the incompletely released FM dye by the presence of the membrane-impermeant extracellular tracer bromophenol blue (BPB) (Harata et al. 2006), capacitance measurements of giant synapses (He et al. 2006), and recycling of quantum dots trapped in synaptic vesicles (Zhang et al. 2009). It is important to note that even studies that support kiss and run acknowledge the occurrence of parallel endocytic pathways.

What mechanism(s) could account for kiss and run of synaptic vesicles? A highly curved bilayer, such as that of synaptic vesicles, would be expected to immediately flatten in the plasma membrane on fusion, unless there were mechanisms to favor bilayer curvature retention. Such mechanisms must have come into play to impose curvature when the vesicle was generated. Thus, the fate of the vesicle after fusion will depend on whether determinants of membrane curvature are retained on the membrane after vesicle budding. Current evidence suggests that curvature is imposed by core and accessory components of the clathrin coat, although other mechanisms, such as lipid bilayer asymmetries or roles of wedge-shaped membrane proteins, cannot be excluded. Thus, uncoating is predicted to leave behind a "stressed" membrane with a propensity to flatten on exocytosis. Mechanisms that could prevent flattening of a "stressed" membrane could include (1) an only incomplete fusion of the two bilayers, with a flickering of the fusion pore that allows escape of the small neurotransmitter molecules, but does not proceed to full fusion; (2) the presence of a collar at the site of fusion that prevents neck opening; and (3) a rigid structure of the active zone plasma membrane that cannot accommodate a flattening of the "extra" membrane added by exocytosis. Whether any of these mechanisms comes into play at synapses is not known.

Compelling evidence for the occurrence of kiss-and-run fusion (also called cavicapture, to indicate the recovery of a cavity) was obtained for peptide- and amine-containing secretory granules of neuroendocrine cells and for large secretory vesicles of other cells (Albillos et al. 1997; Holroyd et al. 2002; Taraska et al. 2003). It should be noted, however, that the time courses of fusion-fission events for these types of vesicles (seconds) (Albillos et al. 1997) are much longer than those involved in the fusion of synaptic vesicles (milliseconds). The different physicochemical properties of these granules, such as lower curvature of the membrane and presence of a protein core that undergoes slow solubilization, are factors that may favor the flickering of the fusion pore.

Y. Saheki and P. De Camilli

BULK ENDOCYTOSIS

This form of synaptic vesicle endocytosis operates most prominently under strong stimulatory conditions, when a large number of synaptic vesicles fuse with the plasma membrane within a short time interval. Excess plasma membrane is rapidly recaptured via the formation of plasma membrane infoldings, which then undergo fission to generate intracellular vacuoles and cisternae (endosome-like intermediates) (Heuser and Reese 1973; Miller and Heuser 1984; Holt et al. 2003; Paillart et al. 2003; Wu and Wu 2007; Clayton et al. 2008; Hayashi et al. 2008). This process is a nonselective mechanism of membrane uptake. However, the resulting endosome-like intermediates may be enriched with intrinsic synaptic vesicle membrane proteins given the abundance of these proteins in the plasma membrane in response to a burst of synaptic vesicle exocytosis (Heuser and Reese 1973). The vacuoles subsequently disappear as new vesicles appear, indicating a precursor-product relationship between these organelles and new synaptic vesicles (Heuser and Reese 1973; Teng et al. 2007; Clayton et al. 2008).

The molecular mechanisms underlying bulk endocytosis remain largely unknown. The increase in surface to volume ratio resulting from massive exocytosis may help to buckle the plasma membrane, leading to the generation of deep infoldings (Torri-Tarelli et al. 1987). However, their fission from the plasma membrane implies an active mechanism. Such a mechanism appears to be dynamin independent because it occurs robustly even in the absence of dynamin 1 (by far the most abundant neuron-specific dynamin), when clathrin-mediated endocytosis is severely impaired (Ferguson et al. 2007; Hayashi et al. 2008; and our unpublished observations). The actin cytoskeleton and proteins that couple membrane deformation to actin dynamics such as BAR superfamily proteins (Itoh and De Camilli 2006) may be involved in this form of endocytosis. Supporting this possibility, the F-BAR protein syndapin was implicated in this process (Andersson et al. 2008; Clayton et al. 2009). Additionally, a form of bulk endocytosis was recently reconstituted in vitro using isolated plasma membrane lawns and brain cytosol, and this process was shown to involve both actin and F-BAR proteins (Wu et al. 2010).

