

CELL BIOLOGY OF THE PRESYNAPTIC TERMINAL

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■ **Abstract** The chemical synapse is a specialized intercellular junction that operates nearly autonomously to allow rapid, specific, and local communication between neurons. Focusing our attention on the presynaptic terminal, we review the current understanding of how synaptic morphology is maintained and then the mechanisms in synaptic vesicle exocytosis and recycling.

INTRODUCTION

Synaptic transmission involves fusion of neurotransmitter-containing vesicles with the plasma membrane and activation of postsynaptic receptors. The process of transmitter release through vesicle exocytosis is typically repeated many times a minute, and this rate is maintained for the lifetime of the synapse. To prevent depletion of the finite number of synaptic vesicles by sustained release, an intricate web of recycling events restocks the synapse with vesicles. Remarkably, freshly released vesicles can be recycled and rereleased in just a few seconds under some conditions (Pyle et al. 2000). In this review, we present a cell biological view of synaptic function, emphasizing a mechanistic understanding of the organization of the presynaptic terminal and the dynamics of synaptic vesicles. For details of the molecular basis of specific aspects of synaptic transmission, the reader is referred to several recent reviews (Hanson et al. 1997, Fernandez-Chacon & Sudhof 1999, Lin & Scheller 2000, De Camilli et al. 2001, Rizo & Sudhof 2002, Chapman 2002).

THE ORGANIZATION OF THE PRESYNAPTIC TERMINAL

A typical presynaptic bouton is a specialized portion of the axon. It is characterized by an active zone, a region where the presynaptic plasma membrane comes in close contact with the postsynaptic plasma membrane and an associated cluster of vesicles (De Camilli et al. 2001). A few synaptic vesicles are adjacent to the

active zone and are referred to as docked vesicles. Vesicle exocytosis occurs at the active zone; subsequent endocytic retrieval of vesicular components may occur both at the active zone and in a peri-active zone area (Roos & Kelly 1999). At least a fraction of this endocytic uptake is mediated by vesicles that mature directly into new synaptic vesicles, whereas an additional membrane pool may travel through an intermediate endosomal station where sorting of components occurs. Vesicle maturation involves acidification of the lumen, loading with neurotransmitter, association with peripheral membrane proteins needed for exocytosis, and recapture into the vesicle cluster. The vesicle cluster is embedded in an actin-rich area and is generally located next to mitochondria, which provide energy for the vesicle cycle and neurotransmitter dynamics and to the endoplasmic reticulum whose function includes the regulation of local cytosolic calcium. Synapses are typically also surrounded by glial cells, which might perform auxiliary roles such as secretion of neuromodulatory factors, regulation of extracellular ion concentration, and uptake of neurotransmitters (Haydon 2001).

We start with a description of the structure of the synapse and then address the different stages in the life of a synaptic vesicle.

SYNAPTIC ADHESION—KEEPING THE PARTS TOGETHER

The synapse is an intercellular junction whose structural organization shares similarities with other types of junctions such as the immunological synapse or the intercellular junctions of epithelial cells (Dustin & Colman 2002, Jamora & Fuchs 2002). The close apposition and precise alignment of the presynaptic release site and the postsynaptic receptive site prompts several basic questions: (*i*) Which part of the synapse—the active zone or the periaxial zone—is involved in adhesion; (*ii*) what are the molecular glues that cause the adhesion; and (*iii*) how is the contact between the pre- and postsynaptic membrane transduced into clustering of vesicles presynaptically and of neurotransmitter receptors postsynaptically. There is evidence for the involvement of both the active zone and the surrounding region in cell adhesion, based mainly on the ultrastructural localization of putative adhesion molecules (Uchida et al. 1996, Phillips et al. 2001). Functionally, adhesive interactions between the active zone and the postsynaptic density will ensure close proximity of release and receptive sites. On the other hand, adhesion at the edges of the active zone may bring the two membranes together while freeing up the active zone itself for other components. Adhesion at the boundary can also be dynamic, allowing for expansion or contraction of the active zone (Tanaka et al. 2000, Colicos et al. 2001). The asymmetric nature of the synapse suggests the presence of different molecules in the presynaptic and the postsynaptic membranes in the initial stages of synapse formation. Yet, despite the functional polarization of the synaptic junction, symmetry in the organization of pre- and postsynaptic compartments is increasingly being recognized.

Several molecules have been identified in relation to synaptic adhesion, including cadherins (Shapiro & Colman 1999, Yagi & Takeichi 2000), protocadherins (Frank & Kemler 2002), neural cell-adhesion molecule (NCAM), L1, fasciclin II (Davis et al. 1997), apCAM (Mayford et al. 1992), synCAM (Biederer et al. 2002), nectin (Mizoguchi et al. 2002), DsCAM (Schmucker et al. 2000), neuexins and neuroligin (Missler & Sudhof 1998), integrins (Chavis & Westbrook 2001), syndecans (Hsueh & Sheng 1999), and sidekicks (Yamagata et al. 2002). Some of these putative adhesion molecules have also been implicated in regulating the size of the presynaptic specialization (Broadie & Richmond 2002). All of them are transmembrane proteins generally expressed in both pre- and postsynaptic membranes, and the adhesive properties are thought to result from interaction of their extracellular domains, either with each other or with components of the extracellular matrix. Perhaps not surprising, most of these molecules contain multiple immunoglobulin-like (IgG) domains in their extracellular regions or a related structural feature in the case of cadherins. In addition, some of them have cytosolic sequence motifs that bind to PDZ domains typically present in multimodular proteins that function as molecular scaffolds (Sheng & Sala 2001). In all cases except neuroligin, homophilic interactions occurring in the *trans* configuration mediate adhesion. The multiplicity of these adhesion proteins, further enhanced by the existence of a large number of splice variants, raises the possibility that they may play essential roles in the specification of synaptic connectivity (Sanes & Yamagata 1999, Yagi & Takeichi 2000, Clandinin & Zipursky 2002).

Some of the putative postsynaptic adhesion molecules, neuroligin and synCAM for example, can induce synapse formation by cocultured neurons when expressed in heterologous cells (Scheiffele et al. 2000, Biederer et al. 2002). Although it is unclear if these molecules are the real trigger for synaptogenesis in intact circuits, the ability of a single postsynaptically expressed molecule to induce presynaptic differentiation is remarkable and might allow a rational dissection of the steps in synapse formation.

What is the relationship of these proteins to underlying cytosolic scaffolds? Cadherins provide a natural link from adhesion to the actin cytoskeleton through their interaction with β -catenins (Gottardi & Gumbiner 2001). Similarly, integrins are connected to the actin cytoskeleton, and several proteins typical of focal adhesion are present at the presynaptic terminal (Qualmann & Kelly 2000, Di Paolo et al. 2002). Adhesion molecules that have PDZ domain-interacting motifs might couple to the cytoskeleton through the PDZ domain-containing proteins (Sheng & Sala 2001). Actin is concentrated primarily around the active zone and vesicle clusters (Dunaevsky & Connor 2000, Colicos et al. 2001, Shupliakov et al. 2002), and the Rho family GTPases may play a key role in their regulation (Sone et al. 2000). In contrast to the presence of actin, presynaptic specializations are microtubule-free zones, speaking against a role of microtubules in synaptic vesicle traffic within the presynaptic bouton. Microtubule-associated proteins, however, have been recently implicated in synaptic growth and retraction at *Drosophila* neuromuscular junction (Eaton et al. 2002, Roos et al. 2000).

In addition to molecules with a primary role in adhesion, pre- and postsynaptic proteins, whose interactions play a role in signaling, are also present at the synapse. At the neuromuscular junction, cross-junctional signaling mediated through agrin and the postsynaptic transmembrane tyrosine kinase MuSK appear to be critical for the proper formation of this synapse (Glass & Yancopoulos 1997, Sanes & Lichtman 2001). A similar cross talk mediated by tyrosine kinases is likely to play a role at central synapses. For example, ephrins binding to their receptors may contribute to signaling as well as synaptic adhesion (Kullander & Klein 2002). Heparan sulfate proteoglycans such as agrin, dystroglycan, and syndecan are present at many synapses and are likely to have important roles in synaptic function (Yamaguchi 2001).

THE ACTIVE ZONE AND HOW IT CAPTURES VESICLES

The active zone is the specialized region of the cortical cytoplasm of the presynaptic nerve terminal that directly faces the synaptic cleft. It has a dense appearance under the electron microscope reflecting the presence of a protein scaffold—the presynaptic grid—within which docked vesicles are nested (Pfenninger et al. 1969). Functionally, the vesicles that are docked, or a subset of them, comprise the readily releasable pool of vesicles (Murthy et al. 2001, Schikorski & Stevens 2001). Scaffolding proteins play a role both in recruiting vesicles to the plasma membrane as well as in the regulation of release. This arrangement ensures the exocytotic release of neurotransmitter in close proximity to their postsynaptic receptors and in setting the release process to the appropriate dynamic range. Recently, the structure of the active zone was examined in detail at the frog neuromuscular junction using electron microscopy tomography, and several interesting features such as pegs, ribs, and beams were described (Harlow et al. 2001). Similar principles of organization are likely to be maintained at central synapses in spite of differences in the fine structure.

The identification of protein components of active zones has lagged behind the characterization of synaptic vesicle proteins. This is explained by the much greater abundance of synaptic vesicles than of active zone components at synapses as well as by the difficulty of solubilizing active zone structures. Pre- and postsynaptic scaffolds are tightly linked together in an insoluble matrix that can be disrupted only by very harsh treatments (Dresbach et al. 2001, Phillips et al. 2001). Immunological and genetic techniques, more recently combined with yeast two hybrid studies and proteomic analysis, have begun to provide some molecular insight into these structures. Important classes of active zones components include the members of the rab3-interacting molecule or RIM (Wang et al. 1997), piccolo/aczonin (Fenster et al. 2000, Wang et al. 1999), and bassoon protein families (tom Dieck et al. 1998). Piccolo and bassoon are two very large proteins with multiple domains involved in protein-protein interaction as well as signaling. RIM is a smaller protein that shares blocks of homology with both piccolo and bassoon and which was identified as an

interactor of rab3. Rab3 is a small GTPase of the rab family present on synaptic vesicles (Geppert et al. 1994a). RIM, like Piccolo, has a PDZ (postsynaptic density-95/discs-large/ZO-1) domain and multiple C2 domains that could provide a direct linkage to plasma membrane proteins (Wang et al. 1997). The GTP-dependent interaction of RIM with Rab3 makes RIM a particularly interesting component of active zones because it could mediate the tethering of synaptic vesicles at release sites. However, RIM appears to have a role in synaptic dynamics independent of its interaction with rab3a (Schoch et al. 2002). In both worms and mice, genetic disruption of RIM function does not produce obvious morphological defects; yet, the efficacy of release is significantly lower in these animals, suggesting a role in the regulation of release rather than in defining structural parameters of the synapse (Koushika et al. 2001, Schoch et al. 2002). The regulatory role of RIM in release may be mediated in part by its interaction with munc13, a critical player in the priming of synaptic vesicle for exocytosis (Betz et al. 2001).

