Molecular Circuitry of Endocytosis at Nerve Terminals

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Abstract
Presynaptic terminals are specialized compartments of neurons responsible for converting electrical signals into secreted chemicals. This self-renewing process of chemical synaptic transmission is accomplished by the calcium-triggered fusion of neurotransmitter-containing vesicles with the plasma membrane and subsequent retrieval and recycling of vesicle components. Whereas the release of neurotransmitters has been studied for over 50 years, the process of synaptic vesicle endocytosis has remained much more elusive. The advent of imaging techniques suited to monitor membrane retrieval at presynaptic terminals and the discovery of the molecules that orchestrate endocytosis have revolutionized our understanding of this critical trafficking event.
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## INTRODUCTION

Chemical communication between cells was harnessed for both distributing and controlling multicellular behavior early on in the development of complex life forms. In the nervous system, synapses encompass a transduction machine whereby propagating electrical signals, usually in the form of action potentials, are transformed into chemical messages in the form of packets of neurotransmitters that are secreted onto a neighboring cell, which in turn are transduced back into both chemical and electrical signals in this cell. The past 60 years have witnessed an intense scientific effort to understand how synapses work, how they form and are controlled, how their function changes over time scales from milliseconds to months, and how they are built at the molecular level. Increasingly, as molecular detail has emerged, it has overlapped with the recognition that this information is crucial for understanding a variety of diseased states of neuronal function. Here we focus our attention on the first half of the synaptic transmission problem, the cell biology of the presynaptic nerve terminal and, in particular, the current understanding of endocytosis and synaptic vesicle (SV) recycling.

## THE SYNAPSE

The basic structural organization of synaptic terminals appears to be relatively well conserved across evolution. Neurotransmitters are...
The synaptic vesicle (SV) cycle. An SV fuses with the plasma membrane (a) and is subsequently rebuilt through the assembly of a clathrin-coated pit (b,c) followed by budding (d), and fission and uncoating of the endocytic coat proteins (e). The vesicle is refilled with neurotransmitter and returned to the vesicle pool for further rounds of exocytosis (f). Several SV proteins are depicted including synaptobrevin/VAMP (green), synaptotagmin (purple), and the vacuolar ATPase (blue). Syntaxin is shown (red rods), and the assembled SNARE complex is represented (red-green coils). Other proteins depicted here are clathrin triskelia (gray), adaptins (red ellipses), dynamin, and endophilin (red helices).

packaged into small, clear vesicles with a diameter that ranges from ~30 to 40 nm (depending on the species). Typically a few dozen to a few hundred SVs are maintained in a cluster near the active zone, a dedicated intracellular location on the presynaptic plasma membrane where SVs, upon the arrival of an action potential stimulus, will fuse with the plasma membrane and release their content through exocytosis (Figure 1). These preferential sites of exocytosis are typically found juxtaposed to a postsynaptic specialization of neurotransmitter-gated ion channels in the receiving cell. As nerve terminals are located along or at the end of axons very distal to the cell body and the total number of SVs is limited, ongoing synaptic transmission is maintained by local recycling of SVs. The mechanisms of how vesicles are rebuilt following exocytosis for reuse are the focus of this review.

THE SYNAPTIC VESICLE: A LOCAL CURRENCY FOR INFORMATION FLOW AT THE SYNAPSE

SVs act as key intermediates in converting electrical to chemical information at synapses.
and represent a currency of information flow in the nervous system. Because their size is tightly regulated (see below), it has been possible to purify these organelles to near homogeneity. Such purified vesicles were the starting point for identifying SV proteins (Jahn et al. 1985, Trimble et al. 1988, Perin et al. 1990) and provided the basis for generating the first draft of an SV proteome carried out by the lab of Reinhard Jahn (Takamori et al. 2006). SVs are ∼50% protein and 50% lipid, with a 1:1 ratio of phospholipid to cholesterol, similar to generic cellular plasma membranes. Following exocytosis, the transmembrane proteins of SVs become transiently incorporated into the plasma membrane and need to be endocytosed to rebuild the SV. Figure 2 illustrates the major transmembrane proteins associated with SVs as well as their average copy number deduced from quantitative proteomics. There are roughly nine types of transmembrane proteins that are specifically enriched in SVs and of these, only four types have been assigned clear-cut functions. These are (a) the proton pump (vacuolar ATPase), which provides the proton-motive force for driving neurotransmitter uptake through (b) the vesicular neurotransmitter transporter (Edwards 2007), (c) synaptotagmin I, the calcium sensor for fast calcium-triggered exocytosis (Chapman 2008), and (d) synaptobrevin 2/VAMP2, the vesicle-associated SNARE that provides one of the helices required to catalyze membrane fusion through formation of a four-helix bundle with the specific plasma membrane SNARE proteins syntaxin and SNAP-25 (Rizo & Rosenmund 2008). It is noteworthy that the relative abundance of these different proteins varies significantly: Some proteins such as the proton pump, whose functional role is unequivocal and required for filling vesicles with transmitter, appear to be expressed at single-copy levels. In contrast, synaptophysin is at least one order of magnitude more abundant but is dispensable, at least in terms of animal viability (McMahon et al. 1996) and basic synaptic function (Abraham et al. 2006). Presently little is known about variability in the stochiometric relationship of these different proteins across individual vesicles.

**SYNAPTIC VESICLE RETRIEVAL AT NERVE TERMINALS**

Typical CNS synapses contain a limited number of SVs, usually a few hundred or less. As nerve terminals are typically located great distances from their cell bodies, the biosynthetic engines of cells, the proteins and lipids that make up a SV, must be recaptured from the plasma membrane following exocytosis, refashioned into a SV, and refilled with neurotransmitters (De Camilli et al. 2001). This currency of synaptic communication is in fact in relatively limited supply. The mechanism of vesicle retrieval in the SV cycle has been a subject of intense interest and debate for over 35 years. The first descriptions of vesicle recycling began with ultrastructural observations at neuromuscular junctions (NMJ), where uptake of extrasynaptic horse radish peroxidase demonstrated conclusively the existence of the SV recycling
pathway (Heuser & Reese 1973). These studies also provided the first evidence that vesicle retrieval occurred through a clathrin-mediated pathway. To visualize vesicle recycling at the EM level, it was necessary to provide a sufficiently rapid and intense stimulus while catching exo- or endocytic events in the act through rapid freezing. This approach led to the first kinetic description of endocytosis (Miller & Heuser 1984) because the time between stimulus and freezing could be varied (Figure 3a,b). The relatively long time scales observed (1 min for completion of vesicle endocytosis) fueled speculation that a faster, more direct route must be operational, and they provided grist for the idea that endocytosis may occur by reversal of fusion at the active zone, first proposed by Ceccarelli and colleagues (Ceccarelli et al. 1973); this pathway was later coined kiss-and-run. During the past 10–15 years, efforts to understand the mechanisms of endocytosis of SVs have been concentrated in two approaches. One is the examination and discovery of the molecular machinery responsible for endocytosis at nerve terminals; the other is the biophysical probing of the time scales and molecular behavior of the endocytic process at the synapse.

