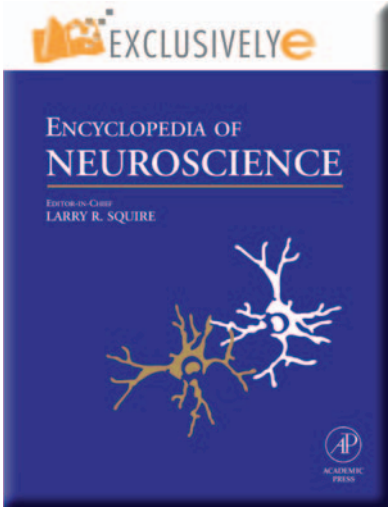

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Endocytosis and Presynaptic Scaffolds

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Introduction

Neurons communicate with each other by the temporally and spatially controlled release of secretory molecules (the so-called neurotransmitters) via regulated exocytosis. Following diffusion across the synaptic cleft the released nonpeptide neurotransmitters and neuroactive peptides bind to and activate postsynaptic receptors, which then elicit a response within the postsynaptic cell. In the case of most fast-acting transmitters, such as glutamate, γ-aminobutyric acid (GABA), or acetylcholine, signaling is elicited by ligand-gated ion channels, which are clustered at morphologically discernible zones specialized for chemical neurotransmission termed postsynaptic densities (PSDs). The PSD is opposed to a corresponding presynaptic element including the 'active zone' at which presynaptic neurotransmitter-bearing vesicles (SVs) are clustered. Presynaptic active zones are characterized by an electron-dense grid of scaffolding proteins interconnected with an actin-rich cytoskeleton, which among other functions helps to maintain a pool of vesicles docked at the presynaptic plasmalemma. To sustain chemical neurotransmission under conditions of high activity and to counter-balance net insertion of membrane by exocytic vesicle fusion, SVs undergo activity-driven cycles of calcium-triggered exocytosis and endocytosis within nerve terminals, commonly referred to as the SV cycle. Cycling of SVs must allow them to retain their specific biochemical identity, including the ability to store neurotransmitter by proton pump-driven neurotransmitter transporters, and to undergo further rounds of calcium-induced fusion with the presynaptic plasmalemma. The observed tight coupling between exocytic neurotransmitter release by vesicle fusion and compensatory endocytosis has resulted in a long and still unresolved debate regarding the precise molecular mechanisms involved in SV cycling. Here, we provide a brief summary of the pathways of SV cycling, the role of clathrin and its partner proteins in maintaining SV pools, and the temporal and spatial cues provided by scaffolding proteins and membrane lipids in maintaining presynaptic exocytic–endocytic membrane traffic.

Pathways of SV Cycling

Clathrin-Mediated Endocytic Cycling of Presynaptic Vesicles

Landmark studies in the early 1970s by Heuser and Reese showed that following fusion by complete collapse into the plasmalemma, SVs are retrieved by compensatory 'clathrin-dependent endocytosis' at specialized endocytic areas just outside the active zone. An overwhelming amount of genetic, morphological, biochemical, and physiological data suggests that clathrin-mediated endocytosis indeed constitutes an essential pathway of SV recycling, at least on the organismic level and over extended periods of time. Mutants in clathrin coat components, including the AP-2 complex or accessory and adaptor proteins such as dynamin, AP180, stoned B (the *Drosophila* ortholog of mammalian stonins 1 and 2), eps15, synaptojanin, endophilin, amphiphysin, or intersectin/DAP160, all display defects in neurotransmission owing, at least in part, to impaired SV endocytosis (Table 1). The most dramatic phenotypes have been observed following injection of dominant-negative domains or inhibitory peptides into the giant reticulospinal synapse of the lamprey. Following electrical stimulation, distinct endocytic intermediates and vacuolar structures accumulate within and around the active zones in conjunction with a partial or complete depletion of the recycling vesicle pool. Some of these intermediates resemble structures seen in neuromuscular junctions following intense stimulation or after genetic perturbation of protein function in temperature-sensitive alleles. The mechanistic details of this pathway will be discussed later.