Mutations that affect active zone structure and localization have been identified in model organisms. Mutations of the *highwire/RPM1* gene in *Drosophila* and *Caenorhabditis elegans* result in excessive arborization and abnormally large synaptic size (Chang & Balice-Gordon 2000, Wan et al. 2000). *Highwire* is localized at periaxonal zones, suggesting an indirect role on the active zone structure (Wan et al. 2000). The protein encoded by the *Highwire/RPM1* gene contains a ubiquitin ligase domain, which raises interesting questions on the role of protein ubiquitination in synaptic ontogenesis (DiAntonio et al. 2001). Mutation of liprin- α in *C. elegans* leads to abnormal active zones that are longer and less electron dense (Zhen & Jin 1999); similar effects are observed in *Drosophila* (Kaufmann et al. 2002). Liprins interact with RIM and receptor tyrosine phosphatases, but how they are involved in organizing the active zone is unclear. The active zone, the vesicle clusters, and the overall size of the presynaptic compartment need to be regulated in a concerted manner to set the size of the synapse. In *Drosophila*, several genes, including *scribble* (Roche et al. 2002), *futsch* (Roos et al. 2000), *spinster* (Sweeney & Davis 2002), *dynactin* (Eaton et al. 2002), and *VAP33* (Pennetta et al. 2002), have been implicated in the control of synaptic size, as well as function. In NCAM-deficient mice, the transition from immature to mature vesicle clusters was affected, indicating an important cross talk between adhesion molecules and mechanisms in vesicle clustering (Polo-Parada et al. 2001). A role for NCAM in stabilizing Golgi-derived vesicles at nascent synapses has been suggested by a recent study (Sytnyk et al. 2002).

Other components of the active zone include the protein CAST [cytomatrix-of-the-active-zone-associated structural protein, (Ohtsuka et al. 2002)] and the tripartite protein complex comprising Mint, CASK, and Veli (Butz et al. 1998). CASK is a member of the membrane-associated guanylate kinase family of proteins, which interacts with calcium channels, beta-neurexins, mint, and Veli (Butz et al. 1998). In addition to adhesion molecules discussed above, membrane components of the active zone include calcium channels (Robitaille et al. 1993), which are thought to be localized at synaptic vesicle docking sites, and the t-SNAREs

(soluble N-ethylmaleimide-sensitive factor attachment protein receptors) syntaxin and SNAP25 (Garcia et al. 1995). Calcium channels and syntaxin interact with each other, which may contribute to the regulation of the channels and to the triggering of fusion in their proximity (Catterall 1999). Syntaxin and SNAP25, however, are not concentrated at the active zone (Garcia et al. 1995). Other plasma membrane proteins that play critical roles in presynaptic function, such as transporters involved in neurotransmitter uptake, a variety of ion channels, receptors for growth factors, and neurotransmitters, may not be selectively concentrated at the active zone.

Synapses that release transmitter on a continuous basis in response to graded changes in membrane potential and without precise synchronization by action potentials have structures called ribbons or dense bodies instead of conventional active zones (Lenzi & von Gersdorff 2001). These are electron-dense particles with vesicles attached to them. In addition to concentrating vesicles at release sites, ribbons and dense bodies may function as conveyer belts that deliver vesicles to the plasma membrane (Zenisek et al. 2000). Ribbon synapses can sustain very high rates of release, and ribbons may be specially designed to guarantee a constant supply of vesicles during intense stimulation (Lenzi & von Gersdorff 2001). Many of the components of the active zones, including piccolo, bassoon, and RIM, are found in the ribbons and dense bodies (Lenzi & von Gersdorff 2001).

Beyond a static description of vesicles at the active zone, there are many questions regarding the dynamics of the interactions leading to docking. In neurosecretory cells, granules leaving putative docking sites without undergoing exocytosis have been observed directly using total internal reflection microscopy (Steyer et al. 1998). At synaptic terminals, newly endocytosed vesicles can gain access to the docking sites within a few minutes even in the absence of significant emptying of the docking sites due to exocytosis (Murthy & Stevens 1999). Therefore, it appears that over a timescale of minutes, docking may be reversible. It will be important to define at the precise sequence of events occurring at docking sites the rate constants of these events and the stage, if any, at which a docked vesicle becomes irreversibly committed to fusion.

VESICLE CLUSTERS OR WHAT KEEPS VESICLES TOGETHER?

Synaptic vesicles docked at active zones represent only a small fraction of vesicles present in nerve terminals (De Camilli et al. 2001). At most synapses, there appears to be an abrupt transition in the density of synaptic vesicles at the edge of the clusters, suggesting the presence of either a “glue” that keeps them together or a restraining cage around them. Although early hypotheses focused on actin as a potential scaffold to which vesicles are anchored (Landis et al. 1988, Hirokawa et al. 1989), recent studies have shown that the bulk of presynaptic actin is localized

around the cluster (Dunaevsky & Connor 2000, Shupliakov et al. 2002), possibly helping in preventing vesicle dispersion or in the recapture of endocytic vesicles. Very large proteins with long flexible regions, such as piccolo and bassoon, may be part of a matrix holding vesicles together (Dresbach et al. 2001). Such a role would be similar to that of the very long flexible proteins that anchor small vesicles in the region of the Golgi complex (Nakamura et al. 1995). Accordingly, piccolo immunoreactivity is seen not exclusively at the cell surface but also within the core of vesicle clusters (Cases-Langhoff et al. 1996).

Peripheral vesicle membrane proteins that dissociate from vesicles during the endocytic phase of their cycle may function as protein adaptors in vesicle clustering. One potential candidate is rab3 (Geppert et al. 1994a). However, although work in worms has shown a dispersion of synaptic vesicles in rab3 mutant synapses (Nonet et al. 1997), no such effects were observed at mammalian synapses defective in rab3 function (Geppert et al. 1994a). A series of interactions between rab3 and other scaffolding proteins has been described. For example, rab3a interacts with the protein Pra1, which in turn interacts with Piccolo and the vesicle protein synaptobrevin (Fenster et al. 2000). Another peripheral vesicle protein that has received considerable attention is synapsin (De Camilli et al. 1990, Pieribone et al. 1995, Rosahl et al. 1995). Synapsins bind to each other and to actin filaments, and these interactions are regulated by phosphorylation (Greengard et al. 1993). Disrupting synapsin function results in a reduction in the size of the vesicle cluster and a corresponding reduction of the reserve pool of vesicles, which suggests an important but not essential role of these proteins in vesicle clustering (Pieribone et al. 1995, Rosahl et al. 1995). Real-time observation of synapsins labeled with green fluorescent protein has demonstrated a correlation between the kinetics of its dissociation from vesicles and the mobilization rate of vesicles (Chi et al. 2001), but the precise cause-effect relationship of these events remains unclear. An important open question is whether the ability of synapsin to bind and hydrolyze ATP (Hosaka & Sudhof 1998) reflects an enzymatic activity critical for vesicle function. The protein α -synuclein, which has been implicated in some forms of Parkinson's disease, is a vesicle-associated protein of unknown function (Abeliovich et al. 2000). Recently, some results have pointed to a role for this protein in vesicle clustering. Genetic deletion of α -synuclein in mice leads to a reduction in the reserve pool of vesicles, without a significant change in the docked vesicles (Cabin et al. 2002, Murphy et al. 2000).

The mechanisms holding vesicles together appear to be already in place prior to synapse formation. Small clusters of recycling synaptic vesicles, which contain most components of mature presynaptic vesicle clusters and may define a minimal synaptic vesicle recycling unit, are already present in developing axons before they contact a target. These clusters are not stationary but move along the axon until they are recruited to nascent synapse where they coalesce into large clusters (Kraszewski et al. 1996, Ahmari et al. 2000, Zhai et al. 2001). These findings indicate that formation of a vesicle cluster is not necessarily the result of a cascade of interactions that starts at the active zone of a synapse.

At mature synapses, vesicle motility within clusters may be limited, as inferred from studies using the method of fluorescence recovery after photobleaching (Betz & Henkel 1994, Kraszewski et al. 1996). Newly reformed vesicles, in contrast, appear to be recaptured at random by the cluster (Ryan & Smith 1995, Schikorski & Stevens 2001). Application of okadaic acid, a generic inhibitor of phosphatases to living neurons disperses vesicle clusters both in immature neurons and mature synapses (Betz & Henkel 1994). Conversely, treatment with the broad spectrum kinase inhibitor staurosporine inhibits the recruitment of the vesicles from the reserve pool (Becherer et al. 2001) and retards the return of endocytosed vesicles back into the vesicle cluster (Li & Murthy 2001). These observations point to a role of phosphorylation of unknown substrates in vesicle dynamics. Genetic studies in *C. elegans* have identified Sad-1, a protein kinase related to the PAR-1 kinase that regulates cell polarity, as an important regulator of synaptic vesicle clusters (Crump et al. 2001). A similar phenotype, which also includes a partial loss of axonal and dendritic polarity, was observed in Syd-1 mutants in *C. elegans* (Hallam et al. 2002).

EXOCYTOSIS

The mechanism of exocytosis has been a subject of intense research. In very basic terms, the question has been whether bilayer fusion is mediated simply by lipids or whether intrinsic membrane proteins play a critical, causal role. It is now well established that efficient membrane fusion *in vivo* requires the interaction of small cytoplasmically exposed membrane proteins called SNAREs (Söllner et al. 1993). For synaptic vesicle exocytosis, the relevant SNAREs are synaptobrevin/VAMP 1 and 2, syntaxin 1, and SNAP-25. Synaptobrevins/VAMPs are localized primarily on synaptic vesicles, syntaxin and SNAP25, primarily on the plasma membrane. Fusion is driven by the progressive zippering of vesicle and plasma membrane SNAREs to form a four-helix bundle (Sutton et al. 1998). Although many other proteins appear to have critical roles in synaptic vesicle exocytosis, it seems likely that the SNAREs represent the minimal machinery for fusion (Weber et al. 1998). Once assembled, SNARE complexes are disassembled by NSF, which functions in conjunction with SNAP proteins. Block of NSF action results in the accumulation of fusion-incompetent vesicles owing to a progressive loss of SNARE proteins available for the formation of productive *trans*-SNARE complexes (Söllner et al. 1993, Hanson et al. 1997).

The SNARE hypothesis of membrane fusion, and of synaptic vesicle fusion in particular, was first formulated based on studies of membrane fusion *in vitro* (Söllner et al. 1993, Rothman 2002) and complemented by results of genetic studies in yeast (Schekman 2002). Compelling support for this hypothesis came from the identification of synaptobrevin/VAMP, syntaxin, and SNAP25 as targets for the proteolytic action of clostridial neurotoxins, which are potent blockers of neurotransmitter release (Blasi et al. 1993, Montecucco 1998, Schiavo et al. 1992).

Each of these toxins acts by selectively cleaving one of the synaptic SNAREs at a specific site. Treatment of synapses with various clostridial neurotoxins results in an increase in the number of docked vesicles, probably as a result of block in fusion (Hunt et al. 1994, Neale et al. 1999). This observation not only indicates that SNAREs are not critical determinants of vesicle docking but also demonstrates a surplus of docking sites for synaptic vesicles. The SNARE hypothesis has now been further validated by a large number of genetic studies in a variety of organisms and by the demonstration that SNARE complexes are sufficient to drive fusion of artificial membrane bilayers. This research has been summarized in many recent reviews (Lin & Scheller 2000, Rothman 2002, Schekman 2002, Jahn et al. 2003).

Although zippering of SNARE proteins may be the final trigger for membrane fusion, a variety of other factors play critical roles in exocytosis by controlling SNARE function, as emphasized by the growing list of SNARE-interacting proteins. Many of these regulatory factors occur as specialized isoforms at synapses. Two such factors, *unc/munc13* and *unc/munc18* (a member of the Sec1 family), are particularly important given the dramatic effect produced by their absence: a complete block of synaptic vesicle exocytosis (Richmond et al. 1999, Verhage et al. 2000, Varoqueaux et al. 2002).