THE TIMING OF ENDOCYTOSIS AT NERVE TERMINALS: EVIDENCE FOR MULTIPLE TIME SCALES OF RETRIEVAL

Synaptic endocytosis kinetics measurements became possible with the advent of new technologies as well as suitable synaptic preparations. Electrical capacitance recordings, which, in principle, provide direct measurements of cell surface area, allow one to measure the net balance of exocytosis and endocytosis. This has been useful, however, only at select giant synaptic preparations because SVs have a very small surface area (3000–5000 nm²) and sites of fusion are usually quite distant from the cell body, where the recording electrode makes electrical contact. Three different types of giant synaptic preparations have proven useful in this regard. The first direct real-time measurements of vesicle retrieval following a burst of exocytosis were made in retinal bipolar cells (von Gersdorff & Matthews 1994a) and auditory hair cells (Parsons et al. 1994). Refinement of these measurements (Beutner et al. 2001, Neves & Lagnado 1999) revealed that, generally, two distinct kinetic components of endocytosis are apparent (with ~1-s and ~10-s time constants, respectively) where the relative proportion of retrieval via the faster component was sensitive to calcium influx (Beutner et al. 2001, Neves et al. 2001). Given that this calcium-driven fast component was sensitive to the calcium chelator BAPTA (and occurred even when calcium elevations were spatially confined), the authors concluded that the faster form was likely occurring near active zones. Similar observations were made in the giant auditory brainstem calyx of the Held synapse (Wu et al. 2005).

The appearance of distinct kinetic components leads to the question of how each participates in regenerating SVs. Interestingly, the detailed ultrastructural kinetic study by Heuser and colleagues (Miller & Heuser 1984) also revealed ultrastructural components with distinct kinetic signatures following stimulation at the NMJ. Freeze-fracture views of presynaptic terminal membrane showed that following stimulation, non-clathrin-coated endocytic vacuole-like structures formed near the active zone without concentrating cargo molecules and disappeared within approximately 1 s (Figure 3a,b). In retrospect, it seems likely that the fast component seen with capacitance measurements could be the same fast pathway identified by Heuser at the NMJ. Consistent with this interpretation, this component was shown to be insensitive to clathrin perturbation (Jockusch et al. 2005). At the calyx of Held, however, the appearance of fast endocytosis was independent of neurotransmitter release (Yamashita et al. 2005). Pretreatment of nerve terminals with botulinum toxin blocked neurotransmitter release without eliminating a capacitance transient with fast recovery. Thus, this rapid membrane response may represent a homeostatic response to acute calcium elevation independent of SV endocytosis.
Figure 3
Kinetics of endocytosis from 1984 to 2007 (a) The first measurements of the kinetics of synaptic vesicle (SV) endocytosis were obtained via pulse-chase freeze fracture experiments at the frog neuromuscular junction (NMJ) by Miller & Heuser (1984). An image that was obtained by quick freezing an NMJ 30 s after action potential stimulation in 4-AP is shown. The white circles (diameter of 110 nm) indicate membrane deformations that correspond to clathrin-coated pits. Image adapted courtesy of John Heuser. (b) The histogram of the accumulation and disappearance of the membrane invaginations obtained by examining freeze-fracture pictures taken at many different times with respect to the action potential stimulus. Redrawn from Miller & Heuser (1984). (c) Single-vesicle exocytosis and endocytosis as revealed by following the vesicular glutamate transporter (vGlut1) tagged with pHluorin in a luminal loop. Adapted from Balaji & Ryan (2007). Upon fusion, pHluorin becomes deprotonated and its fluorescence dequenched. After a variable delay (\( t_{\text{dwell}} \)), the SV protein is endocytosed and the fluorescence becomes quenched as the vesicle lumen reacidifies over a 3- to 4-s time scale. Four different examples displaying the range of \( t_{\text{dwell}} \) are shown. (d) The histogram of \( t_{\text{dwell}} \) values obtained from approximately 200 events shows that the timing of endocytosis is dictated by an exponential process (red curve) whose mean time is approximately 13 s.
pHluorin-based measurements of the vesicle cycle. pHluorin is a modified form of green fluorescent protein (GFP). GFPs, in general, are excellent proton sensors, as the fluorescence is quenched in an all-or-none fashion upon protonation, which is well described by a 1-proton binding equilibrium (Haupts et al. 1998). The pKa of pHluorin is \( \sim 7.1 \), one log unit more alkaline than GFP and, when resident within the acidic lumen of a synaptic vesicle, is quenched \( \sim 97\% \) of the time. Redrawn from Sankaranarayanan et al. (2000).

The introduction of optical tracers has proven particularly powerful for examining endocytosis at small nerve terminals. Pulse-chase application of the fluorescent amphipathic molecule FM 1-43 (Betz & Bewick 1992) allowed the first kinetic dissection of endocytosis in dissociated hippocampal neurons (Ryan et al. 1993, Ryan & Smith 1995). Vesicle retrieval following a large burst of activity indicated that endocytosis decayed over a 60-s time scale. Although useful for labeling recycling vesicles pools and measuring pool turnover kinetics, tracers such as FM 1-43 do not directly provide real-time endocytosis readouts. The advent of pHluorins (see Figure 4) provided higher-fidelity readout where specific vesicle proteins fates can be followed. Initial measurements of VAMP2 endocytosis for relatively large stimuli showed that the recovery time scale depended on the amount of exocytosis (Sankaranarayanan & Ryan 2000). Improvements in these approaches allowed detection of endocytosis following single-action-potential stimuli (Balaji & Ryan 2007, Granseth et al. 2006), which showed endocytic recovery occurred with a time constant of approximately 15 s. Analysis of the delay time between exocytosis and endocytosis for individual vesicles showed that endocytosis times varied stochastically from vesicle to vesicle, ranging from instantaneous to tens of seconds with an exponentially distributed dwell time (Balaji & Ryan 2007), which indicates that a single rate-limiting stochastic step determines the time scale of endocytosis.
MODULATION OF ENDOCYTOSIS: CALCIUM AND EXOCYTIC LOAD

A central goal of research in presynaptic function is to understand the regulation of different vesicle cycling steps. Prior to assigning specific molecular roles, one must understand how the conditions under which the measurements were performed impact the biophysical observations. Two main variables associated with driving exocytosis at synapses are the extent to which intracellular calcium becomes elevated and the total endocytic burden that ensues from successful exocytosis. These two variables are closely entwined as greater elevations in intracellular calcium generally lead to higher exocytic rates, more material that must be recaptured. Given the importance of intracellular calcium in signaling, determining calcium’s role in endocytosis is of significant interest. Early attempts to disentangle calcium elevations from exocytosis used α-latrotoxin, a potent secretagog from black widow spider venom, to stimulate exocytosis in the absence of extracellular calcium (Ceccarelli & Hurlbut 1980). After several hours of stimulation under these conditions, NMJs became severely depleted of SVs and the plasma membrane developed large enfoldings, indicating that endocytosis was blocked. Ceccarelli & Hurlbut (1980) concluded that intracellular calcium elevation was necessary for SV endocytosis. It was impossible, however, to distinguish the extent to which calcium might simply be modulating rates as opposed to playing a requisite role in the biochemistry of endocytosis. A number of different experiments have shown that, following exocytosis, calcium entry and endocytosis can be decoupled as the presence of extracellular calcium is no longer necessary for endocytosis to proceed (Gad et al. 1998, Ryan et al. 1996) and that bulk calcium levels typically decay faster than the completion of endocytosis (Wu & Betz 1996). At the other extreme, persistent photolysis of caged calcium led to complete arrest of endocytosis in giant bipolar terminals so long as bulk cytoplasmic calcium was greater than approximately 1 μM (von Gersdorff & Matthews 1994b). Although peak intracellular calcium levels near exocytosis sites transiently reach tens of micromolar, average sustained intracellular calcium levels remain well below 1 μM at most nerve terminals following a single-action potential or low-frequency stimulation. Thus, it is unclear if such inhibition plays a role under physiological conditions.