Kiss-and-Run Mode

Based on an apparent lack of correlation between the number of morphologically distinct stable endocytic intermediates and the synaptic endocytic activity, Ceccarelli and colleagues proposed an alternative model according to which SVs release their contents through the controlled opening of a narrow fusion pore, followed by rapid closure and refilling with neurotransmitter (Figure 1). This 'kiss-and-run' mode of regulated secretion has been convincingly demonstrated to occur by combined electrophysiological recordings and membrane capacitance measurements in neuroendocrine cells, which mostly secrete peptide hormones or biogenic amines from large secretory granules (SGs; also termed large dense core vesicles (LDCVs)). In the case of SG exocytosis, flickering of

Table 1 Endocytic proteins involved in presynaptic vesicle cycling and their interaction partners

Endocytic protein	*Domains and motifs*	*Interaction partner(s)*	*Proposed function(s)*
Abp1	ADF homology domain	F-actin	Linking actin cytoskeleton with endocytosis and CAZ
	SH3 domain	PRDs of synaptojanin, dynamin, Piccolo, and synapsin1	
AP-2 (α, β2, μ2, σ2 subunits)	Trunk	$PI(4,5)P_2$	Plasmalemmal recruitment
	α-Appendage	FXDXF/DXW, WVXF motif proteins	Coat assembly
	β2-Appendage	$[DE]_nX_{1-2}FXX[FL]XXR$ motif proteins	Cargo selection and coat assembly
	β2-Hinge	Clathrin terminal domain	Scaffolding
	C-μ2	Yxx$\varnothing$ and basic motif membrane cargo; $PI(4,5)P_2$	Membrane cargo selection
Amphiphysins	N-BAR domain	(Acidic) phospholipids; dimerization	Membrane curvature induction/sensing; scaffolding
	PWXXW, LLDLD motifs	Clathrin terminal domain	Coat assembly
	FXDXF, DXF motifs	AP-2 via α-appendage	
	SH3 domain	PRDs of dynamin (& synaptojanin)	Vesicle fission
AP180	ANTH domain	$PI(4,5)P_2$	Plasmalemmal recruitment
	FXDXF, DXF motifs	AP-2 via α-appendage	Coat assembly
	DLL motifs	Clathrin	Scaffolding
Auxilin	DnaJ domain	Hsc70	Stimulation of ATPase
	DPF, WDW motifs	AP-2 via α-appendage	Coated pit recruitment
	WDW, DLL motifs	Clathrin	
Clathrin (HC, LCa/b)	HC-terminal domain	PWXXW, LLDLD, DLL motif proteins	Assembly of scaffold
	LC	HIP1, calmodulin, Hsc70	Diverse
Dynamin 1	GTPase domain	GDP/GTP	Control of membrane fission
	GED domain	Dynamin-GED	Self-assembly; stimulation of GTPase activity
	PH domain	$PI(4,5)P_2$	Plasmalemmal recruitment
Endophilin	N-BAR domain	(Acidic) phospholipids; dimerization	Membrane curvature induction/sensing
	SH3 domain	PRD of dynamin and synaptojanin	Coated pit maturation; fission and uncoating

Epsins	ENTH domain	$PI(4,5)P_2$	Membrane curvature induction; coat assembly
	DPW motifs	AP-2 via α-appendage	
	LLDLD motifs	Clathrin terminal domain	Scaffolding
	UIMs	Ubiquitin (Ub)	Ub-dependent cargo endoctyosis
Eps15, Eps15R	EH domains	NPF motif proteins	Endocytic protein network formation
	DXF motifs	AP-2 via α-appendage	Coat assembly (edges of CCPs)
	UIMs	Ubiquitin (Ub)	Ub-dependent cargo endoctyosis
HIP1, HIP1R	ANTH domain	$PI(4,5)P_2$	Plasmalemmal recruitment
	FXDXF, DXF motifs	AP-2 via α-appendage	Coat assembly
	LLDLD motif	Clathrin terminal domain	Scaffolding; links actin with endocytosis
Intersectin	EH domains	NPF motif proteins	Endocytic protein network formation
	SH3 domains	PRD domain proteins	CDC42-mediated actin polymerization
	Rho-GEF domain	CDC42	
Numb, Numb-like	PTB	$PI(4,5)P_2$	Plasmalemmal recruitment; coat assembly
	DXF motifs	AP-2 via α-appendage	
PIPK type Iγ	PIP kinase domain	PI(4)P, ATP; Arf6-GTP	Localized $PI(4,5)P_2$ formation; regulation of
	WYSPL tail peptide	FERM domain of talin	$PI(4,5)P_2$ synthesis at cell adhesion sites
Stonin 2	WVXF motifs	AP-2 via α-appendage	Coat assembly; coated pit recruitment
	Stonin-homology domain	?	?
	μ-Homology domain	Synaptotagmin (1,2,9)	AP-2-dependent recycling
Synaptojanin	SAC1 domain	4′-phosphate-containing phosphoinositides	4′-phosphoinositide phosphate hydrolysis
	5′-Phosphatase domain	$PI(4,5)P_2$	$PI(4,5)P_2$ hydrolysis, CCV uncoating coat assembly; coated pit recruitment
	FXDXF, WVXF motifs	AP-2 via α-appendage	
Synaptotagmin	C2A	Ca^{2+}, acidic phospholipids	Ca^{2+}-triggered membrane fusion membrane fusion; SV recycling
	C2B	$PI(4,5)P_2$; AP-2 via subdomain B of μ2	
	C2AB	Stonin 2 via μ-homology domain	SV recycling
Syndapins	BAR	(Acidic) phospholipids; dimerization	Membrane curvature sensing actin and dynamin-mediated fission
	SH3	PRD of N-WASP and dynamin	