Munc18/Sec1 acts by regulating syntaxin function as initially shown by the property of yeast syntaxins (the SSO proteins) to suppress mutations in *Sec1* (Aalto et al. 1993). The N-terminal region of syntaxin 1 contains a three-helix domain that folds back onto the SNARE motif forming a closed conformation. *Munc18* interacts strongly with this conformation of syntaxin, suggesting an inhibitory role on fusion (Misura et al. 2000). Yet, the *munc18* family of proteins is needed for fusion as demonstrated, for example, by the complete absence of synaptic vesicle exocytosis events in *Munc18-1* knockout mice (Verhage et al. 2000). Therefore, *munc18* may also have a second role downstream to syntaxin inhibition, as supported by the property of yeast *Sec1* to bind the assembled yeast SNARE complex (Carr et al. 1999). How syntaxin shifts from a closed *munc18*-bound state to the open state capable of forming SNARE complexes is not understood. The displacement of *munc18* might be achieved by other proteins known to be important for priming: *munc13* and RIM (Richmond et al. 2001, Rizo & Sudhof 2002). Evidence in support of this idea comes from *C. elegans*, where loss of exocytosis due to mutant *unc13* or RIM could be overcome by expression of the open form of syntaxin (Koushika et al. 2001, Richmond et al. 2001).

Priming of Vesicles

Fusion competence follows vesicle docking with some delay. Acquisition of fusion competence may require multiple steps and may involve both lipid and protein changes, but it is defined collectively as priming. *Munc13* is now thought to play a central role in the priming reaction. Initially identified in a *C. elegans* screen (*unc13*), its function is highly conserved from worms to mice (Richmond et al.

2001, Varoqueaux et al. 2002). The virtually complete absence of synaptic vesicle exocytosis produced by deletion of munc13 in mice is not accompanied by a reduction in docked vesicle number (Geppert et al. 1994a, Augustin et al. 1999, Richmond et al. 2001, Varoqueaux et al. 2002). Munc13 has a phorbol ester binding site, whose activation potentiates transmitter release (Rhee et al. 2002). This interaction is likely to reflect the property of munc13 to interact with diacylglycerol in the plasma membrane. It will be of interest to determine whether and how this diacylglycerol pool is regulated. Munc13 interacts with the active zone protein RIM, and this interaction is critical for priming (Augustin et al. 1999). Furthermore, deletion of an abundant isoform of RIM in mice causes an alteration in the efficacy of vesicle release without significant alteration in the number of docked vesicles (Schoch et al. 2002), which points to a potential role of RIM itself in priming. In a related observation, the absence of rab3, a protein that interacts with RIM, appears to enhance calcium-triggered exocytosis (Geppert et al. 1997).

Calcium-Triggered Exocytosis

Synaptic vesicles fuse with the plasma membrane constitutively under resting conditions, but the probability of vesicle fusion is increased dramatically by elevations in cytosolic Ca^{2+} . The rapid effect of Ca^{2+} (fraction of a millisecond) suggests the existence of a Ca^{2+} -induced conformational change of a component of a prefusion complex, rather than an indirect effect mediated by a Ca^{2+} -sensitive enzyme such as a protein kinase. The Ca^{2+} -dependence of exocytosis most likely reflects the synergistic action of several Ca^{2+} sensors, among which some isoforms of synaptotagmin are likely to play an important role (Chapman 2002, Fernandez-Chacon et al. 2002). Synaptotagmin is an integral vesicle membrane protein with two C2 domains that bind Ca^{2+} , SNARE proteins, and phospholipids. There are several isoforms of synaptotagmins with differential intracellular localization and tissue expression patterns. Synaptotagmin I is selectively enriched in synaptic vesicles (Geppert et al. 1994b), and its absence causes a severe loss of Ca^{2+} -dependent synchronous release (Geppert et al. 1994b, Fernandez-Chacon et al. 2002). The function of synaptotagmin has been the object of a systematic biochemical, genetic, and physiological analysis. A current model suggests that Ca^{2+} binding induces the interaction of one or both C2 domains with proteins and lipids in the plasma membrane, thus helping to catalyze SNARE-mediated membrane fusion (Chapman 2002, Fernandez-Chacon et al. 2002).

In addition to synaptotagmin, other proteins with tandem C2 domains are present either on the vesicles (for example, Doc2 and rabphilin) or at the active zone (for example, RIM and piccolo) (Rizo & Sudhof 1998). These proteins, together with other calcium-binding proteins of the presynaptic terminal, may contribute to Ca^{2+} -sensitivity of exocytosis. Studies of dense core granule release from chromaffin cells suggest a role of SNAP-25 in Ca^{2+} -triggered exocytosis, possibly via its Ca^{2+} -dependent interaction with synaptotagmin and SNARE complexes (Banerjee et al. 1996). In addition, synaptobrevin and syntaxin may play an indirect

role in calcium sensitivity of the release process because reducing the levels of these proteins in flies results in a systematic reduction in the cooperative relation between calcium concentration and exocytosis (Stewart et al. 2000). A protein whose absence results in a phenotype very similar to synaptotagmin deletion is complexin (Reim et al. 2001). Complexins (also called synaphins) are cytosolic proteins that bind to SNARE complexes. In a study of the squid giant synapse, disrupting complexin-SNARE complex interaction prevented SNARE complex oligomerization and decreased release (Tokumaru et al. 2001). Deletion of complexin I and II in mouse resulted in a severe deficit in Ca^{2+} -triggered release of neurotransmitter (Reim et al. 2001). The functionally defined, readily releasable pool and the number of docked vesicles were, however, unaltered (Reim et al. 2001).

Cysteine string proteins (CSPs), first identified and studied in *Drosophila*, appear to have a role in the Ca^{2+} sensitivity of exocytosis (Dawson-Scully et al. 2000). At synapses lacking CSPs, the Ca^{2+} dependence of exocytosis shifted to higher Ca^{2+} concentrations, without a change in the Ca^{2+} -cooperativity (Dawson-Scully et al. 2000). CSPs have a DNAJ domain, typically present in proteins that have a role as molecular chaperones. Accordingly, CSPs associate with the ATPase hsc70 involved in uncoating (Tobaben et al. 2001), and both CSP and hsc70 interact with another protein, SGT, to form a complex with putative molecular chaperone function (Tobaben et al. 2001).

In summary, exocytosis at synapses shares many features with other intracellular membrane fusion events, but it also has some unique features related to the exquisite spatial and temporal control of release. New interacting partners for the proteins described above continue to be discovered, pointing to a complex regulation of the basic fusion machinery.

ENDOCYTOSIS

The concept of synaptic vesicle recycling was conclusively established in the early 1970s (Holtzman et al. 1971, Ceccarelli et al. 1973, Heuser & Reese 1973), but the mode of endocytosis has been a topic of continued investigation and discussion (Ceccarelli et al. 1973, Heuser & Reese 1973, Fesce et al. 1994, Cremona & De Camilli 1997, Brodin et al. 2000). Studies from Heuser & Reese led to the formulation of the classical model of endocytosis involving clathrin coats and a sorting endosome. Although more recent studies have suggested that the endosomal station may be bypassed, the role of clathrin-mediated endocytosis in the recycling of synaptic vesicles is now well established. There is, however, evidence for additional modes of vesicle retrieval.

Kiss-and-Run

A mode of endocytosis referred to as “kiss-and-run” has attracted considerable interest (Ceccarelli et al. 1973, Fesce et al. 1994). In this model, vesicles release neurotransmitter via a transient fusion pore. Newly reformed vesicles may then stay

in place, be reloaded, and undergo a new round of exocytosis or may de-dock and allow other vesicles to take their place. To date, kiss-and-run events have been directly demonstrated only for dense-core granules in neuroendocrine cells (Breckenridge & Almers 1987, Albillos et al. 1997, Graham et al. 2002, Holroyd et al. 2002, Taraska et al. 2003). At neuronal synapses, where these events would have to occur with much faster kinetics, evidence for kiss-and-run is only indirect. The most rapid endocytosis measured electrophysiologically at central synapses has a time constant of around 50 msec (Sun et al. 2002), but whether this represents kiss-and-run remains unclear. A main line of evidence relies on the use of styryl dyes, such as FM1-43, as tracers for exo- and endocytosis (Klingauf et al. 1998, Pyle et al. 2000, Stevens & Williams 2000). During exocytosis from dye-labeled nerve terminals, vesicles lose dye either through lateral diffusion (and mixing with the plasma membrane) and/or via direct partitioning into solution. Experiments with dyes with different partitioning rates have indicated that, under some conditions, exocytosis occurs in a manner that retains a significant portion of the dye in the vesicle. These results have been interpreted as reflecting rapid endocytosis without full collapse of the vesicle membrane, thus leading to retention of the styryl dye. Notably, at the only synapse where exocytosis was directly visualized, dye could escape from vesicles readily in the plane of the membrane (Zenisek et al. 2002).

Studies of *Drosophila* endophilin mutants have been invoked to support the “kiss-and-run” model of neurotransmitter release (Verstreken et al. 2002). Disruption of the *Drosophila endophilin* gene, which codes for an important endocytic protein, resulted in synapses that have very few vesicles but nevertheless sustain synaptic transmission at low frequencies (Guichet et al. 2002, Rikhy et al. 2002, Verstreken et al. 2002). At these synapses, release events were not followed by detectable uptake of styryl dyes. These findings, together with evidence for an impaired clathrin-mediated recycling pathway, led the authors to propose the occurrence of a very rapid form of endocytosis, independent of clathrin, dynamin, and endophilin—perhaps kiss-and-run events (Verstreken et al. 2002). Studies of the kiss-and-run events in endocrine cells, however, have demonstrated that they are not simply the reversal of fusion and have implicated the GTPase dynamin, an endophilin-interacting protein, in fusion pore closure (Graham et al. 2002, Holroyd et al. 2002). Thus, the occurrence of kiss-and-run, and the mechanisms underlying it, remain open questions. If kiss-and-run exists, it will be important to determine the factors that prevent collapse of the vesicle into the plasma membrane. Lack of collapse could be explained either by the presence of proteins that promote and maintain membrane curvature on the vesicle membrane or by a molecular complex that prevents fusion pore dilation, for example, a collar of dynamin and its interacting proteins. A problem posed by SNARE-mediated fusion is the need to dissociate SNAREs prior to endocytosis in order to avoid excessive intermixing of v- and t-SNAREs. Yet, the speed invoked for kiss-and-run events of synaptic vesicles (msecs) seems incompatible with NSF-mediated SNARE complex disassembly.

Clathrin-Mediated Endocytosis

Clathrin-mediated endocytosis at the synapse is a specialized form of the house-keeping process that plays a general role in the internalization of receptors and their ligands in all cells (Kirchhausen 1993). Clathrin coats and their accessory factors function as molecular sorters that select proteins to be internalized and provide the driving force for membrane invagination (Schmid 1997). Their structure and putative functions have been summarized in many recent reviews (Marsh & McMahon 1999, Kirchhausen 2000, Pearse et al. 2000). Clathrin coats have two protein layers: an outer clathrin layer and an inner layer composed of clathrin adaptors. These adaptors are a set of proteins that function as molecular linkers between the membrane and the clathrin layer and that also have clathrin assembly properties. At synapses, the main adaptors are the heterotetrameric complex AP-2 and AP180 (Zhang et al. 1998, Kirchhausen 1999, Morgan et al. 1999, Collins et al. 2002). Other proteins, such as epsin (De Camilli et al. 2002, Ford et al. 2002), HIP/HIPR (Engqvist-Goldstein et al. 2001), and amphiphysin (Slepnev et al. 2000) have the expected properties of clathrin adaptors. They are, however, not enriched in purified clathrin-coated vesicle fractions, which suggests that they are bound to vesicles less tightly than other adaptors or that they participate in early stages of clathrin-mediated endocytosis and are then shed as clathrin-coated vesicles mature (Slepnev & De Camilli 2000). Major steps in clathrin coat dynamics are summarized below.