Approaches that allowed direct measurements of endocytosis kinetics made it possible to examine the relationship between the amount of exocytosis and the speed of endocytosis, as well as how elevations in intracellular calcium might modulate this process. Experiments in hippocampal nerve terminals and at the calyx of Held showed that increases in exocytosis led to progressively longer endocytosis time scales (Sankaranarayanan et al. 2000, Sun et al. 2002), in spite of the fact that endocytosis was accelerated under conditions that led to elevated calcium in these preparations (Sankaranarayanan & Ryan 2001, Wu et al. 2005). This slowing of endocytosis with greater exocytosis may result from an underlying saturation of the total endocytic capacity of the synapse. It is notable that such saturation behavior is not observed in other synaptic preparations such as NMJs in mice (Tabares et al. 2007) and flies (Poskanzer et al. 2006), suggesting that it is not a fundamental property of endocytosis at nerve terminals. This paradox between acceleration of endocytosis due to intracellular calcium versus slowing due to increased accumulation, and the absence of either in certain preparations, was recently resolved. Taking advantage of single-vesicle sensitivity to monitor endocytosis kinetics systematically over a 40-fold range in exocytosis, Balaji et al. (2008) demonstrated that endocytosis recovery follows a single exponential time course (15 s) over a wide range of exocytosis but begins to slow significantly only above a threshold accumulation of SVs on the plasma membrane. These data imply that until this threshold is reached, endocytosis of different vesicles is likely happening in parallel, each with an
average time scale of 15 s. Beyond this threshold, the synapse can no longer assemble sufficient endocytic machinery to handle greater endocytic loads, thereby defining the endocytic capacity of the synapse. Importantly, for sufficiently small amounts of exocytosis, the endocytosis time course was insensitive to manipulations of intracellular calcium. Balaji et al. (2008) observed that the endocytic capacity was increased by stimulus conditions that elevated residual calcium in the nerve terminal. These data thus help reconcile the original observations at the frog NMJ as well. Persistent stimulation in the absence of any calcium elevation (as achieved in α-latrotoxin in zero calcium for prolonged periods) could lead to a situation where the amount of exocytosis occurring greatly exceeds the endocytic capacity, eventually leading to persistent depletion of vesicle pools. Furthermore, this hypothesis offers an explanation as to why modulation of endocytosis by activity may not be observed: The detailed tuning of calcium entry, buffering, and extrusion, along with the specific abundance of the necessary endocytic proteins, may be such that one never operates in an exocytosis regime where the endocytic capacity becomes limiting. For example, at physiological temperatures, the time constant for endocytosis decreases to 6 s (Balaji & Ryan 2007) in hippocampal nerve terminals. This increase in clearance time coupled with the fact that release probability is lower at this temperature lead to a situation where the endocytic capacity is rarely exceeded. Thus, for three different central nervous system synapses examined (bipolar terminals, the calyx of Held, and dissociated hippocampal neurons), a common time scale of 10 to 15 s (at room temperature) appears to prevail in SV recovery unless significant accumulation of SV components can be driven to the cell surface.

KISS-AND-RUN: A LONG-RUNNING DEBATE

Beginning with the first observations of vesicle recycling, there has been speculation of a rapid recycling pathway whereby the vesicle maintains its identity and is retrieved intact at the active zone. This idea was originally fueled by disparity in the time scales of exo- and endocytosis observed at the NMJ. Endocytosis took approximately 1 min (Miller & Heuser 1984), whereas exocytosis took place on the submillisecond time scale. In comparing the time scale for exo- and endocytosis, however, one should consider the time for the entire recycling vesicle pool to fuse with the plasma membrane, not just a single vesicle. At hippocampal synapses at 37°C, exocytosis of the entire recycling pool requires approximately 20 s for stimulation at 10 Hz (Fernandez-Alfonso & Ryan 2004), which should be compared to the 6-s time constant for endocytosis under these conditions (Balaji et al. 2008). Thus, it is possible for exocytosis to outrun endocytosis, but only under relatively high-frequency stimulation.

The most direct evidence for transient reversible fusion pores arose from capacitance recordings of single-vesicle fusion events in chromaffin cells coupled with simultaneous measurements of release of catecholamine using carbon fiber amperometry (Albillos et al. 1997). In the majority of events, the appearance of a small fusion pore was followed by an irreversible expansion and complete loss of catecholamine. However, in 5–10% of events, the pore closed without expanding. At conventional synapses, this dual approach of capacitance recordings and amperometry has not been possible, but single-vesicle fusion events have been recorded using capacitance measurements alone at the calyx of Held. Wu and colleagues reported that 17% of events appeared to be transient in nature (He et al. 2006). There are two important caveats in interpreting these results. First, this approach required examining exocytosis at random locations on the synaptic surface that were not necessarily active zones. For technical reasons, the events could be evoked only using nonphysiological stimuli. Second, the appearance of exocytosis followed soon after by endocytosis may simply represent a narrow sampling of the select fast events of an exponential distribution of time scales as predicted by a stochastic process (Balaji & Ryan
2007). Verification that such events are insensitive to perturbations of elements in the clathrin-mediated pathway will be necessary to assign them to a distinct endocytosis pathway.

Less direct biophysical methods have also been used to ascertain the presence of kiss-and-run. One approach that has been used extensively at hippocampal synapses was to examine the efficiency of escape of lipophilic tracers from SVs during exocytosis. The idea is that a lipophilic dye potentially would dissociate from the vesicle lumen membrane too slowly to escape during a kiss-and-run event, but that it might fully dissociate during a full fusion event. This approach, first introduced more than a decade ago (Ryan et al. 1996), has been used in one variant or another to argue both for (Aravanis et al. 2003, Harata et al. 2006, Klingauf et al. 1998, Pyle et al. 2000, Richards et al. 2005) and against (Fernandez-Alfonso & Ryan 2004, Ryan et al. 1996, Zenisek et al. 2002) the presence of kiss-and-run. Examination of dye loss at the single-vesicle level, however, is quite challenging, as one is effectively asking whether losses in fluorescence are well quantized. A number of subtle artifacts can potentially lead to loss of fidelity of the fluorescence measurements. Chief among these is that lipophilic tracers are added to the outside of the synapse during activity to load recycling vesicles; however, other recycling membranes in neighboring glia and postsynaptic dendrites, as well as other presynaptic compartments, can become labeled. Such spurious labeling can strongly contribute to background fluctuations in fluorescence intensity. Recent experiments designed to control for some of these possible complications concluded that lipophilic dyes destain completely during action-potential stimuli (Chen et al. 2008) and suggest either that kiss-and-run does not occur at these synapses or that one cannot use these dyes reliably to detect such transient fusions.

Recently, a novel form of molecular tracer was introduced for examining the frequency of full fusion during exocytosis in cultured neurons. In this new technique, a recycling vesicle endocytoses a quantum dot (Qdot) (Zhang et al. 2007) whose optical properties allow continuous optical recordings without photobleaching. Because of its large size [hydrodynamic radius of nearly 15 nm (Larson et al. 2003)], the internalized Qdot escapes only during full-fusion exocytosis (Zhang et al. 2007). Zhang et al. (2007) found that stimulus-dependent unloading of Qdots followed a slower time course than an FM dye loaded into the same synapses, suggesting that some fusion events release FM dye but not Qdots. A significant caveat with this approach comes from the large size of Qdots relative to the vesicles that contain them. If the Qdot interacts with the vesicle lumen and stabilizes the curvature of a fusing vesicle, perhaps an otherwise rare partial-fusion event could be made more probable.