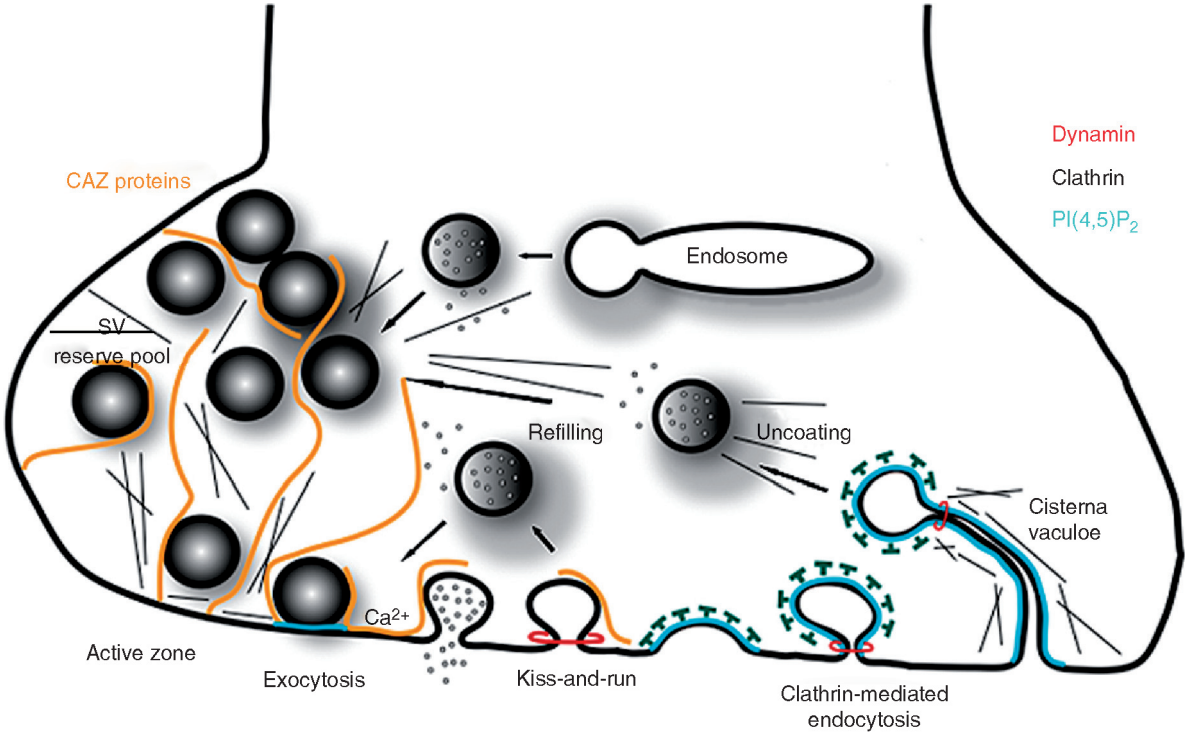

Figure 1 Pathways of SV recycling. Schematic depiction of various proposed modes of synaptic vesicle (SV) recycling: a fast 'kiss-and-run' mechanism, where the vesicle connects only briefly to the plasma membrane without full collapse ('kiss-and-run') or a slow clathrin-mediated pathway, which either operates from large vacuolar infoldings (cisternae) or by direct recovery of vesicle membrane from plasmalemmal CCPs ('clathrin-mediated endocytosis'). SVs may also arise from endosomes. Components of the cytomatrix assembled at the active zone (CAZ) together with actin function as molecular scaffolds in the spatial organization of the active zone. The SV cycle is paralleled by a cycle of phosphorylation and dephosphorylation of phosphoinositides, including $PI(4,5)P_2$, that couple exocytosis and endocytosis.