NUCLEATION OF THE COAT The bulk of clathrin-mediated endocytosis occurs constitutively in nonneuronal cells, and constitutive clathrin-mediated membrane recycling may also occur at a low rate in nerve terminals. Superimposed on this house-keeping recycling, however, is a compensatory form of clathrin-mediated endocytosis that mediates recapture of synaptic vesicle membrane after exocytosis. Clathrin-coated profiles are seldom observed in resting nerve terminals but are prominent following stimulation, revealing a large reservoir of clathrin coat proteins (Heuser & Reese 1973). In a study of the giant lamprey axon where clathrin-mediated endocytosis was inhibited by removal of Ca^{2+} after an exocytosis-dependent depletion of synaptic vesicles, the reintroduction of Ca^{2+} led to a massive transient wave of clathrin-mediated endocytosis (Gad et al. 1998). This finding demonstrates a tight correlation between coat nucleation and presence in the plasma membrane of vesicle proteins to be internalized.

If synaptic vesicle exocytosis is the trigger for clathrin coat assembly, the vesicle membrane must contain docking sites for clathrin adaptors. Accordingly, synaptotagmin and tyrosine-based endocytic motif of synaptic vesicle proteins were shown to bind cooperatively the clathrin adaptor AP-2 (Li et al. 1995, Haucke & De Camilli 1999). Yet, binding does not occur prior to exocytosis, indicating that exocytosis represents a critical switch in triggering clathrin coats. Clathrin adaptors and many clathrin accessory factors share a key property: an interaction with acidic phospholipids in general, and specifically with PI(4,5)P2 (Gaidarov & Keen 1999; Itoh et al. 2001; Ford et al. 2001, 2002; Collins et al. 2002), a phosphoinositide

selectively concentrated in the plasma membrane. In vitro studies and crystallographic analysis indicate that the interaction of AP-2 with PI(4,5)P2 is synergistic with the interaction with transmembrane proteins (Haucke & De Camilli 1999, Collins et al. 2002). Thus, the efficient recruitment of clathrin may require the cooperation of protein docking sites in the vesicle membrane with PI(4,5)P2—a conjunction that will occur only after exocytosis. The cytoplasmic tail of synaptotagmin also binds PI(4,5)P2, which raises the possibility that the incorporation of synaptotagmin in the plasma membrane may expose a cryptic binding site for the adaptors, thus contributing to the switch (Chapman 2002). Finally, membrane tension has been shown to regulate endocytosis in some nonneuronal cells (Raucher & Sheetz 1999). Whether a relaxation of plasma membrane tension following exocytosis plays a role in compensatory endocytosis at the synapse remains an open question.

Changes in protein phosphorylation may help in the coating reaction. The activity of an AP-2-associated serine-threonine kinase (Conner & Schmid 2002) that phosphorylates μ adaptin and possibly other accessory factors is thought to help in coat assembly. Conversely, the reciprocal interaction of several clathrin accessory factors is enhanced by their Ca^{2+} /calmodulin- and calcineurin-dependent dephosphorylation, which is triggered by nerve terminal depolarization when a burst of endocytic activity is needed (Cousin & Robinson 2001, Slepnev et al. 1998). There is evidence for the regulation of endocytosis by Ca^{2+} (Artalejo et al. 1996, Gad et al. 1998). Calmodulin and calcineurin are likely to be only two of several mediators of this regulation.

Studies of other coat-mediated budding reactions have demonstrated a critical role of small GTPases, such as Sar1 and Arf1, as switches that trigger coat assembly (Schekman 2002). Their effects are mediated by GTP-dependent interactions with coat proteins and, in the case of Arf, by the independent recruitment and stimulation of lipid metabolizing enzymes (Cremona & De Camilli 2001). The potent stimulatory effect of $\text{GTP}\gamma\text{S}$ on coat recruitment suggests an important role of GTPases also in endocytic clathrin coat assembly. However, no direct interactions of small GTPases with endocytic coat proteins have been identified so far. The effect of GTPases on endocytic coat recruitment may be indirect and mediated, at least to some extent, by a stimulation of PI(4,5)P2 synthesis by Arf and Rho family GTPases (Chong et al. 1994, Brown et al. 2001).

As discussed below, new synaptic vesicles may mature directly from the uncoating of clathrin-coated vesicles. Thus, clathrin-mediated budding in nerve terminals must ensure the presence on the vesicle of all the critical intrinsic membrane components of synaptic vesicles. Synaptic vesicle proteins may remain partially associated after exocytosis either via the occurrence of large multiprotein complexes or via the clustering in specific lipid microdomains. Coat proteins and their accessory factors, however, are likely to play a major role in selecting all the appropriate cargo proteins in the nascent vesicle bud.

INVAGINATION OF THE PIT It has been proposed that curved clathrin coats arise from the restructuring of hexagonal flat lattices into icosahedral structures (Heuser

1989). This change implies a very large number of intermolecule arrangements of clathrin triskelia but is supported by GFP-clathrin photobleaching experiments, indicating that clathrin triskelia do exchange with assembled lattices (Wu et al. 2001). It remains unknown, however, whether the curvature of the clathrin lattice drives, or is the passive result, of the formation of a bud. Many recent studies have suggested that the driving force for membrane invagination may actually be provided by clathrin adaptors and by the clathrin accessory factors that have the intrinsic ability to generate membrane curvature (Farsad et al. 2001, Ford et al. 2002). The rapidly expanding list of these factors, whose lipid deforming properties are supported by *in vitro* and *in vivo* studies, include dynamin, amphiphysin, endophilin, and epsin (Hinshaw & Schmid 1995, Takei et al. 1999, Farsad et al. 2001, Ford et al. 2002). These proteins act by binding and partially penetrating the lipid bilayer. For epsin 1, the interaction of its ENTH domain with the head of PI(4,5)P₂ results in the formation of an amphipathic helix, which may account for bilayer penetration (Ford et al. 2002). In the case of endophilin, its role in curvature acquisition by nascent clathrin-coated pits has been strongly suggested by ultrastructural observations of mutant *Drosophila* synapses and lamprey giant axons injected with anti-endophilin antibodies (Ringstad et al. 1999, Guichet et al. 2002).

Synaptic clathrin adaptors and clathrin accessory factors not only promote clathrin recruitment to the membrane and invagination but also help to produce homogeneously sized vesicles, possibly by inducing the highest possible degree of membrane curvature (Zhang et al. 1998, Morgan et al. 1999, Takei et al. 1999, Ford et al. 2002). There is evidence that these factors act at different stages of clathrin coat invagination (Brodin et al. 2000). Thus, it will be of interest to elucidate their relative contribution to the opposite curvatures of the bud dome and of its neck.

FISSION The fission of a deeply invaginated endocytic bud to generate a free vesicle is a step mechanistically and kinetically distinct from invagination. Several experimental manipulations produce an arrest or delay of synaptic vesicle endocytosis at the stage of deeply invaginated bud. The first known example of a block of endocytosis at this stage was the block observed in *shibire* mutants of *Drosophila* (Koenig & Ikeda 1983), which harbor mutations in the *dynamin* gene. Subsequent studies have shown a block at a similar stage by other experimental manipulations known to affect the function of this GTPase, such as GTP γ S, or dynamin sequestration by the SH3 domains (Hinshaw & Schmid 1995, Koenig & Ikeda 1983, Takei et al. 1995). These observations have conclusively linked dynamin to fission, although its precise mechanism of action remains unclear.

Dynamin alone, or together with some of its binding partners, can oligomerize into rings *in vitro* (Hinshaw & Schmid 1995, Takei et al. 1999, Schweitzer & Hinshaw 1998). Dynamin-containing rings are observed at the neck of deeply invaginated pits following a block of dynamin function (Takei et al. 1995). In addition, dynamin rings can undergo constriction and produce a cleavage of the underlying tubular membrane upon GTP hydrolysis *in vitro*. These observations point to a role for dynamin as a GTP hydrolysis-dependent "pinchase" (Schweitzer & Hinshaw 1998, Marks et al. 2001). Other studies, however, have suggested that

GTP hydrolysis may not be required for the fission reaction and that, like other GTPases, dynamin may function as a switch that recruits downstream effectors in a GTP-dependent fashion (Sever et al. 2000). So far, no protein that binds dynamin selectively in its GTP-bound state has been identified (other than dynamin itself). However, many studies have demonstrated a very close link between dynamin and actin, raising new questions both on the mechanism of action of dynamin and on the role of actin in endocytosis. For example, nearly all interactors of the COOH-terminal region of dynamin are proteins that directly or indirectly participate in regulating the actin cytoskeleton; dynamin isoforms are present at sites of actin dynamics, and dynamin mutants were shown to affect some parameters of actin dynamics (Qualmann & Kelly 2000, Slepnev & De Camilli 2000). Observation of clathrin-coated pit dynamics using total internal reflection microscopy has indicated that, during fission, dynamin recruitment to coated pits is followed rapidly by recruitment of actin (Merrifield et al. 2002). Finally, perturbation of actin function at synapses disrupts the endocytic reaction with the accumulation of coated pits with wide neck (Shupliakov et al. 2002). It remains to be seen whether these effects are indirect or whether they reflect a direct role of actin in invagination and fission.

UNCOATING Fission of clathrin-coated vesicles is rapidly followed by clathrin uncoating. Key players in this step are molecular chaperone Hsc70 and its cofactor auxilin, a DNAJ protein (Schmid 1997). These proteins bind directly to clathrin and disrupt triskelia interactions. As for the dissociation of clathrin adaptors, hydrolysis of PI(4,5)P₂ by the polyphosphoinositide phosphatase synaptojanin may play a role. This hypothesis is supported by the synaptic vesicle exocytosis-dependent accumulation of clathrin-coated vesicles in nerve terminals following disruption of synaptojanin function (Cremona et al. 1999). It remains unclear why blocking synaptojanin function also affects clathrin dissociation. Possibly, persistence of adaptors on the membrane prevents a catastrophic loss of the clathrin coat upon Hsc70 action. Clathrin uncoating is probably timed to occur after vesicle fission and occurs very rapidly after endocytosis. Perhaps a hole or “scar” left where membrane fission occurred represents a site where uncoating begins, explaining the close coupling between fission and uncoating.

FATE OF UNCOATED VESICLES AND BULK ENDOCYTOSIS

In the classical model of clathrin-mediated endocytosis, clathrin-coated vesicles fuse with early endosome after uncoating and authentic synaptic vesicles bud from endosomes. The large expansion of an endosome-like compartment visible in nerve terminals after strong stimulation was thought to reflect this pathway (Heuser & Reese 1973). At least some of the endosome-like structures are now thought to result from deep invagination of the plasma membrane (Gad et al. 1998, Takei et al. 1996), which is consistent with the occurrence in all cells of “bulk” forms of endocytosis, including Ca²⁺-triggered rapid endocytosis (Artalejo et al. 1995). Nonspecific membrane internalization by bulk endocytosis may prevent excess

expansion of the cell surface under conditions in which clathrin-mediated endocytosis becomes rate limiting. Clathrin-coated buds are present not only at the plasma membrane but also on these endosome-like structures, which suggests a parallel pathway for the formation of clathrin-coated synaptic vesicle precursors (Takei et al. 1996). Styryl dye uptake and release experiments are consistent with this possibility (Murthy & Stevens 1998). Under moderate conditions of stimulation a same unit of dye is taken up by endocytosis and then released by exocytosis, which argues against a sorting station where the dye would unavoidably be diluted (Murthy & Stevens 1998). The potential role of bona fide endosomes in synaptic vesicle recycling deserves further investigation.