Investigations into the molecules that underlie endocytosis have yielded little evidence in support of kiss-and-run mechanisms to date (see below). Furthermore, the observation that vesicle components exchange with counterparts resident on the cell surface during endocytosis over a wide range of stimulus conditions (Fernandez-Alfonso et al. 2006, Wienisch & Klingauf 2006) indicates that vesicles typically lose some of their components following exocytosis. Finally, detailed measurements of the time between exocytosis and endocytosis for individual vesicles showed that, although vesicles do occasionally endocytose quickly, the frequency of such events is well predicted by a stochastic (single-exponential) process with a mean endocytic time of 15 s (Balaji & Ryan 2007). Taken together, the biophysical evidence weighs largely against significant vesicle recycling via a kiss-and-run mechanism. Furthermore, as discussed below, most experiments aimed at explicitly targeting clathrin or associated machinery at the synapse support a major role for the classical endocytic pathway in vesicle retrieval.

THE MOLECULAR MACHINERY OF SYNAPTIC VESICLE ENDOCYTOSIS

Molecular and genetic insights over the past 25 years from a variety of model systems have
culminated in a formidable list of potential proteins and lipids involved in endocytosis. Many of the proteins share common binding partners and exhibit overlapping functions within the endocytosis machinery. This property of interconnectivity may be important in explaining an emerging dilemma in the field of SV endocytosis: Gene knockouts and RNA interference knockdown of endocytic proteins generally do not eliminate SV endocytosis and, in many cases, have remarkably subtle effects (Di Paolo et al. 2002, Ferguson et al. 2007, Gu et al. 2008, Sato et al. 2009). This situation starkly contrasts with SV exocytosis, where removal of any one of a small collection of essential proteins eliminates SV fusion (Südhof 2004). If altered SV endocytosis is observed after a particular endocytic protein is removed, there are three possible considerations for the residual endocytosis. First, this perturbation may uncover a second, independent pathway with distinct underlying molecular mechanisms. Second, the measured endocytosis is identical to the wild-type process except that a functional paralog has taken the place of the deleted molecule, although it may not function with the same efficacy. Third, the impaired endocytosis proceeds via the identical molecular pathway but with lower efficacy because the deleted molecule played a contributory rather than obligatory role in the process. In this case, the network of endocytic proteins is robust to deletions of individual members.

Consistent with the third scenario, experiments to date have demonstrated that no single protein appears to be absolutely essential to the process of SV endocytosis. Perhaps particular components of the synaptic endocytic network play a larger or smaller role in different animal phyla, but all the components are generally found in presynaptic specializations of metazoa. Furthermore, these components show a high degree of conservation, both at the sequence level and in terms of interactions with other proteins and phospholipids (Lloyd et al. 2000). In the following section of this review, we introduce 15 proteins/protein complexes that compose the SV endocytic network (Figures 6 and 7). These proteins are separated into three layers of the network on the basis of their function, binding interactions, and protein domain content. At the core of the endocytic network is the capacity to gather up cargo into a local patch of plasma membrane and deform this patch into a separate compartment destined for internalization. A second layer of proteins acts to stabilize the core proteins while recruiting additional effector molecules that both catalyze and terminate the process. These effectors make up the third layer, and they function in numerous cellular trafficking processes in addition to SV endocytosis.

A CLATHRIN-MEDIATED PATHWAY FOR VESICLE REBUILDING

The first details of the molecular basis of SV recycling arose with the pioneering studies of Heuser & Reese (1973), who noted that endocytosis following stimulation appeared to occur via the newly discovered clathrin coats. Since these early discoveries, progress in our understanding of the molecular basis of SV recycling has benefited from a number of approaches in different model systems. Although the relevance of the finding that clathrin-coated pits could be found at synapses originally was debated, the evidence that this pathway predominates as the route of vesicle recovery has become overwhelmingly strong in the past 10 years.

Three types of experiments have been performed, all of which support a major role for the clathrin reuptake pathway. First, microinjections of peptides or antibodies that interfere with different steps of endocytosis were followed by electron microscopy and functional assays. Second, genetic ablations of endocytic proteins were followed by kinetic assays and electron microscopy. Third, the effects of photoinactivation of clathrin in Drosophila NMJs were followed by functional and ultrastructural assays. At synapses such as in the lamprey giant reticulospinal axons, injections of peptides and antibodies designed to interrupt dynamin function (see below), followed by activity-driven
The accumulation of clathrin-coated pits at nerve terminals during activity in the absence of dynamin-1. Strong evidence for synaptic vesicle (SV) endocytosis normally proceeding through a clathrin-coated pit pathway has been obtained from a number of experiments in which molecular perturbations led to trapping vesicle endocytosis at an intermediate stage. Here, an example of the ultrastructure of a hippocampal synapse derived from a dynamin-1 knockout mouse taken from Ferguson et al. (2007) is shown. (a) Single section of a tomogram taken from a synapse where spontaneous activity led to the development of profound invaginations studded with clathrin-coated profiles. The small arrows indicate the presence of interconnected clathrin-coated buds, the asterisk shows an evagination of an adjacent cell into this nerve terminal, and the large arrow shows a small cluster of heterogeneously sized synaptic vesicles. (b) 3D tomographic reconstruction shows that these clathrin-coated profiles were associated with plasma membrane invagination.

**Figure 5**

The accumulation of clathrin-coated pits at nerve terminals during activity in the absence of dynamin-1. Strong evidence for synaptic vesicle (SV) endocytosis normally proceeding through a clathrin-coated pit pathway has been obtained from a number of experiments in which molecular perturbations led to trapping vesicle endocytosis at an intermediate stage. Here, an example of the ultrastructure of a hippocampal synapse derived from a dynamin-1 knockout mouse taken from Ferguson et al. (2007) is shown. (a) Single section of a tomogram taken from a synapse where spontaneous activity led to the development of profound invaginations studded with clathrin-coated profiles. The small arrows indicate the presence of interconnected clathrin-coated buds, the asterisk shows an evagination of an adjacent cell into this nerve terminal, and the large arrow shows a small cluster of heterogeneously sized synaptic vesicles. (b) 3D tomographic reconstruction shows that these clathrin-coated profiles were associated with plasma membrane invagination.