A key question that remains unsolved is how the large vacuoles (endocytic intermediates) that form by bulk endocytosis eventually convert to new synaptic vesicles (see Fig. 1A). Several potential scenarios can be considered. A first possibility is the back fusion of these vacuoles with the plasma membrane for piecemeal recapture by clathrin-mediated endocytosis. In this scenario, one can envisage only one pathway to the generation of synaptic vesicles, clathrin-dependent endocytic budding. However, robust conversion of the vacuoles was observed even at synapses that lack dynamin 1, in which clathrin-mediated endocytosis is severely impaired, thus questioning this possibility (Ferguson et al. 2007).

A second possibility is that new vesicles reform by a coat-mediated mechanism from the endocytic intermediates. Based on studies of broken cell preparations it had been proposed that such a reaction might occur via the same clathrin coat that mediates budding from the plasma membrane (Takei et al. 1996). On the other hand, more recent studies have shown an obligatory role of $PI(4,5)P_2$ in the assembly of such coats, and our studies suggest that endocytosis is coupled to $PI(4,5)P_2$ dephosphorylation, thus implicating absence of $PI(4,5)P_2$ from endocytic membranes in living cells (Cremona et al. 1999; Di Paolo and De Camilli 2006). Accordingly, EM tomographic studies have shown that the overwhelming majority of clathrin-coated pits present in intact nerve terminals are connected to the plasma membrane, even when they appear to be emerging from endosomes in single EM sections (Fig. 4) (Ferguson et al. 2007; Hayashi et al. 2008; Raimondi et al. 2011). Thus, if coats are involved, such coats should be different from endocytic clathrin coats. AP-3 and AP-1, heterotetrameric complexes similar to AP-2 that may function independently of clathrin, are potential candidates (Faundez et al. 1998; Nakatsu et al. 2004; Kim and Ryan 2009; Newell-Litwa et al. 2009; Glyvuk et al. 2010). However, although both AP-3 and AP-1 were implicated in synaptic vesicle recycling
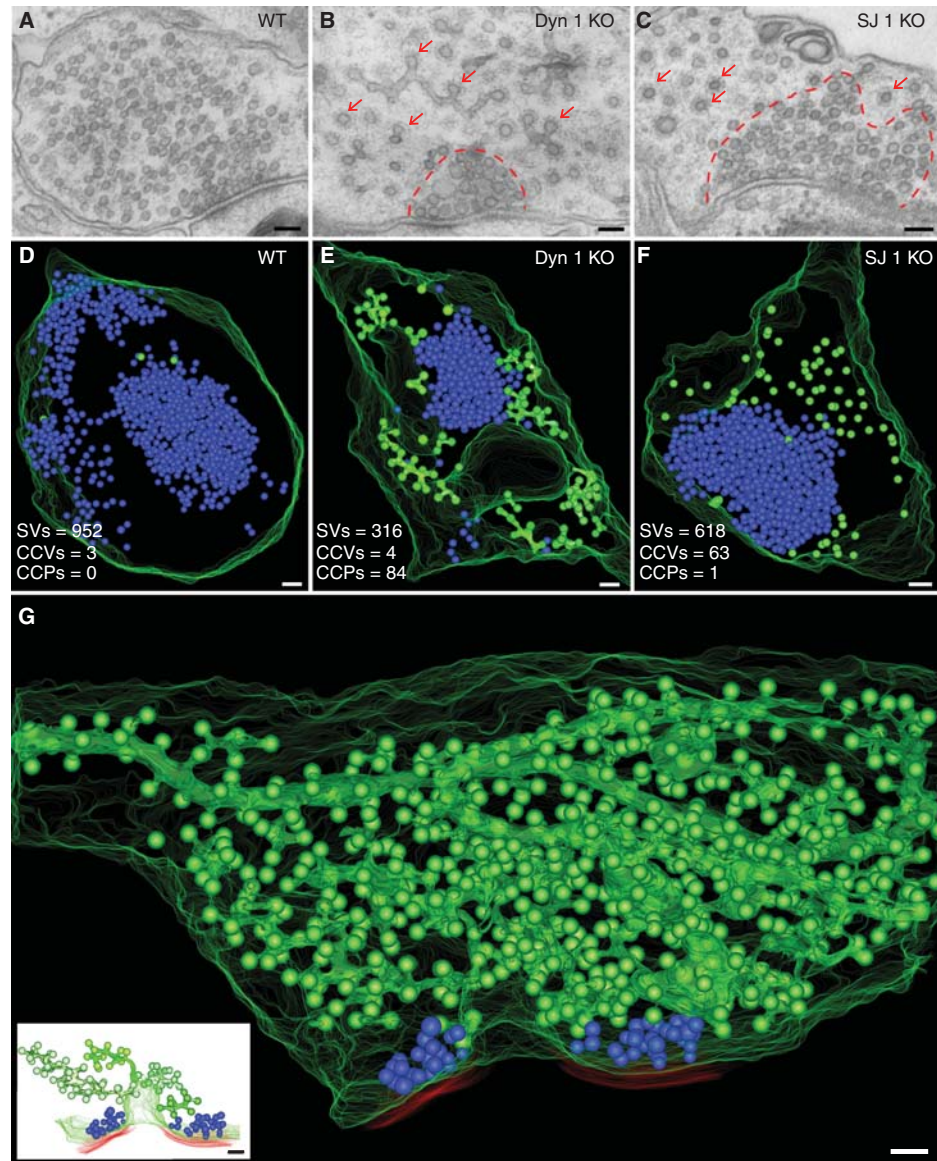
Figure 4. Ultrastructure and 3D models of wild-type (WT), dynamin 1 knockout (KO), and synaptojanin 1 KO synapses demonstrating the accumulation of clathrin-coated intermediates in the KO synapses. (*A−C*) Conventional electron micrographs of synapses from mouse primary cortical cultures of the indicated genotypes. Note the abundance of clathrin-coated profiles (arrows) in dynamin 1 and synaptojanin 1 KO nerve terminals and the less dense packing of these structures relative to the packing of synaptic vesicles (SVs) (enclosed by dashed red lines). (*D−F*) 3D models from the EM tomograms of 250-nm-thick sections of WT and mutant nerve terminals showing that the bulk of coated profiles (green) are buds that are located that at the tip of branched tubules in dynamin 1 KO synapses and free vesicles in synaptojanin 1 KO synapses. Synaptic vesicles are shown in blue. (*G*) 3D model from the EM tomogram of an 800-nm-thick volume of a dynamin 1 KO nerve terminal. Synaptic vesicles are blue spheres, the plasma membrane is outlined by a series of green curves, and red lines indicate postsynaptic membranes. The overwhelming majority of coated structures (green) are buds originating from tubular plasma membrane invaginations (four such invaginations are shown separately in the *inset*). (Scale bars, 100 nm.) (Panel *G* from Hayashi et al. 2008; reprinted, with permission, from the author. Bottom *inset* from Ferguson et al. 2007; reprinted, with permission, from *Science* © 2007.)