a transient fusion pore precedes complete degranulation. However, in contrast to small clear SVs that undergo local recycling, SGs need to pass through the *trans*-Golgi network in order to allow refilling with secretory peptides generated from larger precursor proteins. The recent development of lipophilic fluorescent styryl dyes (FM dyes) that rapidly partition into membranes and exhibit a large increase in fluorescence within this hydrophobic environment and of pH-sensitive fluorescent proteins (so-called 'pHluorins'; Figure 2) has provided the means to follow exocytic–endocytic cycling of SVs in real-time. FM1–43 dye-based single-vesicle tracking in dissociated hippocampal neurons in culture has revealed the existence of at least two types of release: small-amplitude events that show tightly clustered rate constants of dye release, and larger events with a more scattered distribution. The small-amplitude partial release events have been attributed to a pool of vesicles that undergoes cycling by rapid closure of a narrow, approximately 1 nm diameter, fusion pore. One would therefore have to assume that vesicles are targeted for partial release by specific factors that prevent the dilation and thus the complete opening of the fusion pore. Whether vesicles undergoing transient opening and closure of the fusion pore remain docked ('kiss-and-stay') or undergo local cycling (as depicted in Figure 1) is under debate. The balance between partial kiss-and-run-type and full fusion events that may be followed by clathrin-dependent compensatory endocytosis can be shifted depending on the frequency of stimulation. While kiss-and-run exocytosis may prevail under conditions of low activity, high-frequency stimulation results in predominantly complete fusion events. Membrane capacitance measurements of giant terminals (e.g., goldfish retinal bipolar cells or the calyx of Held) have also provided evidence for two kinetically distinguishable cycling vesicle pools. However, all of these studies suffer from the lack of information on specific factors that target SVs for fast kiss-and-run exocytosis–endocytosis and that allow the application of genetic or biochemical tools to molecularly distinguish the proposed kiss-and-run mode from other pathways of SV endocytosis.

Vacuolar Bulk Retrieval and Synaptic Endosomes

Extensive stimulation of the presynaptic neuron results in the massive insertion of SV membrane into