POSTENDOCYTOTIC TRANSPORT OF VESICLES AND RECAPTURE IN THE VESICLE CLUSTER

Newly formed vesicles are immediately acidified and translocated away from the cell surface, probably through restricted or assisted movement rather than by free diffusion. A region of polymerized actin that surrounds the vesicle cluster and overlaps with the endocytic periaxial zone is clearly visible at large synapses (Dunaevsky & Connor 2000, Shupliakov et al. 2002). Further, as discussed above, there is evidence for a role of actin in vesicle fission, raising the possibility that actin may also propel vesicles away from the cell surface (Merrifield et al. 2002, Shupliakov et al. 2002). Such a mechanism may involve actin tails, myosin-based transport along actin cables, or the trapping of the vesicles in a treadmill meshwork of actin that assembles at the plasma membrane. Some striking electron microscopic images obtained at lamprey reticular spinal synapses suggest that actin cables around the synapse represent tracks for targeting endocytic vesicles back to the vesicle cluster (Shupliakov et al. 2002). Accordingly, actin disruption drastically affects recycling at these synapses (Shupliakov et al. 2002). At hippocampal synapses, however, the disruption of actin does not seem to have a major effect on recycling (Li & Murthy 2001, Morales et al. 2000, Sankaranarayanan et al. 2003). These synapses may be less dependent upon actin for recycling owing to their different structure.

Recycled vesicles are eventually recaptured by the vesicle cluster through an unknown mechanism. Vesicles must reassociate with a variety of cytosolic factors that are shed upon exocytosis but are needed in preparation for it. These include synapsin, rab3, and rabphilin. It is likely that changes of both lipids and proteins at the surface of vesicles participate in the "maturation" process leading to reclustering. It is also not known at which stage newly reformed synaptic vesicles are loaded with neurotransmitters. Transporters present in the vesicle membrane use the electrical and chemical potential generated by the proton pump to concentrate nonpeptide neurotransmitters inside vesicles (Reiman et al. 1998). A regulation of the pump and of neurotransmitter transporters could contribute to control strength of a synapse by determining the quantal size.

A surprising observation, based on the uptake of styryl dyes, is that even during sustained stimulation only a fraction of the vesicles in the cluster participate in

recycling (Murthy & Stevens 1999, Harata et al. 2001). A slow exchange between the normally recycling vesicles and normally resting vesicles may induce a complete mixing over longer timescales. At the frog neuromuscular junction, there is evidence that vesicles retrieved slowly after tetanic stimulation reach a deeper pool of vesicles from which release occurs more reluctantly (Richards et al. 2000). It is unclear whether this functional difference within the synaptic vesicle pool reflects an intrinsic heterogeneity of the vesicles or heterogeneity in the matrix that clusters them. Staurosporine affects the degree of vesicle mixing, which supports a role for phosphorylation in this regulation.

VECTORIALITY IN THE VESICLE CYCLE

An important question in synaptic vesicle recycling is what mediates the vectorial progression of the vesicle membrane through the different stations of the cycle. While some steps seem to be reversible (for example, docking or even an early stage of SNARE complex zippering), other steps are clearly unidirectional. Some first understanding of the molecular switches that underlie this vectoriality has emerged both from studies of synapses as well as from more general studies of intracellular membrane vesicle traffic. Vectoriality appears to be controlled at least in part by the rab proteins, which belong to a family of small GTPases. Each member associates with a specific set of intracellular membranes and interacts in a GTP-dependent fashion with multiple effectors (Zerial & McBride 2001). The effectors of a given rab act cooperatively to mediate a specific transport reaction (Zerial & McBride 2001). In some cases, effectors include proteins that trigger the recruitment and activation of the rab involved in the next step of transport (Ortiz et al. 2002). Rab3 may contribute to programming synaptic vesicle for exocytosis via its interaction with rabphilin and RIM. Conversely, rab5, which is also found on synaptic vesicle membranes (Fischer von Mollard et al. 1994) and is a regulator of the early endocytic pathway, may be involved in programming vesicles for endocytosis. A sequential link between the two processes may be mediated by the interaction of rabphilin with rabaptin 5 (Ohya et al. 1998). Rabphilin functions both as an effector and as an activator of Rab5 and is negatively regulated by Rab3 (Ohya et al. 1998, Coppola et al. 2001). It is surprising, however, that the phenotype of rab3 and rabphilin knockout mice is relatively mild, but their function may be partially redundant with that of other Rab proteins on synaptic vesicles (Geppert et al. 1994a, Schluter et al. 1999). As discussed earlier, a potential mediator of rab3 association with membrane is Pra1, which also interacts with piccolo and synaptobrevin/VAMP (Fenster et al. 2000). Loading of rab3 with GTP is mediated by rab3 GEFs, GRAB/Rabin 3, and Rab3-GEF/Aex-3 (Wada et al. 1997, Iwasaki et al. 2000, Saiardi et al. 2002,). It will be of interest to identify the upstream regulators of these proteins.

In addition to synaptic proteins, membrane lipids, in particular phosphoinositides, may also control vectoriality. As discussed above, the selective localization (or concentration) of PI(4,5)P₂ in the plasma membrane may explain why clathrin coating occurs only after exocytosis, whereas a synaptojanin-mediated cleavage

of PI(4,5)P₂ may act in uncoating. Synaptojanin can degrade PI(4,5)P₂ to phosphatidylinositol. A PI4-kinase activity has been described on synaptic vesicles (Wiedemann et al. 1998, Guo et al. 2003). Generation PI(4)P on the uncoated vesicle could be one of the steps required for its maturation into a new synaptic vesicle. If so, it will be important to determine the identity of the PI4-kinase, its regulation, and potential PI(4)P-interacting proteins. As also discussed above, PI(4,5)P₂ generation is partially under the control of Arf and Rho family GTPases (Chong et al. 1994, Brown et al. 2001), and the activation of these GTPases is regulated by phosphoinositides via PH domain containing GTP/GDP exchange factors (Jackson & Casanova 2000). This complex regulatory cascade involving lipids and proteins requires further study.

CONCLUDING REMARKS

The basic mechanisms in vesicle mobilization, docking, priming, exocytosis, and endocytosis probably involve a minimum set of core proteins, but they are then regulated by a complex array of accessory elements, including kinases and phosphatases. As we begin to better understand the different steps in the vesicle cycle, investigation into short-term and long-term regulation of these steps will increasingly move to center stage. Synaptic modification is thought to underlie many forms of learning and experience-dependent plasticity. Although much of current work on synaptic plasticity is focused on changes in postsynaptic function (Sheng & Kim 2002), it is likely that regulation of presynaptic structure and function contributes to the adaptation of neural networks. Conversely, there is now evidence for vesicle recycling in the postsynaptic side of the synapse, where vesicle trafficking participates in receptor regulation and therefore in synaptic plasticity. Perhaps this serves to remind us that the synapse is a bicellular unit, and its structural and functional polarity may have evolved from the fundamentally symmetric characteristics of most intercellular junctions.

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LITERATURE CITED

- Aalto MK, Ronne H, Keranen S. 1993. Yeast syntaxins Sso1p and Sso2p belong to a family of related membrane proteins that function in vesicular transport. *EMBO J.* 12:4095–104
- Abeliovich A, Schmitz Y, Farinas I, Choi Lundberg D, Ho WH, et al. 2000. Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron* 25:239–52
- Ahmari SE, Buchanan J, Smith SJ. 2000. Assembly of presynaptic active zones from

- cytoplasmic transport packets. *Nat. Neurosci.* 3:445–51
- Albillos A, Dernick G, Horstmann H, Almers W, Alvarez de Toledo G, Lindau M. 1997. The exocytotic event in chromaffin cells revealed by patch amperometry. *Nature* 389:509–12
- Artalejo CR, Elhamdani A, Palfrey HC. 1996. Calmodulin is the divalent cation receptor for rapid endocytosis, but not exocytosis, in adrenal chromaffin cells. *Neuron* 16:195–205
- Artalejo CR, Henley JR, McNiven MA, Palfrey HC. 1995. Rapid endocytosis coupled to exocytosis in adrenal chromaffin cells involves Ca^{2+} , GTP, and dynamin but not clathrin. *Proc. Natl. Acad. Sci. USA* 92:8328–32
- Augustin I, Rosenmund C, Südhof TC, Brose N. 1999. Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles. *Nature* 400:457–61
- Banerjee A, Kowalchuk JA, DasGupta BR, Martin TFJ. 1996. SNAP-25 is required for a late postdocking step in Ca^{2+} -dependent exocytosis. *J. Biol. Chem.* 271:20,227–30
- Becherer U, Guatimosim C, Betz WJ. 2001. Effects of staurosporine on exocytosis and endocytosis at frog motor nerve terminals. *J. Neurosci.* 21:782–87
- Betz A, Thakur P, Junge HJ, Ashery U, Rhee JS, et al. 2001. Functional interaction of the active zone proteins Munc13-1 and RIM1 in synaptic vesicle priming. *Neuron* 30:183–96
- Betz WJ, Henkel AW. 1994. Okadaic acid disrupts clusters of synaptic vesicles in frog motor nerve terminals. *J. Cell Biol.* 124:843–54
- Biederer T, Sara Y, Mozhayeva M, Atasoy D, Liu X, et al. 2002. SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science* 297:1525–31
- Blasi J, Chapman ER, Link E, Binz T, Yamasaki S, et al. 1993. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature* 365:160–63
- Breckenridge LJ, Almers W. 1987. Currents through the fusion pore that forms during exocytosis of a secretory vesicle. *Nature* 328:814–17
- Broadie K, Richmond J. 2002. Establishing and sculpting the synapse in *Drosophila* and *C. elegans*. *Curr. Opin. Neurobiol.* 12:491–98
- Brodin L, Low P, Shupliakov O. 2000. Sequential steps in clathrin-mediated synaptic vesicle endocytosis. *Curr. Opin. Neurobiol.* 10:312–20
- Brown FD, Rozelle AL, Yin HL, Balla T, Donaldson JG. 2001. Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J. Cell Biol.* 154:1007–17
- Butz S, Okamoto M, Südhof TC. 1998. A tripartite protein complex with the potential to couple synaptic vesicle exocytosis to cell adhesion in brain. *Cell* 94:773–82
- Cabin DE, Shimazu K, Murphy D, Cole NB, Gottschalk W, et al. 2002. Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein. *J. Neurosci.* 22:8797–807
- Carr CM, Grote E, Munson M, Hughson FM, Novick PJ. 1999. Sec1p binds to SNARE complexes and concentrates at sites of secretion. *J. Cell Biol.* 146:333–44
- Cases-Langhoff C, Voss B, Garner AM, Appeltauer U, Takei K, et al. 1996. Piccolo, a novel 420 kDa protein associated with the presynaptic cytomatrix. *Eur. J. Cell Biol.* 69:214–23
- Catterall WA. 1999. Interactions of presynaptic Ca^{2+} channels and snare proteins in neurotransmitter release. *Ann. NY Acad. Sci.* 868:144–59
- Ceccarelli B, Hurlbut WP, Mauro A. 1973. Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. *J. Cell Biol.* 57:499–524
- Chang Q, Balice-Gordon RJ. 2000. Highwire, rpm-1, and futsch: balancing synaptic growth and stability. *Neuron* 26:287–90
- Chapman ER. 2002. Synaptotagmin: a Ca^{2+} sensor that triggers exocytosis? *Nat. Rev. Mol. Cell Biol.* 3:498–508
- Chavis P, Westbrook G. 2001. Integrins mediate functional pre- and postsynaptic maturation at a hippocampal synapse. *Nature* 411:317–21