MECHANISMS FOR SORTING THE APPROXIMATELY NINE CARGO PROTEIN TYPES

An important unsolved question in SV recycling is, how is each of the nine different types of SV transmembrane proteins resorted into SVs following exocytosis? As retrieval occurs through clathrin-mediated endocytosis, the expectation is that known mechanisms for receptor-mediated endocytosis will be manifest here. For instance, perhaps there are interactions of sorting motifs in vesicle protein cytoplasmic tails with the plasma membrane adaptor protein complex AP2. AP2, a heterotetrameric complex that additionally binds clathrin, is thought to coordinate clathrin assembly with cargo recognition during endocytosis (Keen 1987). At present, only a single putative sorting motif has been functionally identified in SV proteins. Mutation of a dileucine-like motif in the vesicular glutamate transporter (vGlut1) slows vGlut1 internalization at nerve terminals (Voglmaier et al. 2006). Synaptotagmin has also been identified as a binding partner to AP2 (Grass et al. 2004, Haucke & De Camilli 1999, Haucke & Krauss 2002, Haucke et al. 2000, Zhang et al. 1994). In the absence of Synaptotagmin 1 (Jorgensen et al. 1995, 2000; Morgan et al. 2000, 2003), consistent with a depletion of available vesicles due to a block in endocytosis. At hippocampal synapses, the kinetics of endocytosis can be dramatically slowed when clathrin is removed by siRNA (Granseth et al. 2006). Finally, photoinactivation of fluorescently labeled clathrin, which offers the most temporally precise molecular interruption, showed that acutely inactivating clathrin’s inability to assemble in Drosophila NMJs undergoing exocytosis leads to the formation of large dead-end vacuoles (Heerssen et al. 2008, Kasprowicz et al. 2008) and the loss of any further vesicle recycling. Thus, data using several distinct approaches and examining multiple different types of synapses all argue that clathrin assembly normally plays a critical role in SV endocytosis.
One proposed scenario for efficiently re-capturing SV proteins is that they remain stably associated after exocytosis and directly nucleate clathrin-coat formation. Although this may occur for certain SV proteins, this is not the case for VAMP2 or synaptotagmin. At hippocampal nerve terminals, these proteins diffuse onto the axonal surface (Li & Murthy 2001, Sankaranarayanan & Ryan 2000), and they exchange with surface counterparts during endocytosis (Fernandez-Alfonso et al. 2006, Wienisch & Klingauf 2006). Currently, the issue of how SV proteins are retargeted into SVs during endocytosis remains largely unresolved.

**CURVING MEMBRANES: INITIATION OF SYNAPTIC VESICLE ENDOCYTOSIS**

In addition to recollecting and sorting SV proteins, the endocytosis machinery must locally curve the plasma membrane and eventually pinch off 3000 to 5000 nm² of bilayer in the form of a 30- to 40-nm-diameter vesicle. This process can be broken into two parts: deformation of membrane into a vesicular or tubular shape and scission of the stalk connecting this structure to the plasma membrane.

The process of membrane deformation begins with the organization of endocytic coat proteins on the inner leaflet of the synaptic membrane. This assembly is thought to be driven in part by the generation of phosphoinositol-(4,5)-bis-phosphate (PIP₂), which recruits several endocytic proteins to the plasma membrane including AP2, epsin, dynamin, endophilin, amphiphysin, and AP180. At synapses, activity-dependent PIP₂ synthesis is largely carried out by phospho-inositol-5-kinase Iγ (Di Paolo et al. 2004), which presumably drives rapid recruitment of endocytic factors during repetitive exocytosis. In addition to providing a scaffold for concentrating appropriate cargo proteins destined for endocytosis, endocytic coat proteins are thought to regulate the area destined for internalization in a manner that leads to a precise SV size.
On the basis of biochemical, structural, and genetic evidence, several protein domains have been implicated in the curving of membranes. ENTH (epsin N-terminal homology) domains found at the amino termini of epsin, AP180, and CALM are phospholipid-binding modules (Hurley 2006, Itoh & De Camilli 2006). ENTH domains interact preferentially with PIP2-containing phospholipids and tubulate liposomes in vitro as well as plasma membranes of living cells when overexpressed (Ford et al. 2002, Itoh et al. 2001). Structural studies of ENTH domains suggest that an N-terminal amphipathic alpha helix inserts into the lipid bilayer upon binding to PIP2 (Itoh et al. 2001). The canonical example of an ENTH domain protein at the synapse is epsin. Thought to function as a clathrin adaptor, epsin binds clathrin heavy chain and AP2. These associations position epsin as part of the endocytic core module. Epsin contains several NPF (asparagine-proline-phenylalanine) motifs near its carboxy terminus that interact with Eps15 homology (EH) domain modules found in the secondary scaffolds Eps15 and intersectin/Dap160 (Figure 6). Epsin also contains ubiquitin-interacting motifs, which may function in the recognition of ubiquitinated cargo proteins. In yeast, expression of the ENTH domain is sufficient to rescue viability of the Ent1/2 double mutant (the two yeast epsin orthologs), suggesting that this domain has an essential role irrespective of its adaptor functions (Wendland et al. 1999). Under some conditions, epsin and clathrin triskelia, but not clathrin alone, can invaginate lipid monolayers (Ford et al. 2002). Disruption of epsin function by antibody binding in the lamprey giant synapse decreased SV numbers and led to an accumulation of large coated pits following stimulation (Jakobsson et al. 2008). These data suggested that SV endocytosis was trapped at an early stage in the absence of functional epsin. In contrast, loss of the Drosophila epsin ortholog Liquid Facets did not appear to impair SV endocytosis, although synapse architecture was significantly affected (Bao et al. 2008). Epsin is also found in other cellular compartments, suggesting that it may have a general role in clathrin-mediated endocytosis (Horvath et al. 2007).

AP180 and CALM share an ENTH-like module at their amino termini along with clathrin and AP2-interacting domains within their unstructured carboxy termini. However, interaction of these proteins with PIP2 is distinct from epsin’s ENTH domain interaction and the N-terminal domain contains additional alpha helices (Itoh & De Camilli 2006). These biochemical and structural differences led investigators to call these ANTH (AP180 N-terminal homology) domains. ANTH domains do not appear to deform lipids on their own, but they may influence curvature indirectly through interactions with other membrane-binding proteins. AP180 is predominantly found at presynaptic terminals, whereas CALM is ubiquitous and also possesses multiple NPF motifs (which are not found in AP180) near its carboxy terminus (Itoh & De Camilli 2006). C. elegans and Drosophila each have a single AP180/CALM ortholog.

Figure 6
Categories of endocytosis proteins. Schematic diagrams of protein domain organization are shown for the core module (AP2, clathrin, stonin 2, epsin, and amphiphysin), secondary scaffolds (intersectin and Eps15), and secondary effectors (endophilin, syndapin, dynamin, synaptopjanin, N-WASP, CALM, and stonin 1). The domain abbreviations are as follows: PH, pleckstrin homology domain; ANTH, AP180 N-terminal homology domain; ENTH, epsin N-terminal homology domain; BAR, bin amphiphysin rvs; EH, eps15 homology domain; PRD, proline-rich domain; C-C, coiled-coil domain. NPF represents any of five possible motifs that interact with EH: NPF, WW, FW, and H(ST)F. AP2/Clath represents two AP2-binding motifs [WXXF and DP(WF)] and three clathrin-binding motifs [D(LI)(LFQ), L(FWY)(X(FWY)(DE), and PWXXW]. The diagram depicts these as one localized domain, but these motifs are actually distributed throughout the polypeptide. Two isoforms of intersectin are shown: long and short. Dynamic properties are depicted as either positive feedback (polymerization, enzymatic catalysis) or avidity (multiple binding sites linked together).
that presumably fulfills the duties of both proteins. In both of these model systems, loss of AP180/CALM substantially broadened the distribution of SV sizes and increased the mean diameter by over 30% (Nonet et al. 1999, Zhang et al. 1998). In addition, AP180 and CALM regulate the plasma membrane abundance of synaptobrevin (Dittman & Kaplan 2006, Harel et al. 2008, Nonet et al. 1999, Zhang et al. 1998), suggesting that ANTH domain proteins play a role in both cargo recruitment and size of the endocytic area destined to undergo endocytosis.