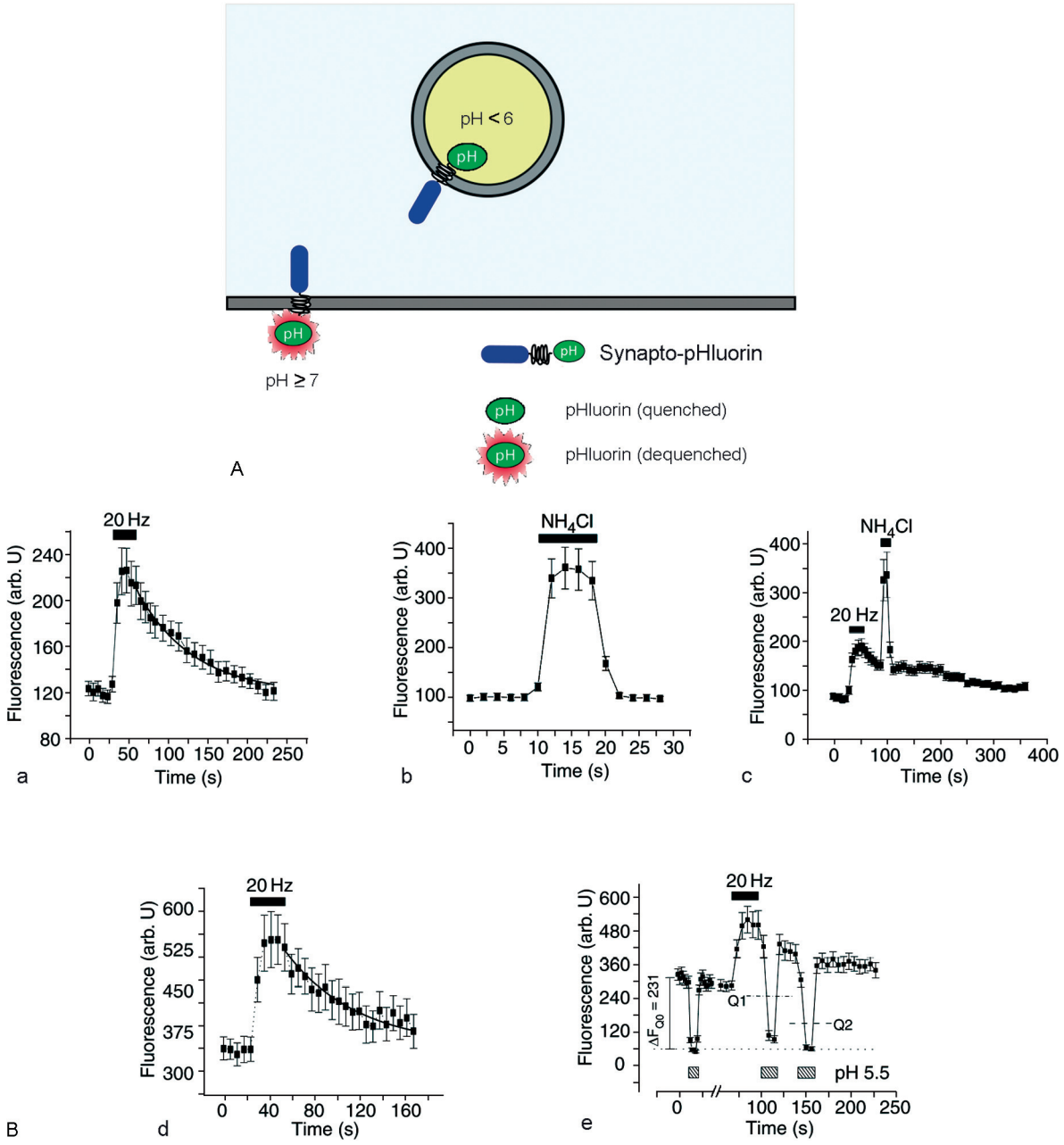

Figure 2 Real-time measurement of SV cycling using pHluorins. Schematic illustration (A) of how synapto-pHluorin can be used to probe SV cycling. Its fluorescence is quenched in the acidic vesicular lumen, but not when residing at the plasmalemma. Synapto-pHluorin signals (B) during firing of action potentials. (a)–(c) synapto-pHluorin (spH) is recycled at boutons. (a) Time course of fluorescence intensity, averaged over 13 boutons expressing spH, following stimulation with a train of 600 action potentials at 20 Hz. The dark bar shows the duration of the stimulus. The decay of fluorescence was fit by a single exponential (solid line) with $\tau = 74$ s. (b) Time course of average spH fluorescence in the same boutons as those used for (a) during alkalinization with NH_4Cl (dark bar). (c) Time course of fluorescence intensity at the same boutons as in (a, b) during train of 600 action potentials followed by exposure to NH_4Cl 30 s after the end of the electrical stimulus. NH_4Cl-induced changes are completely reversible. (d) and (e) Endocytosis, not reacidification, is rate limiting during fluorescence decay. (d) Time course of the fluorescence intensity of spH-positive boutons ($n = 20$) following electrical stimulation (dark bar). Exocytosis of spH causes a rapid increase in fluorescence, followed by a slow decay (solid line is a single exponential fit to the average fluorescence decay, $\tau = 68$ s). (e) Time course of fluorescence intensity during brief exposures to acidic solution (hatched bars below trace) before and after electrical stimulation (dark bar). Exposure to acid during resting periods led to decreases in fluorescence (quenching), indicating the presence of a resistant surface pool of spH. The fluorescence after acid quenches was similar before and after electrical stimulation, indicating that most of the newly endocytosed vesicles were rapidly reacidified. Reprinted by permission from Macmillan Publishers Ltd: *Nature Cell Biology* (Sankaranarayanan S and Ryan TA (2000) Real time measurements of vSNARE recycling in CNS synapses. *Nature Cell Biology* 2: 197–204), copyright 2000.

the presynaptic plasmalemma. It therefore may not be surprising that at least in some experimental systems, such as the neuromuscular junction of frogs and snakes, parts of the presynaptic membrane can be internalized via large vacuolar structures and cisternae, in particular after chemical induction of neurotransmitter release by application of high concentrations of K^+ and calcium. Some of these vacuoles may still exhibit a narrow tubular connection with the plasma membrane and are sometimes seen to contain clathrin-coated buds at their cytoplasmic ends (Figure 1). Whether such cisternal invaginations are eventually consumed by clathrin- and/or dynamin-dependent processes remains unclear. Once having undergone fission from the plasmalemma, cisternae could undergo additional budding steps and thereby constitute a form of a specialized presynaptic endosome. In fact, early endosomal markers including the small GTPase Rab5 and the SNARE protein Vti1aβ are present on SVs. Rab5 mutations in *Drosophila* interfere with efficient release during repetitive stimulation, suggesting that presynaptic endosomes could play an important functional role in maintaining SV pools.