(Voglmaier et al. 2006; Glyvuk et al. 2010), they do not appear to be major and general components of synapses.

A third possibility is that synaptic vesicles may reform from endocytic intermediates by a novel mechanism, either lipid-mediated or protein-mediated, but independent of classical coats (Kirchhausen 2000b; Graham 2004). For example, in vitro studies have shown that some proteins of the BAR superfamily that tubulate liposomes can also fragment them into small vesicles (Gallop et al. 2006; Wang et al. 2009). Although such a mechanism is unlikely to achieve the sorting of membrane cargo, efficient sorting may be dispensable, given the high concentration of synaptic vesicle proteins in these endosome-like intermediates. A problem with a direct, clathrin-independent, reformation of synaptic vesicles from endosomal structures is that such reaction would imply that a "well-defined" organelle, such as a synaptic vesicle, can be generated via two distinct pathways, clathrin-mediated endocytosis and an additional mechanism. Such a hypothesis seems unattractive. It should be noted, however, that synaptic vesicles reformed after a strong stimulus are often more heterogeneous in size than synaptic vesicles present in resting or mildly stimulated nerve terminals (Ferguson et al. 2007; Clayton et al. 2008; our unpublished observations). Thus, following a strong stimulus, the fidelity of synaptic vesicle reformation may be partially lost and a new cycle of exocytosis and clathrin-dependent endocytosis may be needed to reconstitute a vesicle with a well-defined composition and size. The possible occurrence of clathrin-independent mechanisms to support neurotransmission has been raised by recent studies in worms (Gu et al. 2008; Sato et al. 2009). More work is clearly needed to investigate the provocative conclusions of these interesting studies.