Another membrane binding module employed during SV endocytosis is the BAR (Bin, amphiphysin, Rvs) domain (Figure 6). BAR domains found in endophilin, amphiphysin, syndapin, and sorting nexin 9, among others, bind phospholipids as crescent-shaped dimers, and they are thought to generate or stabilize highly curved regions of the membrane (Gallop & McMahon 2005, Itoh & De Camilli 2006, Ren et al. 2006). In vitro, these domains can tubulate liposomes and, in some cases, appear to bind preferentially to particular liposome sizes, suggesting that the BAR domain can sense lipid curvature (Gallop & McMahon 2005). Amphiphysin and endophilin share amino-terminal domains that form amphipathic helices capable of penetrating the cytoplasmic bilayer leaflet and perturbing lipid packing, particularly in the presence of acidic phospholipids. Thus, their N-terminal helix and BAR motif are together termed N-BAR domains. Both amphiphysin and endophilin contain carboxy-terminal SH3 domains, which are thought to interact with the proline-rich domains of dynamin and synaptojanin (Itoh & De Camilli 2006). The combination of N-BAR and SH3 domains makes these proteins ideally suited for the dual task of deforming membranes and recruiting other endocytic molecules to the curved region. Amphiphysin additionally has clathrin- and AP2-binding motifs, linking it to the core endocytic machinery.

The mouse knockout of amphiphysin I displayed a modest decrease in recycling SV pool size as well as a slower recycling rate (Di Paolo et al. 2002). Fly mutants lacking amphiphysin did not appear to have significant transmission defects at the NMJ, and nervous system function is largely intact (Razzaq et al. 2001, Zelhof et al. 2001). Similarly, neither deletion nor RNA interference knockdown of the worm ortholog AMPH-1 impacts nervous system function appreciably (J. Kaplan and J. Bai, personal communication). However, loss of endophilin significantly impairs synaptic transmission in both flies and worms (Rikhy et al. 2002, Schuske et al. 2003, Verstreken et al. 2002). The SV pool is decreased, whereas SV diameter and quantal size are increased. Ultrastructural similarities between endophilin and synaptojanin mutants, such as accumulations of clathrin-coated pits and vesicles, queues of vesicles far from the active zone, and large cisternae, support the hypothesis that endophilin and synaptojanin act together at a similar stage during endocytosis (Schuske et al. 2003, Verstreken et al. 2003).

Syndapin, a member of another class of BAR domain known as F-BAR (formerly known as extended FCH) domain proteins, may play a role during SV endocytosis, particularly during prolonged or intense stimulation (Andersson et al. 2008). Also known as PACSIN, syndapin possesses an N-terminal F-BAR domain, several NPF motifs, and a C-terminal SH3 domain, which interacts with dynamin and N-WASP (Anggono & Robinson 2007, Kessels & Qualmann 2004). Syndapin binds to EH domain proteins via its NPF motifs and participates in trafficking through the recycling endosome pathway (Braun et al. 2005). Fly syndapin mutants failed to display any significant defects in synaptic transmission or SV endocytosis (Kumar et al. 2009). However, antibodies against syndapin caused enhanced synaptic depression and large VAMP2-containing cisternae after sustained stimulation when injected into lamprey giant synapses (Andersson et al. 2008). Because no effect was observed at low stimulus frequencies, syndapin may be part of a secondary pathway for retrieving SV components through endocytic intermediates following prolonged bouts of activity.
SECONDARY SCAFFOLDING FOR RECRUITING GENERAL ENDOCYTIC EFFECTORS

The proteins described thus far can be organized either into a core endocytic module (clathrin, AP2, AP180, stonin 2, epsin, amphiphysin) or into secondary effectors (endophilin and synaptojanin; others mentioned below) based on their interactions (or lack thereof) with vesicle cargo molecules or cargo-binding proteins. Two synaptic proteins bridge this gap between the core module and secondary effectors, acting as recruiters and organizers during SV endocytosis. Eps15 and intersectin/Dap160 are binding partners and each has highly conserved domain structures coupling the endocytic coat to secondary effectors (Montesinos et al. 2005). Eps15 is a 110-kDa protein containing three N-terminal EH domains, a central coiled-coil domain, and clathrin/AP2 binding motifs near its carboxy terminus (Morgan et al. 2003, Salcini et al. 2001). The EH domains mediate interactions with NPF motifs in epsin, stonins, AP180, and synaptojanin, whereas the coiled coil interacts with intersectin (Kelly & Phillips 2005, Morgan et al. 2003). The EH domains of Eps15 in C. elegans results in decreased SV number, particularly at elevated temperatures (Salcini et al. 2001). The Eps15 mutant ehs-1(ok146) genetically interacts with the worm temperature-sensitive dynamin mutant dyn-1(ky51) such that locomotion is severely impaired in the double mutant even at permissive temperatures (Salcini et al. 2001). The Eps15 mutant ehs-1(ok146) genetically interacts with the worm temperature-sensitive dynamin mutant dyn-1(ky51) such that locomotion is severely impaired in the double mutant even at permissive temperatures (Salcini et al. 2001). Eps15 also appears to enhance AP180-mediated clathrin assembly in vitro, suggesting that it may have an early role in building clathrin pits (Morgan et al. 2003). The loss of Eps15 in Drosophila causes substantial decrease in synaptic intersectin, dynamin, stonin, synaptotagmin I, α adaptin, and endophilin (Koh et al. 2007).

Intersectin/Dap160 is a synaptic protein containing two N-terminal EH domains, a central coiled-coil domain, and four to five SH3 domains (depending on the species) at its carboxy terminus (Koh et al. 2004, Marie et al. 2004). A long isoform in vertebrates also contains Dbl homology domain, a pleckstrin homology domain, and a C2 domain after the SH3 repeat. This module can act as a guanine nucleotide exchange factor for Cdc42, regulating actin dynamics through Cdc42 and N-WASP (Hussain et al. 2001). Interestingly, Drosophila and C. elegans intersectin orthologs do not contain this module. The EH domains interact with epsin; the coiled coil binds Eps15; and the SH3 domains bind to dynamin, synaptojanin, and N-WASP (Marie et al. 2004, Montesinos et al. 2005). The loss of intersectin in the fly results in diminished SV endocytosis; decreased synaptic levels of AP180, endophilin, synaptojanin, and dynamin; and increased synaptic depression during prolonged stimulation (Koh et al. 2004, Marie et al. 2004). Eps15 intersectin double mutants exhibit synaptic depression and decreased FM1-43 uptake to a similar degree as either single mutant (Koh et al. 2007). Microinjection of the intersectin SH3 domain in lamprey giant synapses inhibits SV recycling and traps a large number of coated pits in the periactive zone (Evergren et al. 2007). Furthermore, intersectin appears to negatively regulate recruitment of dynamin to periactive zones at this synapse. In C. elegans, ITSN-1 localizes to the periactive zone in NMJs, and loss of ITSN-1 reveals a substantial increase in large, irregular vesicular structures as well as a decrease in spontaneous transmitter release (Rose et al. 2007, Wang et al. 2008).