Clathrin-Mediated SV Endocytosis

Clathrin was first purified by Barbara Pearse more than 30 years ago, using coated vesicles isolated from pig brain. In fact, clathrin is most abundantly expressed in the central nervous system, where it is found to be particularly concentrated in presynaptic nerve terminals. The importance of clathrin for SV recycling is further underscored by the fact that clathrin-coated vesicles (CCVs) isolated from nerve terminals are highly enriched in SV proteins. Clathrin, the heterotetrameric adaptor complex (AP-2), and monomeric adaptors and accessory proteins (including epsin, eps15, AP180, HIP1/HIP1R, amphiphysin, endophilin, stonin 2, etc.) play an early role in coat formation. Recruitment of AP-2 to the plasma membrane is a cooperative and presumably highly regulated process involving interactions with phosphoinositides, membrane cargo, and a variety of AP-2α ear domain-binding partners. Many of these adaptor and accessory proteins also display higher expression levels in brain than in other tissues, perhaps owing to their increased half-lives. In addition, neurons contain endocytic protein isoforms, including splice variants of clathrin light chains and α_A-adaptin, AP180, auxillin, intersectin, and dynamin 1. Much of what we know about the mechanism of CCV formation has been learned from nonneuronal systems or from structural studies on clathrin, adaptor, and accessory proteins or domains thereof. In the following sections, we summarize these data and provide a tentative model for how clathrin, dynamin, and their binding partners could act at nerve terminals.

Early Steps of Clathrin-Coated Pit Formation

CCVs are formed by the coordinated assembly of clathrin triskelia built from three tightly linked heavy and associated light chains onto the plasma membrane. The recruitment and polymerization of the outer clathrin layer is assisted by mono- and heterotetrameric adaptor proteins, which simultaneously bind to clathrin, to membrane lipids, and in many cases to transmembrane cargo proteins. In addition, there is a large reservoir of preassembled flat hexagonal clathrin lattices at the plasma membrane that, however, need to undergo a structural transition involving the formation of clathrin pentagons in order to accommodate a curved membrane bud. The most important clathrin adaptor is the heterotetrameric AP-2 complex comprising two large subunits (α and β2), a medium subunit (μ2), and a small subunit (σ2). The two large subunits together with σ2 and the amino-terminal domain of μ2 (N-μ2) form the trunk or core domain of AP-2, and are joined by extended, flexible 'hinges' to the appendage or ear domains of α- and β2-adaptins. Since AP-2 associates with clathrin, a variety of accessory endocytic proteins, phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2], and membrane cargo proteins, it has been postulated to serve as a main protein interaction hub during coated pit assembly. Many accessory proteins, such as epsins, AP180/CALM, and amphiphysin, also have an adaptor function by linking clathrin assembly to membrane bud formation. These mono- or dimeric adaptors possess a folded lipid-binding domain linked to a more flexible portion of the protein harboring short clathrin- and AP-2-binding motifs, which may aid stabilization of nascent clathrin-coated pits (CCPs) during the assembly process. During CCP assembly transmembrane cargo proteins are recognized by adaptor proteins, most notably the AP-2 complex, which bind to endocytic sorting motifs within their cytoplasmic tails. These motifs include tyrosine-based Yxxϕ (where ϕ is a bulky hydrophobic residue) and acidic cluster di-leucine motifs, which bind directly to distinct sites within the AP-2 core domain. Yxxϕ motifs have been co-crystallized with the carboxy-terminal portion of the AP-2 μ-subunit (C-μ2), to which they bind in an extended conformation. Cargo recognition by AP-2 requires the presence of PI(4,5)P_2, which stabilizes the protein in an open conformation that enables cargo recognition by its μ2-subunit. Consistent with this, clathrin/AP-2-coated pits were

shown to become stabilized in living cells upon encounter of cargo receptors, suggesting that the process of AP-2 recruitment and initiation of plasmalemmal CCPs is highly cooperative.

Despite intense efforts, evidence regarding the presence of canonical Yxxø- or di-leucine-type endocytosis signals within SV protein cytoplasmic domains remains scarce. However, C-μ2 harbors a structurally unresolved binding site for basic internalization motifs found in a variety of multimeric membrane proteins, including the presynaptic vesicle protein synaptotagmin, the presumed calcium sensor in neuroexocytosis. Neuronal synaptotagmin isoforms also interact with the AP-2-binding μ-homology domain containing adaptor protein stonin 2, which is capable of targeting synaptotagmin for clathrin/AP-2-dependent internalization in neurons as well as in transfected fibroblasts. In addition to its interaction with the ear-domain of AP-2α, stonin 2 can bind to eps15 and intersectin, thereby linking synaptotagmin with other components of the clathrin-dependent endocytic machinery in neurons. Stonin 2 (and by analogy, stoned B in *Drosophila*) thus represents the first endocytic adaptor protein identified that is specifically dedicated to the endocytic internalization of a SV protein. Synaptotagmin may thus regulate both the exocytic and endocytic limbs of the SV cycle. In support of this hypothesis, it has been observed that genetic or chemical perturbation of synaptotagmin function by fluorophore-assisted light inactivation in mice, flies, or worms results in recycling defects and a partial depletion of SVs. Whether other SV proteins also interact with specific endocytic adaptor proteins or get co-sorted with synaptotagmin, that is, as part of a membrane microdomain, remains an open question.