- Chi P, Greengard P, Ryan TA. 2001. Synapsin dispersion and reclustering during synaptic activity. *Nat. Neurosci.* 4:1187–93
- Chong LD, Traynor-Kaplan A, Bokoch GM, Schwartz MA. 1994. The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell* 79:507–13
- Clandinin TR, Zipursky SL. 2002. Making connections in the fly visual system. *Neuron* 35: 827–41
- Colicos MA, Collins BE, Sailor MJ, Goda Y. 2001. Remodeling of synaptic actin induced by photoconductive stimulation. *Cell* 107: 605–16
- Collins BM, McCoy AJ, Kent HM, Evans PR, Owen DJ. 2002. Molecular architecture and functional model of the endocytic AP2 complex. *Cell* 109:523–35
- Conner SD, Schmid SL. 2002. Identification of an adaptor-associated kinase, AAK1, as a regulator of clathrin-mediated endocytosis. *J. Cell Biol.* 156:921–29
- Coppola T, Hirling H, Perret-Menoud V, Gattesco S, Catsicas S, et al. 2001. Rabphilin dissociated from Rab3 promotes endocytosis through interaction with Rabaptin-5. *J. Cell Sci.* 114:1757–64
- Cousin MA, Robinson PJ. 2001. The dephosphins: dephosphorylation by calcineurin triggers synaptic vesicle endocytosis. *Trends Neurosci.* 24:659–65
- Cremona O, De Camilli P. 1997. Synaptic vesicle endocytosis. *Curr. Opin. Neurobiol.* 7: 323–30
- Cremona O, De Camilli P. 2001. Phosphoinositides in membrane traffic at the synapse. *J. Cell Sci.* 114:1041–52
- Cremona O, Di Paolo G, Wenk MR, Luthi A, Kim WT, et al. 1999. Essential role of phosphoinositide metabolism in synaptic vesicle recycling. *Cell* 99:179–88
- Crump JG, Zhen M, Jin Y, Bargmann CI. 2001. The SAD-1 kinase regulates presynaptic vesicle clustering and axon termination. *Neuron* 29:115–29
- Davis GW, Schuster CM, Goodman CS. 1997. Genetic analysis of the mechanisms controlling target selection: Target-derived Fasciclin II regulates the pattern of synapse formation. *Neuron* 19:561–73
- Dawson-Scully K, Bronk P, Atwood HL, Zinsmaier KE. 2000. Cysteine-string protein increases the calcium sensitivity of neurotransmitter exocytosis in *Drosophila*. *J. Neurosci.* 20:6039–47
- De Camilli P, Benfenati F, Valtorta F, Greengard P. 1990. The synapsins. *Annu. Rev. Cell Biol.* 6:433–60
- De Camilli P, Chen H, Hyman J, Panepucci E, Bateman A, Brunger AT. 2002. The ENTH domain. *FEBS Lett.* 513:11–18
- De Camilli P, Haucke V, Takei K, Mugnaini E. 2001. The structure of synapses. In *Synapses*, ed. WM Cowan, TC Sudhof, CF Stevens, pp. 89–133. Baltimore, MD: Johns Hopkins Univ. Press
- DiAntonio A, Haghighi AP, Portman SL, Lee JD, Amaranto AM, Goodman CS. 2001. Ubiquitination-dependent mechanisms regulate synaptic growth and function. *Nature* 412:449–52
- Di Paolo G, Pellegrini L, Letinic K, Cestra G, Zoncu R, et al. 2002. Recruitment and regulation of phosphatidylinositol phosphate kinase type Igamma by the FERM domain of talin. *Nature* 420:85–89
- Dresbach T, Qualmann B, Kessels MM, Garner CC, Gundelfinger ED. 2001. The presynaptic cytomatrix of brain synapses. *Cell Mol. Life Sci.* 58:94–116
- Dunaevsky A, Connor EA. 2000. F-actin is concentrated in nonrelease domains at frog neuromuscular junctions. *J. Neurosci.* 20:6007–12
- Dustin ML, Colman DR. 2002. Neural and immunological synaptic relations. *Science* 298: 785–89
- Eaton BA, Fetter RD, Davis GW. 2002. Dynactin is necessary for synapse stabilization. *Neuron* 34:729–41
- Engqvist-Goldstein AE, Warren RA, Kessels MM, Keen JH, Heuser J, Drubin DG. 2001. The actin-binding protein Hip1R associates with clathrin during early stages of

- endocytosis and promotes clathrin assembly in vitro. *J. Cell Biol.* 154:1209–23
- Farsad K, Ringstad N, Takei K, Floyd SR, Rose K, De Camilli P. 2001. Generation of high curvature membranes mediated by direct endophilin bilayer interactions. *J. Cell Biol.* 155:193–200
- Fenster SD, Chung WJ, Zhai R, Cases-Langhoff C, Voss B, et al. 2000. Piccolo, a presynaptic zinc finger protein structurally related to bassoon. *Neuron* 25:203–14
- Fernandez-Chacon R, Konigstorfer A, Gerber SH, Garcia J, Matos MF, et al. 2002. Synaptotagmin I functions as a calcium regulator of release probability. *Nature* 410:41–49
- Fernandez-Chacon R, Sudhof TC. 1999. Genetics of synaptic vesicle function: toward the complete functional anatomy of an organelle. *Annu. Rev. Physiol.* 61:753–76
- Fesce R, Grohovaz F, Valtorta F, Meldolesi J. 1994. Neurotransmitter release: fusion or 'kiss-and-run'? *Trends Cell Biol.* 4:1–4
- Fischer von Mollard G, Stahl B, Walch-Solimena C, Takei K, Daniels L, et al. 1994. Localization of Rab5 to synaptic vesicles identifies endosomal intermediate in synaptic vesicle recycling pathway. *Eur. J. Cell Biol.* 65:319–26
- Ford MG, Mills IG, Peter BJ, Vallis Y, Praefcke GJ, et al. 2002. Curvature of clathrin-coated pits driven by epsin. *Nature* 419:361–66
- Ford MG, Pearse BM, Higgins MK, Vallis Y, Owen DJ, et al. 2001. Simultaneous binding of PtdIns(4,5)P₂ and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science* 291:1051–55
- Frank M, Kemler R. 2002. Protocadherins. *Curr. Opin. Cell Biol.* 14:557–62
- Gad H, Low P, Zotova E, Brodin L, Shupliakov O. 1998. Dissociation between Ca²⁺-triggered synaptic vesicle exocytosis and clathrin-mediated endocytosis at a central synapse. *Neuron* 21:607–16
- Gaidarov I, Keen JH. 1999. Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits. *J. Cell Biol.* 146:755–64
- Garcia EP, McPherson PS, Chilcote TJ, Takei K, De Camilli P. 1995. rbSec1A and B colocalize with syntaxin 1 and SNAP-25 throughout the axon, but are not in a stable complex with syntaxin. *J. Cell Biol.* 129:105–20
- Geppert M, Bolshakov VY, Siegelbaum SA, Takei K, De Camilli P, Sudhof TC. 1994a. The role of rab3A in neurotransmitter release. *Nature* 369:493–97
- Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, et al. 1994b. Synaptotagmin I: a major Ca²⁺ sensor for transmitter release at a central synapse. *Cell* 79:717–27
- Geppert M, Goda Y, Stevens CF, Sudhof T. 1997. The small GTP-binding protein Rab3A regulates a late step in synaptic vesicle fusion. *Nature* 387:810–14
- Glass DJ, Yancopoulos GD. 1997. Sequential roles of agrin, MuSK and rapsyn during neuromuscular junction formation. *Curr. Opin. Neurobiol.* 7:379–84
- Gottardi CJ, Gumbiner BM. 2001. Adhesion signaling: how beta-catenin interacts with its partners. *Curr. Biol.* 11:R792–94
- Graham ME, O'Callaghan DW, McMahon HT, Burgoyne RD. 2002. Dynamin-dependent and dynamin-independent processes contribute to the regulation of single vesicle release kinetics and quantal size. *Proc. Natl. Acad. Sci. USA* 99:7124–29
- Greengard P, Valtorta F, Czernik AJ, Benfenati F. 1993. Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* 259:780–85
- Guichet A, Wucherpfennig T, Dudu V, Etter S, Wilsch-Brauniger M, et al. 2002. Essential role of endophilin A in synaptic vesicle budding at the Drosophila neuromuscular junction. *EMBO J.* 21:1661–72
- Guo J, Wenk MR, Pellegrini L, Onofri F, Benfenati F, De Camilli P. 2003. Phosphatidylinositol 4-kinase type IIa is responsible for the phosphatidylinositol 4-kinase activity associated with synaptic vesicles. *Proc. Natl. Acad. Sci. USA* 100:3995–4000
- Hallam SJ, Goncharov A, McEwen J, Baran R, Jin Y. 2002. SYD-1, a presynaptic protein with PDZ, C2 and rhoGAP-like domains,