FINISHING THE JOB: MEMBRANE FISSION AND COAT DISASSEMBLY

The final step of endocytosis involves severing the endocytic bud from the plasma membrane. In vitro studies have shown that when artificial membrane necks are narrowed to sufficiently small diameter, thermal fluctuations result in a collapse of the neck, presumably the starting point for fission (Bashkirov et al. 2008, Israelachvili 1992). Dynamin, a mechanochemical GTPase, was originally identified as the gene product of the Drosophila shibire mutant, a temperature-sensitive paralytic that becomes
Synaptic vesicle endocytic network. The proteins that compose the endocytic machinery at the synapse are classified in two ways: location in the network (color) and functionality (shape). Blue proteins make up the core module, red proteins are secondary scaffolding, and green proteins are secondary effectors. Proteins found exclusively at the synapse are shown in bold type. Scaffolds are hatched lines, cargo binders are triangles, membrane benders are crescents, and secondary effectors are diamonds. Interactions between proteins are depicted as arrow connectors for clathrin/AP2 binding, SH3 to PRD binding, Eps15 homology domain to NPF binding, and other protein interactions (see figure key for arrow types). Membrane interactions are shown as downward gray arrows, and the PIP2 phosphatase synaptojanin is shown interacting with PIP2 (red arrow). Endophilin (purple) has no known interactions with either the core module or the secondary scaffold and is therefore distinguished from the other secondary effectors.

devoid of SVs during activity at the nonpermissive temperature (Koenig & Ikeda 1989). This protein, which is recruited to the endocytic bud neck, is the minimal essential fission machinery in vitro, provided the membranes undergoing fission are under tension (Roux et al. 2006). Dynamin interacts with a number of SH3-containing proteins such as endophilin, amphiphysin, syndapin, and intersectin via its proline-rich domain (Figures 6 and 7). Together with other BAR domain proteins, dynamin also appears to coordinate the shaping of endocytic buds (Itoh et al. 2005). The evidence for dynamin’s role in endocytosis at the synapse is very strong as many dominant-negative strategies targeting dynamin block SV endocytosis (Koenig & Ikeda 1999, Newton et al. 2006, Yamashita et al. 2005). Surprisingly, genetic ablation of dynamin-1, which encodes ~85% of brain dynamin, resulted in only partial defects in SV endocytosis. Mice lacking dynamin-1 survive about two weeks (Ferguson et al. 2007). Under these conditions, endocytosis appeared to be completely arrested...
only during intense activity, presumably owing to the elevation of intracellular calcium. However, soon after cessation of action potential firing, endocytosis resumed with remarkably normal kinetics. Although synapses lacking dynamin-1 in mouse still contain a small amount of dynamin-3 and even less dynamin-2 (Ferguson et al. 2007), one possible conclusion from this work is that the fission step in endocytosis is not catalyzed by the action of dynamin alone, but it is simply accelerated by this protein.

Once the endocytic vesicle is severed from the plasma membrane, the newly sculpted vesicle must shed all the machinery that was used in rebuilding prior to reuse. Clathrin uncoating occurs via the concerted action of the ATPase Hsc70 and auxilin, which helps recruit this enzyme to the vesicle (Eisenberg & Greene 2007). One important mystery is the identity of the molecular trigger to initiate uncoating, as it would be energetically costly and perhaps detrimental to launch this process prior to fission. A mechanistic clue has arisen from genetic ablation studies of synaptojanin: The loss of this lipid phosphatase leads to a pronounced accumulation of clathrin-coated vesicles at nerve terminals as well as a delay in the availability of newly endocytosed vesicles for additional rounds of exocytosis (Cremona et al. 1999, Mani et al. 2007, Schuske et al. 2003, Verstreken et al. 2003). Synaptojanin is stabilized at endocytic buds through multiple interactions including binding to the SH3 domains of intersectin and endophilin and the EH domains of intersectin and Eps15, as well as direct binding to clathrin and AP2 (Haffner et al. 1997, Marie et al. 2004, Montesinos et al. 2005, Schuske et al. 2003, Verstreken et al. 2003). The 5′ phosphatase domain at the amino terminus of synaptojanin rapidly dephosphorylates PIP2. This depletion of PIP2 decreases the phospholipid binding affinity of the core module and BAR proteins, simultaneously destabilizing the coat assembly. Another possible coupling between membrane fission and synaptojanin arises from the theoretical consideration that local depletion of PIP2 selectively on the vesicle bud causes a transient gradient of PIP2 between the bud and the plasma membrane to which it is connected. If this gradient contributes to a lipid phase separation between these compartments, the interfacial line between the neck and bud tends to be minimized, spontaneously constricting the neck (Liu et al. 2006). Regardless of the details of fission and uncoating, these processes are quite rapid as evidenced by the difficulty of capturing a coated vesicle, even when rapid freeze EM is used.

**CALCIUM SENSORS FOR ENDOCYTOSIS?**

As described, presynaptic calcium affects numerous aspects of SV endocytosis. However, unlike SV exocytosis, in which a single protein (synaptotagmin 1) accounts for much of the calcium dependence of SV fusion (Chapman 2008), it is not clear where calcium acts during endocytosis. Nearly every endocytic protein interacts with the membrane or directly binds to a lipid-binding protein. Because elevated calcium can alter the energetics of these membrane interactions, it is plausible that presynaptic calcium functions to change the efficiency of endocytosis through general effects on protein-lipid interactions rather than through a dedicated calcium sensor. However, it is worth noting that synaptotagmin 1 interacts with the core module and its C2 domains may affect this interaction, at least in regimes where calcium is highly elevated (Diril et al. 2006, Jung et al. 2007, Mohrmann et al. 2008, Walther et al. 2001). Multiple lines of evidence support a role for synaptotagmin in endocytosis. Another candidate mechanism for calcium in endocytosis is through the dephosphins, endocytic proteins such as dynamin, synaptojanin, and amphiphysin, that are dephosphorylated in a calcium-dependent manner by the phosphatase calcineurin (Cousin & Robinson 2001). The significance of these dephosphorylation events has not been fully delineated, although dephosphorylation of dynamin promotes its interaction with syndapin (Anggono et al. 2006). Dephosphins seem to play a greater role...
during conditions that lead to bulk-endocytosis (Clayton et al. 2007), which may happen only rarely under physiological stimuli.

**A ROLE FOR ACTIN DURING SYNAPTIC VESICLE ENDOCYTOSIS?**

The regulation of actin polymerization is a vital part of membrane remodeling and intracellular trafficking, and multiple endocytic proteins mentioned above have a direct impact on actin and the cytoskeleton. For example, the secondary effectors dynamin, syndapin, and N-WASP, as well as intersectin, all connect directly or indirectly to proteins that control actin polymerization (Hussain et al. 2001, Kessels & Qualmann 2004, Koh et al. 2004, Miki & Takenawa 2003, Shin et al. 2007). In yeast, actin plays an essential role in endocytosis (Kaksonen et al. 2006). In vitro membrane scission by dynamin can utilize actin as an anchor to transduce its force on tubules into longitudinal tension (Roux et al. 2006). However, actin does not appear to be important for SV endocytosis per se in hippocampal neurons (Sankaranarayanan et al. 2003), but it may contribute as a secondary scaffold (**Figure 7**). Interestingly, actin seems to have a more pronounced role in the endocytosis of SVs at a giant synapse (Brodin & Shupliakov 2006, Shupliakov et al. 2002). Perhaps the cytoskeletal connections to endocytosis depend on the geometry and size of a synaptic terminal, particularly because regulation of the cytoskeleton is likely to be a major determinant of synapse size.