CCP Maturation and Vesicle Fission

After the clathrin lattice is formed, endophilin, epsin, eps15, amphiphysins, and other proteins are involved in membrane bending and clathrin rearrangements as coated pits progressively invaginate and mature. Partitioning of the amino-terminal amphipathic helix of the ENTH domain protein epsin and perhaps other components (i.e., the small GTPase Arf6) drives the acquisition of membrane curvature. Bin–amphiphysin–Rvs (BAR) domain proteins, such as amphiphysin and endophilin, may aid membrane bending, and function as curvature sensors that signal completion of the process. Through their SH3 domains, both amphiphysin and endophilin also interact with and recruit accessory enzymes, including the large GTPase dynamin and the phosphoinositide phosphatase synaptojanin, to the nascent vesicular bud. Dynamin is required for fission of endocytic membrane vesicles by mechanochemically constricting ('pinchase') the vesicle neck. The eminent role of dynamin for SV recycling is best illustrated by the dramatic phenotype seen in *shibire*^{ts} mutants in *Drosophila*, which exhibit temperature-sensitive paralysis due to the accumulation of unbudded membrane infoldings and endocytic intermediates.

Observation of CCPs dynamics using evanescent wave microscopy indicates that during fission, dynamin recruitment to coated pits is rapidly followed by recruitment of actin. Moreover, perturbation of actin disrupts the endocytic reaction with accumulation of coated pits with wide necks, suggesting a role of actin, actin-binding factors, and dynamin-interacting accessory proteins, such as Abp1 or syndapin, in promoting constriction of the neck and removal of endocytosed vesicles from the membrane. In lamprey, snake, and fly neuromuscular synapse the invagination of the membrane into pits, occurs at distinct 'endocytic zones' surrounding the active zones of exocytosis (termed the peri-active zone). FM1–43 photoconversion and serial section electron microscopy analysis revealed that labeled clathrin-coated endocytic vesicles were clustered significantly near active zones, consistent with local exocytic–endocytic recycling vesicle pools at this synapse. Together with the regulated turnover and synthesis of membrane phosphoinositides, actin and actin-binding proteins may thus provide spatial and temporal landmarks for SV endocytosis (see the next section).

Protein Scaffolds as Spatial Regulators of Vesicle Cycling

Morphologically and functionally, the active zone can be divided into two parts: the core active zone, where regulated exocytosis (and kiss-and-run-type SV retrieval) takes place; and the peri-active zone, where clathrin-mediated endocytosis occurs (see the preceding section). At the ultrastructural level, the core active zone is characterized by the more or less regular array of electron-dense material, called the presynaptic grid, presynaptic particle web, or 'cytomatrix assembled at the active zone' (CAZ). During recent years, multiple molecular components – both CAZ-specific ones and those that are recruited through interaction with CAZ scaffolding proteins – have been identified and characterized.

Functionally, the CAZ is thought to define the site of regulated neurotransmitter release by localizing presynaptic membrane proteins, including voltage-gated calcium channels and cell adhesion molecules, to organize steps of the SV cycle, including tethering and priming of SV, and to link the exocytic machinery