- specifies axon identity in *C. elegans*. *Nat. Neurosci.* 5:1137–46
- Hanson PI, Heuser JE, Jahn R. 1997. Neurotransmitter release—four years of SNARE complexes. *Curr. Opin. Neurobiol.* 7:310–15
- Harata N, Ryan TA, Smith SJ, Buchanan J, Tsien RW. 2001. Visualizing recycling synaptic vesicles in hippocampal neurons by FM 1-43 photoconversion. *Proc. Natl. Acad. Sci. USA* 98:12,748–53
- Harlow ML, Ress D, Stoschek A, Marshall RM, McMahan UJ. 2001. The architecture of active zone material at the frog's neuromuscular junction. *Nature* 409:479–84
- Haucke V, De Camilli P. 1999. AP-2 recruitment to synaptotagmin stimulated by tyrosine-based endocytic motifs. *Science* 285:1268–71
- Haydon PG. 2001. GLIA: listening and talking to the synapse. *Nat. Rev. Neurosci.* 2:185–93
- Heuser J. 1989. The role of coated vesicles in recycling of synaptic vesicle membrane. *Cell Biol. Int. Rep.* 13:1063–76
- Heuser JE, Reese TS. 1973. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. Cell Biol.* 57:315–44
- Hinshaw JE, Schmid SL. 1995. Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. *Nature* 374:190–92
- Hirokawa N, Sobue K, Kanda K, Harada A, Yorifuji H. 1989. The cytoskeletal architecture of the presynaptic terminal and molecular structure of synapsin I. *J. Cell Biol.* 108:111–26
- Holroyd P, Lang T, Wenzel D, De Camilli P, Jahn R. 2002. Imaging direct, dynamin-dependent recapture of fusing secretory granules on plasma membrane lawns from PC12 cells. *Proc. Natl. Acad. Sci. USA.* 99:16,806–11
- Holtzman E, Freeman AR, Kashner LA. 1971. Stimulation-dependent alterations in peroxidase uptake at lobster neuromuscular junctions. *Science* 173:733–36
- Hosaka M, Sudhof TC. 1998. Synapsins I and II are ATP-binding proteins with differential Ca²⁺ regulation. *J. Biol. Chem.* 273:1425–29
- Hsueh YP, Sheng M. 1999. Regulated expression and subcellular localization of syndecan heparan sulfate proteoglycans and the syndecan-binding protein CASK/LIN-2 during rat brain development. *J. Neurosci.* 19:7415–25
- Hunt JM, Bommert K, Charlton MP, Kistner A, Habermann E, et al. 1994. A post-docking role for synaptobrevin in synaptic vesicle fusion. *Neuron* 12:1269–79
- Itoh T, Koshiba S, Kigawa T, Kikuchi A, Yokoyama S, Takenawa T. 2001. Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. *Science* 291:1047–51
- Iwasaki K, Staunton J, Saifee O, Nonet M, Thomas JH. 1997. aex-3 encodes a novel regulator of presynaptic activity in *C. elegans*. *Neuron* 18:613–22
- Jackson CL, Casanova JE. 2000. Turning on ARF: the sec7 family of guanine-nucleotide-exchange factors. *Trends Cell Biol.* 10:60–67
- Jahn R, Lang T, Sudhof TC. 2003. Membrane fusion. *Cell* 112:519–33
- Jamora C, Fuchs E. 2002. Intercellular adhesion, signalling and the cytoskeleton. *Nat. Cell Biol.* 4:E101–8
- Kaufmann N, DeProto J, Ranjan R, Wan H, Van Vactor D. 2002. Drosophila liprin-alpha and the receptor phosphatase Dlar control synapse morphogenesis. *Neuron* 34:27–38
- Kirchhausen T. 1993. Coated pits and coated vesicles—sorting it all out. *Curr. Opin. Struct. Biol.* 3:182–88
- Kirchhausen T. 1999. Adaptors for clathrin-mediated traffic. *Annu. Rev. Cell Dev. Biol.* 15:705–32
- Kirchhausen T. 2000. Three ways to make a vesicle. *Nat. Rev. Mol. Cell Biol.* 1:187–98
- Klingauf J, Kavalali ET, Tsien RW. 1998. Kinetics and regulation of fast endocytosis at hippocampal synapses. *Nature* 394:581–85
- Koenig JH, Ikeda K. 1983. Evidence for a presynaptic blockage of transmission in a temperature-sensitive mutant of *Drosophila*. *J. Neurobiol.* 14:411–19

- Koushika SP, Richmond JE, Hadwiger G, Weimer RM, Jorgensen EM, Nonet ML. 2001. A post-docking role for active zone protein Rim. *Nat. Neurosci.* 4:997–1005
- Kraszewski K, Daniell L, Mundigl O, De Camilli P. 1996. Mobility of synaptic vesicles in nerve endings monitored by recovery from photobleaching of synaptic vesicle-associated fluorescence. *J. Neurosci.* 16:5905–13
- Kullander K, Klein R. 2002. Mechanisms and functions of Eph and ephrin signalling. *Nat. Rev. Mol. Cell Biol.* 3:475–86
- Landis DMD, Hall AK, Weinstein LA, Reese TS. 1988. The organization of cytoplasm at the presynaptic active zone of a central nervous system synapse. *Neuron* 1:201–9
- Lenzi D, von Gersdorff H. 2001. Structure suggests function: the case for synaptic ribbons as exocytotic nanomachines. *Bioessays* 23:831–40
- Li C, Ullrich B, Zhang JZ, Anderson RG, Brose N, Sudhof TC. 1995. Ca(2+)-dependent and -independent activities of neural and non-neural synaptotagmins. *Nature* 375:594–99
- Li Z, Murthy VN. 2001. Visualizing post-endocytic traffic of synaptic vesicles at hippocampal synapses. *Neuron* 31:593–605
- Lin RC, Scheller RH. 2000. Mechanisms of synaptic vesicle exocytosis. *Annu. Rev. Cell Dev. Biol.* 16:19–49
- Marks B, Stowell MH, Vallis Y, Mills IG, Gibson A, et al. 2001. GTPase activity of dynamin and resulting conformation change are essential for endocytosis. *Nature* 410:231–35
- Marsh M, McMahon HT. 1999. The structural era of endocytosis. *Science* 285:215–20
- Mayford M, Barzilai A, Keller F, Schacher S, Kandel ER. 1992. Modulation of an NCAM-related adhesion molecule with long-term synaptic plasticity in *Aplysia*. *Science* 256:638–44
- Merrifield CJ, Feldman ME, Wan L, Almers W. 2002. Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. *Nat. Cell Biol.* 4:691–98
- Missler M, Sudhof TC. 1998. Neurexins: three genes and 1001 products. *Trends Genet.* 14:20–26
- Misura KM, Scheller RH, Weis WI. 2000. Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. *Nature* 404:355–62
- Mizoguchi A, Nakanishi H, Kimura K, Matsubara K, Ozaki-Kuroda K, et al. 2002. Nectin: an adhesion molecule involved in formation of synapses. *J. Cell Biol.* 156:555–65
- Montecucco C. 1998. Protein toxins and membrane transport. *Curr. Opin. Cell Biol.* 10:530–36
- Morales M, Colicos MA, Goda Y. 2000. Actin-dependent regulation of neurotransmitter release at central synapses. *Neuron* 27:539–50
- Morgan JR, Zhao X, Womack M, Prasad K, Augustine GJ, Lafer EM. 1999. A role for the clathrin assembly domain of AP180 in synaptic vesicle endocytosis. *J. Neurosci.* 19:10,201–12
- Murphy DD, Rueter SM, Trojanowski JQ, Lee VM. 2000. Synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons. *J. Neurosci.* 20:3214–20
- Murthy VN, Schikorski T, Stevens CF, Zhu Y. 2001. Inactivity produces increases in neurotransmitter release and synapse size. *Neuron* 32:673–82
- Murthy VN, Stevens CF. 1998. Synaptic vesicles retain their identity through the endocytic cycle. *Nature* 392:497–501
- Murthy VN, Stevens CF. 1999. Reversal of synaptic vesicle docking at central synapses. *Nat. Neurosci.* 2:503–7
- Nakamura N, Rabouille C, Watson R, Nilsson T, Hui N, et al. 1995. Characterization of a cis-Golgi matrix protein, GM130. *J. Cell Biol.* 131:1715–26
- Neale EA, Bowers LM, Jia M, Bateman KE, Williamson LC. 1999. Botulinum neurotoxin A blocks synaptic vesicle exocytosis but not endocytosis at the nerve terminal. *J. Cell Biol.* 147:1249–60
- Nonet ML, Staunton JE, Kilgard MP, Fergestad T, Hartwig E, et al. 1997.

- Caenorhabditis elegans* rab-3 mutant synapses exhibit impaired function and are partially depleted of vesicles. *J. Neurosci.* 17: 8061–73
- Ohtsuka T, Takao-Rikitsu E, Inoue E, Inoue M, Takeuchi M, et al. 2002. Cast: a novel protein of the cytomatrix at the active zone of synapses that forms a ternary complex with RIM1 and munc13-1. *J. Cell Biol.* 158:577–90
- Ohya T, Sasaki T, Kato M, Takai Y. 1998. Involvement of Rabphilin3 in endocytosis through interaction with Rabaptin5. *J. Biol. Chem.* 273:613–17
- Ortiz D, Medkova M, Walch-Solimena C, Novick P. 2002. Ypt32 recruits the Sec4p guanine nucleotide exchange factor, Sec2p, to secretory vesicles; evidence for a Rab cascade in yeast. *J. Cell Biol.* 157:1005–15
- Pearse BM, Smith CJ, Owen DJ. 2000. Clathrin coat construction in endocytosis. *Curr. Opin. Struct. Biol.* 10:220–28
- Pennetta G, Hiesinger P, Fabian-Fine R, Meindertshagen I, Bellen H. 2002. Drosophila VAP-33A directs bouton formation at neuromuscular junctions in a dosage-dependent manner. *Neuron* 35:291–306
- Pfenninger K, Sandri C, Akert K, Eugster CH. 1969. Contribution to the problem of structural organization of the presynaptic area. *Brain Res.* 12:10–18
- Phillips GR, Huang JK, Wang Y, Tanaka H, Shapiro L, et al. 2001. The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution. *Neuron* 32:63–77
- Pieribone VA, Shupliakov O, Brodin L, Hilfiker-Rothenfluh S, Czernik AJ, Greengard P. 1995. Distinct pools of synaptic vesicles in neurotransmitter release. *Nature* 375: 493–97
- Polo-Parada L, Bose CM, Landmesser LT. 2001. Alterations in transmission, vesicle dynamics, and transmitter release machinery at NCAM-deficient neuromuscular junctions. *Neuron* 32:815–28
- Pyle JL, Kavalali ET, Piedras-Renteria ES, Tsien RW. 2000. Rapid reuse of readily releasable pool vesicles at hippocampal synapses. *Neuron* 28:221–31
- Qualmann B, Kelly RB. 2000. Syndapin isoforms participate in receptor-mediated endocytosis and actin organization. *J. Cell Biol.* 148:1047–62
- Raucher D, Sheetz MP. 1999. Membrane expansion increases endocytosis rate during mitosis. *J. Cell Biol.* 144:497–506
- Reim K, Mansour M, Varoqueaux F, McMahon HT, Sudhof TC, et al. 2001. Complexins regulate a late step in Ca²⁺-dependent neurotransmitter release. *Cell* 104:71–81
- Reiman RJ, Fon EA, Edwards RH. 1998. Vesicular neurotransmitter transport and the presynaptic regulation of quantal size. *Curr. Opin. Neurobiol.* 8:405–12
- Rhee JS, Betz A, Pyott S, Reim K, Varoqueaux F, et al. 2002. Beta phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKCs. *Cell* 108:121–33
- Richards DA, Guatimosim C, Betz WJ. 2000. Two endocytic recycling routes fill two vesicle pools in frog motor nerve terminals. *Neuron* 27:551–59
- Richmond JE, Davis WS, Jorgensen EM. 1999. UNC-13 is required for synaptic vesicle fusion in *C. elegans*. *Nat. Neurosci.* 2:959–64
- Richmond JE, Weimer RM, Jorgensen EM. 2001. An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. *Nature* 412:338–41
- Rikhy R, Kumar V, Mittal R, Krishnan KS. 2002. Endophilin is critically required for synapse formation and function in *Drosophila melanogaster*. *J. Neurosci.* 22:7478–84
- Ringstad N, Gad H, Low P, Di Paolo G, Brodin L, et al. 1999. Endophilin/SH3p4 is required for the transition from early to late stages in clathrin-mediated synaptic vesicle endocytosis. *Neuron* 24:143–54
- Rizo J, Sudhof TC. 1998. C2-domains, structure and function of a universal Ca²⁺-binding domain. *J. Biol. Chem.* 273:15,879–82
- Rizo J, Sudhof TC. 2002. Snares and Munc18 in synaptic vesicle fusion. *Nat. Rev. Neurosci.* 3:641–53