NERVE TERMINAL ENDOSOMES

The role of "canonical" early endosomes in nerve terminals (i.e., organelles downstream from clathrin-coated vesicles and of other vesicles that form directly from the plasma membrane

[Helenius et al. 1983]) and the relation of vacuoles generated by bulk endocytosis to such endosomes remain a poorly explored topic. Canonical early endosomes, as defined by the presence of the endosomal markers Rab5 and PI3P (Miaczynska et al. 2004), are expected to be present in axon endings, irrespective of synaptic vesicle traffic, given their ubiquitous importance in the sorting of endocytic traffic of plasma membrane components (including receptors, ion channels, transporters, etc.) (Miaczynska et al. 2004; Sann et al. 2009). Indeed both Rab5- and PI3P-positive organelles are present in nerve terminals (Wucherpfennig et al. 2003; Grill et al. 2007; Brown et al. 2009; Hoopmann et al. 2010). Furthermore, the SNARE Vti1a, which is thought to participate in the fusion of synaptic vesicle membrane with endosomes, is present on synaptic vesicles (Antonin et al. 2000; Ramirez et al. 2012). However, Rab5-and PI3P-positive endosomes are quite few and small, and so far there is no evidence that the large vacuoles generated by bulk endocytosis are positive for these markers. Perturbation of Rab5 with a dominant-negative approach was shown to affect synaptic vesicle recycling only mildly (de Hoop et al. 1994; Wucherpfennig et al. 2003). On the other hand, Rab5 was also detected on synaptic vesicles, thus clouding the interpretation of these results (Fischer von Mollard et al. 1994; Pavlos et al. 2010). It is important to note that the concept of the endosome has been extensively revised in recent years. Endosomes are now considered a highly heterogeneous population of organelles, with a further local heterogeneity within a given organelle being defined by the presence of multiple Rabs, other small G proteins (Arls and Arfs), a wide variety of other peripheral factors, and different phosphoinositides [PI3P, $PI(3,4)P_2$ and $PI(3,5)P_2$] (Miaczynska et al. 2004; Zoncu et al. 2009; Scita and Di Fiore 2010). Stable endosomal populations coexist with endocytic compartments that expand and shrink dependent on the functional state of the cell (Miaczynska et al. 2004; Zoncu et al. 2009; Scita and Di Fiore 2010). A precise functional characterization of nerve terminal endosomes and other endocytic intermediates is an important priority for future studies.

CONCLUDING REMARKS

The uniform size and biochemical composition of synaptic vesicles, which are now the best characterized cellular organelles (Takamori et al. 2006), is a key basis for the quantal property of neurotransmitter release. These organelles are continuously regenerated by endocytic membrane recycling. Although our understanding of this process has greatly advanced in recent years, much remains to be learned. Substantial mechanistic insight has been obtained for clathrin-mediated endocytosis, but little is known about parallel pathways that mediate the endocytic recapture of synaptic vesicles after an exocytic burst, bulk endocytosis in particular. The properties and dynamics of endosomal intermediates, most importantly the mechanisms through which they give rise to new synaptic vesicles, remain poorly understood. The small size of nerve terminals has made difficult the application to nerve terminals of fluorescence live imaging methods that have allowed monitoring the dynamics and fate of specific proteins and individual vesicles in other systems. However, advances in the field of superresolution microscopy and electron tomography methods offer new opportunities in this area. Genetic studies in model organisms and studies of genetic diseases of the human nervous system will continue to yield critical information about proteins involved in synaptic vesicle recycling. It can be anticipated that new progress in this area will not only advance the field of synaptic transmission but also have broad implications in cell biology and medicine.

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