**REBUILDING SYNAPTIC VESICLES: A SINGLE-PASS SORTING AT THE PLASMA MEMBRANE?**

One of the assumptions we have made in discussing vesicle recycling is that the sorting of SV proteins occurs in a single endocytic step, as opposed to having an intermediate organelle after endocytosis at the plasma membrane makes passage through a recycling endosome for further cargo sorting (Koenig & Ikeda 1996). The existence of such a recycling endosome remains a formal possibility, although very few experiments have provided definitive evidence for any functional significance. In the earliest studies of Heuser & Reese (1973), they identified a nonvesicular horse radish peroxidase–positive compartment that appeared only transiently following stimulation. More recent experiments in which serial-sectioning electron microscopy was performed demonstrated that, during intense stimulation, deep invaginations of the plasma membrane often form, which can easily be confused with internal organelles in any single section (e.g., see **Figure 5**), but, in fact, remain topologically connected to the cell surface (Ferguson et al. 2007). Nonetheless, an endosomal intermediate may play a role under some stimulus conditions, particularly following intensive stimulation, when bulk endocytosis has been reported to operate at a number of synapses (Clayton et al. 2008, Holt et al. 2003).

**A MOLECULAR PICTURE FOR THE ENDOCYTIC MACHINE**

Ideally, the molecules described here can be mapped to the phenomenology of SV endocytosis on the basis of their intermolecular interactions, genetics, structure, and cell biological roles in other forms of endocytosis. A host of studies published over the past decade have made apparent a rough version of this molecular map and the dynamics of the endocytic machinery. The core module couples nucleation of an endocytic coat with cargo recruitment. Polymerization of clathrin and membrane interactions with ENTH and N-BAR domains rapidly commence the process of curving the lipid bilayer at the site of endocytosis. Furthermore, assembly of the core may increase the avidity of cargo binding because a multiplicity of binding sites will be brought into a small region of the membrane. In this scenario, cargo capture, scaffold assembly, and membrane curvature are highly coupled coordinated events.
The N-BARs endophilin and amphiphysin contribute to membrane bending and may initiate an additional positive-feedback cycle of binding to curved membrane and self-assembly, driving further membrane curvature. Amphiphysin has direct binding interactions with clathrin and AP2 (placing it in the core module), whereas endophilin has no known interaction with these or other proteins in the core module (qualifying endophilin as a secondary effector). The secondary scaffold molecules Eps15 and intersectin/Dap160 bind to the core module and provide additional binding sites for recruiting the secondary effector molecules: dynamin, synaptojanin, and N-WASP. Both dynamin and synaptojanin convey amplification of a sort within the endocytic complex. Dynamin self-assembles and can utilize some of the energy released by polymerizing to deform membranes. Synaptojanin contains a phospholipase enzymatic domain, and thus a small number of synaptojanins would be expected to deplete PIP_2 rapidly from the membrane. These effectors terminate the endocytic process by irreversibly severing the membrane connection and releasing the endocytic coat with the help of Hsc70 and auxilin. The AP180 paralog CALM, the stonin 2 paralog stonin 1, and the F-BAR syndapin may also contribute to SV endocytosis, but their specific requirements are not well defined. We therefore include these components as potential secondary effectors (Figures 6 and 7).

NETWORK PROPERTIES OF THE ENDOCYTIC MACHINE

There are several advantages to considering the endocytic proteins as a network (Alon 2007, Hintze & Adami 2008, Schmid & McMahon 2007). First, fault-tolerant networks exhibit graceful degradation, because the failure of one component has little effect on the overall performance. Evidence to date suggests that SV endocytosis continues after the removal of any one protein. Second, weak interactions can be transiently strengthened and amplified by positive feedback within the network. Thus, the binding of cargo; polymerization of the core module; and membrane bending by N-BARs, epsins, and dynamin all collaborate to create a fast, high-fidelity endocytic process. Third, modularity within the network allows the basic endocytic machinery to be utilized in many separate types of trafficking processes within a neuron. For instance, most of the SV endocytosis machinery is likely to be involved in postsynaptic receptor trafficking, intracellular trafficking between organelles, and membrane remodeling during synapse development (Bushlin et al. 2008, Gong & De Camilli 2008, Itoh & De Camilli 2006, Koh et al. 2007, Montesinos et al. 2005). Another consequence of a densely connected network is that the removal of one protein can have repercussions on the stability of many others. For instance, in fly Eps15 mutant synapses, many of the core proteins have significantly decreased abundance along with the Eps15-binding partner intersectin/Dap160 (Koh et al. 2004, 2007). This interdependence of protein stability emphasizes the importance of surveying the abundance of all endocytic proteins following a molecular deletion.

Parsing the endocytic proteins into three layers (Figure 7) is not absolute in that members of the core module and the N-BARs also assist in recruiting secondary effectors (Schuske et al. 2003, Takei et al. 1999, Verstreken et al. 2003), whereas Eps15 and intersectin also help stabilize the core module (Koh et al. 2007, Morgan et al. 2003). The relative impact of removing particular proteins does not necessarily correspond to the location within this endocytic network. For instance, the core module proteins AP2, clathrin, and amphiphysin all have a relatively small effect on SV endocytosis when removed individually (Di Paolo et al. 2002, Gu et al. 2008, Sato et al. 2009). These observations would be predicted in a redundant network such as this, because secondary scaffolds and effectors functionally compensate for the loss of these core proteins. Perhaps double deletions that specifically target redundant function may have a more dramatic effect on SV endocytosis. For example, removal of clathrin and intersectin simultaneously would be predicted...
to impact the scaffolding function of the network. Concomitant removal of ENTH- and BAR-domain proteins may eliminate the ability to deform the membrane.

CONCLUDING REMARKS

In the half century following Katz’s quantum hypothesis at the nerve terminal (Del Castillo & Katz 1954), advances in molecular, imaging, and physiological techniques have conspired to produce a fairly detailed understanding of the SV cycle. The process of manufacturing, trafficking, and recycling SVs involves the coordinated efforts of hundreds of proteins discovered over the past few decades. As the list of molecular players grows, so does the need to synthesize and organize this collection. As with many aspects of modern cell biology, the detailed properties of SV endocytosis may be emergent properties of a network. One of the impediments to understanding this emergence is that we still lack in-depth information about the precise location of most of these molecules during various stages of the SV cycle. Quantitative studies of the dynamic interactions, as well as application of new super-resolution techniques, should provide a rich new level of detail to understand the functioning of the endocytic network.

SUMMARY POINTS

1. Clathrin-mediated endocytosis accounts for the majority of SV endocytosis under normal conditions, whereas bulk endocytosis may contribute following high-frequency stimulation.

2. SV proteins are endocytosed stochastically such that their dwell time on the plasma membrane obeys an exponential distribution with a 6-s time constant at 37°C (13-s time constant at room temperature).

3. The proteins of endocytosis can be organized in a simple hierarchical network based on protein interactions, domains, and genetic ablation studies.

4. No single component of the SV endocytosis machinery appears to be essential: The network of endocytic proteins appears robust to these molecular perturbations.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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LITERATURE CITED


First demonstration that the regulation of phosphoinositides is important for the SV cycle through examination of a mouse synaptojanin knockout.

Measured kinetics of single-vesicle retrieval and described the single-mode stochastic nature of SV cargo dwell time on the surface.


This seminal work employed electron microscopy to visualize exo- and endocytosis at the frog NMJ.

The culmination of multiple studies on a temperature-sensitive fly mutant (dynamin) that blocked endocytosis.


This EM study provided the first direct measurement of synaptic vesicle endocytosis kinetics.


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Errata

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