- Robitaille R, Garcia ML, Kaczorowski GJ, Charlton MP. 1993. Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release. *Neuron* 11:645–55
- Roche JP, Packard MC, Moeckel-Cole S, Budnik V. 2002. Regulation of synaptic plasticity and synaptic vesicle dynamics by the PDZ protein Scribble. *J. Neurosci.* 22:6471–79
- Roos J, Hummel T, Ng N, Klambt C, Davis GW. 2000. Drosophila Futsch regulates synaptic microtubule organization and is necessary for synaptic growth. *Neuron* 26:371–82
- Roos J, Kelly RB. 1999. The endocytic machinery in nerve terminals surrounds sites of exocytosis. *Curr. Biol.* 9:1411–14
- Rosahl TW, Spillane D, Missler M, Herz J, Selig DK, et al. 1995. Essential functions of synapsins I and II in synaptic vesicle regulation. *Nature* 375:488–93
- Rothman JE. 2002. The machinery and principles of vesicle transport in the cell. *Nat. Med.* 8:1059–62
- Ryan TA, Smith SJ. 1995. Vesicle pool mobilization during action potential firing at hippocampal synapses. *Neuron* 14:983–89
- Saiardi A, Sciambi C, McCaffery JM, Wendland B, Snyder SH. 2002. Inositol pyrophosphates regulate endocytic trafficking. *Proc. Natl. Acad. Sci. USA* 99:14,206–11
- Sanes JR, Lichtman JW. 2001. Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat. Rev. Neurosci.* 2:791–805
- Sanes JR, Yamagata M. 1999. Formation of lamina-specific synaptic connections. *Curr. Opin. Neurobiol.* 9:79–87
- Sankaranarayanan S, Atluri PP, Ryan TA. 2003. Actin has a molecular scaffolding, not propulsive, role in presynaptic function. *Nat. Neurosci.* 6:127–35
- Scheiffele P, Fan J, Choij J, Fetter R, Serafini T. 2000. Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 101:657–69
- Schekman R. 2002. SEC mutants and the secretory apparatus. *Nat. Med.* 8:1055–58
- Schiavo G, Benfenati F, Poulain B, Rossetto O, Polverino de Laureto P, et al. 1992. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature* 359:832–35
- Schikorski T, Stevens CF. 2001. Morphological correlates of functionally defined synaptic vesicle populations. *Nat. Neurosci.* 4:391–95
- Schluter OM, Schnell E, Verhage M, Tzonooulos T, Nicoll RA, et al. 1999. Rabphilin knock-out mice reveal that rabphilin is not required for rab3 function in regulating neurotransmitter release. *J. Neurosci.* 19:5834–46
- Schmid SL. 1997. Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.* 66:511–48
- Schmucker D, Clemens JC, Shu H, Worby CA, Xiao J, et al. 2000. Drosophila Dscam is an axon guidance receptor exhibiting extraordinary molecular diversity. *Cell* 101:671–84
- Schoch S, Castillo PE, Jo T, Mukherjee K, Gepfert M, et al. 2002. RIM1alpha forms a protein scaffold for regulating neurotransmitter release at the active zone. *Nature* 415:321–26
- Schweitzer SM, Hinshaw JE. 1998. Dynamin undergoes a GTP-dependent conformational change causing vesiculation. *Cell* 93:1021–29
- Sever S, Damke H, Schmid SL. 2000. Garrotes, springs, ratchets, and whips: putting dynamin models to the test. *Traffic* 1:385–92
- Shapiro L, Colman DR. 1999. The diversity of cadherins and implications for a synaptic adhesive code in the CNS. *Neuron* 23:427–30
- Sheng M, Kim MJ. 2002. Postsynaptic signaling and plasticity mechanisms. *Science* 298:776–80
- Sheng M, Sala C. 2001. PDZ domains and the organization of supramolecular complexes. *Annu. Rev. Neurosci.* 24:1–29
- Shupliakov O, Bloom O, Gustafsson JS, Kjaerulff O, Low P, et al. 2002. Impaired recycling of synaptic vesicles after acute perturbation of the presynaptic actin cytoskeleton. *Proc. Natl. Acad. Sci. USA* 99:14,476–81
- Slepnev VI, De Camilli P. 2000. Accessory

- factors in clathrin-dependent synaptic vesicle endocytosis. *Nat. Rev. Neurosci.* 1:161–72
- Slepnev VI, Ochoa GC, Butler MH, De Camilli P. 2000. Tandem arrangement of the clathrin and AP-2 binding domains in amphiphysin 1 and disruption of clathrin coat function by amphiphysin fragments comprising these sites. *J. Biol. Chem.* 275:17,583–89
- Slepnev VI, Ochoa GC, Butler MH, Grabs D, Camilli PD. 1998. Role of phosphorylation in regulation of the assembly of endocytic coat complexes. *Science* 281:821–24
- Söllner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE. 1993. A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation and fusion. *Cell* 75:409–18
- Sone M, Suzuki E, Hoshino M, Hou D, Kuromi H, et al. 2000. Synaptic development is controlled in the periaxonal zones of *Drosophila* synapses. *Development* 127:4157–68
- Stevens CF, Williams JH. 2000. “Kiss and run” exocytosis at hippocampal synapses. *Proc. Natl. Acad. Sci. USA* 97:12,828–33
- Stewart BA, Mohtashami M, Trimble WS, Boulianne GL. 2000. SNARE proteins contribute to calcium cooperativity of synaptic transmission. *Proc. Natl. Acad. Sci. USA* 97:13,955–60
- Steyer JA, Horstmann HA, Almers W. 1998. Transport, docking and exocytosis of single secretory granules in live chromaffin cells. *Nature* 388:474–78
- Sun JY, Wu XS, Wu LG. 2002. Single and multiple vesicle fusion induce different rates of endocytosis at a central synapse. *Nature* 417:555–59
- Sutton RB, Fasshauer D, Jahn R, Brunger AT. 1998. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature* 395:347–53
- Sweeney ST, Davis GW. 2002. Unrestricted synaptic growth in spinster—a late endosomal protein implicated in TGF- β -mediated synaptic growth regulation. *Neuron* 36:403–16
- Sytnyk V, Leshchynska I, Delling M, Dityateva G, Dityatev A, Schachner M. 2002. Neural cell adhesion molecule promotes accumulation of TGN organelles at sites of neuron-to-neuron contacts. *J. Cell Biol.* 159:649–61
- Takei K, McPherson PS, Schmid SL, De Camilli P. 1995. Tubular membrane invaginations coated by dynamin rings are induced by GTP- γ S in nerve terminals. *Nature* 374:186–90
- Takei K, Mundigl O, Daniell L, De Camilli P. 1996. The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin. *J. Cell Biol.* 133:1237–50
- Takei K, Slepnev VI, De Camilli P. 2001. Interactions of dynamin and amphiphysin with liposomes. *Methods Enzymol.* 329:478–86
- Takei K, Slepnev VI, Haucke V, De Camilli P. 1999. Functional partnership between amphiphysin and dynamin in clathrin-mediated endocytosis. *Nat. Cell Biol.* 1:33–39
- Tanaka H, Shan W, Phillips GR, Arndt K, Bozdagi O, et al. 2000. Molecular modification of N-cadherin in response to synaptic activity. *Neuron* 25:93–107
- Taraska JW, Perrais D, Ohara-Imaizumi M, Nagamatsu S, Almers W. 2003. Secretory granules are recaptured largely intact after stimulated exocytosis in cultured endocrine cells. *Proc. Natl. Acad. Sci. USA* 100:2070–5
- Tobaben S, Thakur P, Fernandez-Chacon R, Sudhof TC, Rettig J, Stahl B. 2001. A trimeric protein complex functions as a synaptic chaperone machine. *Neuron* 31:987–99
- Tokumaru H, Umayahara K, Pellegrini LL, Ishizuka T, Saisu H, et al. 2001. SNARE complex oligomerization by synaphin/complexin is essential for synaptic vesicle exocytosis. *Cell* 104:431–32
- tom Dieck S, Sanmarti-Vila L, Langnaese K, Richter K, Kindler S, et al. 1998. Bassoon, a novel zinc-finger CAG/glutamine-repeat protein selectively localized at the active zone of presynaptic nerve terminals. *J. Cell Biol.* 142:499–509
- Uchida N, Honjo Y, Johnson KR, Wheelock MJ, Takeichi M. 1996. The catenin/cadherin

- adhesion system is localized in synaptic junctions bordering transmitter release zones. *J. Cell Biol.* 135:767–79
- Varoqueaux F, Sigler A, Rhee JS, Brose N, Enk C, et al. 2002. Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. *Proc. Natl. Acad. Sci. USA* 99:9037–42
- Verhage M, Maia AS, Plomp JJ, Brussaard AB, Heeroma JH, et al. 2000. Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science* 287:864–69
- Verstreken P, Kjaerulff O, Lloyd TE, Atkinson R, Zhou Y, et al. 2002. Endophilin mutations block clathrin-mediated endocytosis but not neurotransmitter release. *Cell* 109:101–12
- Wada M, Nakanishi H, Satoh A, Hirano H, Obaishi H, et al. 1997. Isolation and characterization of a GDP/GTP exchange protein specific for the Rab3 subfamily small G proteins. *J. Biol. Chem.* 272:3875–78
- Wan HI, DiAntonio A, Fetter RD, Bergstrom K, Strauss R, Goodman CS. 2000. Highwire regulates synaptic growth in *Drosophila*. *Neuron* 26:313–29
- Wang X, Kibschull M, Laue MM, Lichte B, Petrasch-Parwez E, Kilimann MW. 1999. Aczonin, a 550-kD putative scaffolding protein of presynaptic active zones, shares homology regions with Rim and Bassoon and binds profilin. *J. Cell Biol.* 147:151–62
- Wang Y, Okamoto M, Schmitz F, Hofmann K, Sudhof TC. 1997. Rim is a putative Rab3 effector in regulating synaptic-vesicle fusion. *Nature* 388:593–98
- Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, et al. 1998. SNAREpins: minimal machinery for membrane fusion. *Cell* 92:759–72
- Wiedemann C, Schafer T, Burger MM, Sihra TS. 1998. An essential role for a small synaptic vesicle-associated phosphatidylinositol 4-kinase in neurotransmitter release. *J. Neurosci.* 18:5594–602
- Wu X, Zhao X, Baylor L, Kaushal S, Eisenberg E, Greene LE. 2001. Clathrin exchange during clathrin-mediated endocytosis. *J. Cell Biol.* 155:291–300
- Yagi T, Takeichi M. 2000. Cadherin superfamily genes: functions, genomic organization, and neurologic diversity. *Genes Dev.* 14:1169–80
- Yamagata M, Weiner J, Sanes J. 2002. Sidekicks. Synaptic adhesion molecules that promote lamina-specific connectivity in the retina. *Cell* 110:649–60
- Yamaguchi Y. 2001. Heparan sulfate proteoglycans in the nervous system: their diverse roles in neurogenesis, axon guidance, and synaptogenesis. *Semin. Cell Dev. Biol.* 12:99–106
- Zenisek D, Steyer JA, Almers W. 2000. Transport, capture and exocytosis of single synaptic vesicles at active zones. *Nature* 406:849–54
- Zenisek D, Steyer JA, Feldman ME, Almers W. 2002. A membrane marker leaves synaptic vesicles in milliseconds after exocytosis in retinal bipolar cells. *Neuron* 35:1085–97
- Zerial M, McBride H. 2001. Rab proteins as membrane organizers. *Nat. Rev. Mol. Cell Biol.* 2:107–17
- Zhai RG, Vardinon-Friedman H, Cases-Langhoff C, Becker B, Gundelfinger ED, et al. 2001. Assembling the presynaptic active zone: a characterization of an active one precursor vesicle. *Neuron* 29:131–43
- Zhang B, Koh YH, Beckstead RB, Budnik V, Ganetzky B, Bellen HJ. 1998. Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. *Neuron* 21:1465–75
- Zhen M, Jin Y. 1999. The liprin protein SYD-2 regulates the differentiation of presynaptic termini in *C. elegans*. *Nature* 401:371–75