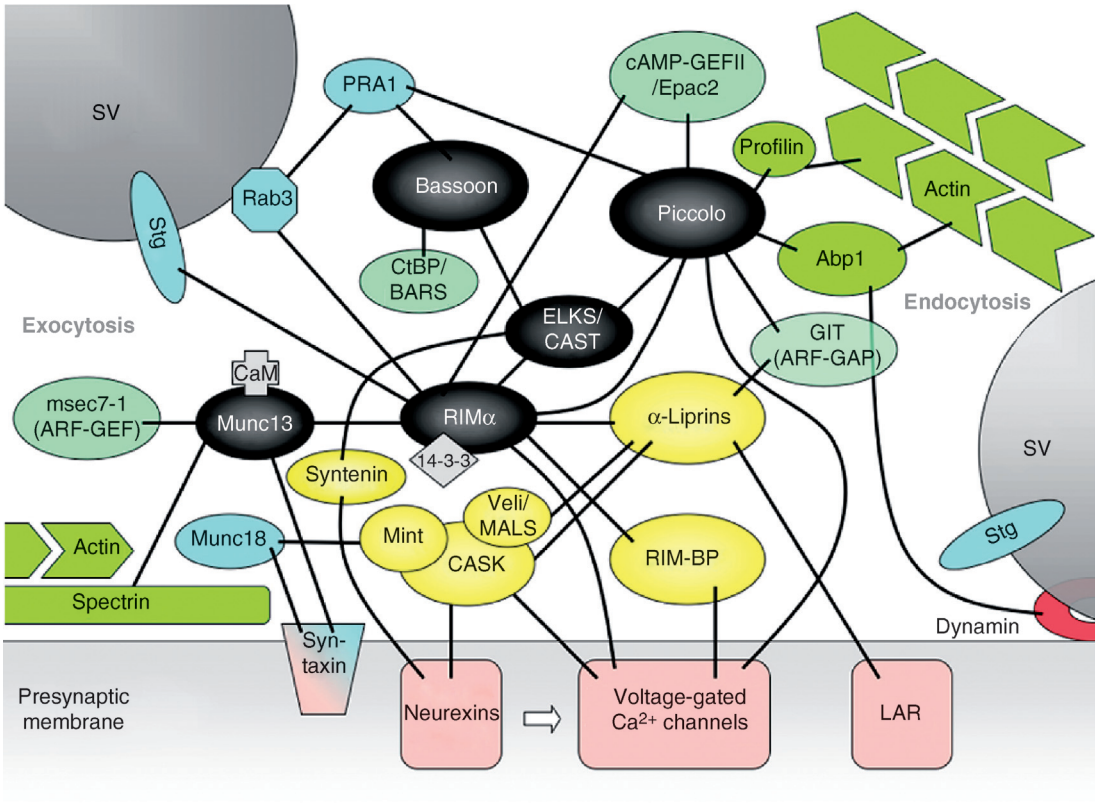

Figure 3 Molecular organization of the cytomatrix at the active zone (CAZ). The scheme depicts observed physical interactions between active zone-specific scaffolding proteins (black), associated proteins with putative structural functions (yellow), effector proteins (blue-green), actin cytoskeletal and associated elements (green), small modulatory molecules (gray), proteins involved in SV exocytosis (blue) and endocytosis (red), as well as presynaptic membrane proteins (pink). Some of the interactions for Piccolo and RIM were discovered in pancreatic β-cells and will have to be confirmed for the CAZ. For further details see **Table 2**. Note: the diagram neither reflects the relative sizes of the proteins nor their exact topographic localization within the presynaptic bouton. The arrow indicates that neurexins (α-forms) are involved in the localization of calcium channels.

with elements of the endocytic zone and with the surrounding actin cytoskeleton (Figure 3). In addition, CAZ elements turned out to be essential mediators of presynaptic plasticity.

Molecular Organization of the CAZ

Relatively few CAZ-specific structural and effector proteins have been identified to date that are believed to constitute the scaffold of the CAZ and to mediate its structural and functional organization (black in Figure 3). These proteins belong to four different protein families: the Rab3-interacting molecules (RIMs), the mammalian Unc13 proteins (Munc13s), the two related giant CAZ scaffolding proteins Bassoon and Piccolo, and the ELKS/CAST proteins (Figure 3; Table 2).

RIMs are multidomain proteins that were identified as effectors of Rab3, a small GTPase associated with SVs. In particular, the α-forms of RIM1 and RIM2 are important scaffolding molecules that interact with multiple other presynaptic proteins. These include isoforms of Munc13, a liaison that might be involved in making SVs fusion-competent, as well as ELKS/CAST and Piccolo. Analysis of RIM1α-deficient mouse mutants revealed that this protein is involved in short- and long-term forms of presynaptic plasticity. For example, long-term potentiation at hippocampal mossy fiber terminals or parallel fiber synapses of the cerebellum involve protein kinase A-dependent phosphorylation of RIM1α and phosphorylation-dependent binding of 14-3-3 proteins. Further interactions of αRIMs point to a central role of these proteins in active zone organization. They can bind presynaptic voltage-gated Ca^{2+} channels, either directly or via their binding to RIM-binding proteins, and the Ca^{2+} sensor synaptotagmin, which is involved in both exocytosis and endocytosis of SVs. The interaction with α-liprins, originally identified as a cytoplasmic adaptor for the receptor tyrosine phosphatase LAR, might serve the formation and maintenance of active zones, as suggested by work in invertebrates.

Munc13 isoforms are involved in SV priming and the regulation of synaptic plasticity. They can link

Table 2 Protein components of the cytomatrix assembled at the active zone

Protein family *Relevant members and synonymous names*	*Domains and motifs*	*Interacting protein*	*Proposed function(s)*
RIMs – Rab3-interacting molecules	Zn finger	Rab3	Link to SV, tethering of SV, presynaptic plasticity
		Munc13-1, ubMunc13-2	SV priming
RIM1α; RIM2α,β,γ; RIM3γ; RIM4γ; UNC10 (*C. elegans*)	Region between Zn finger and	14-3-3	Presynaptic plasticity
	PDZ domain	cAMP-GEFII / Epac2	Insulin secretion in pancreatic β-cells
	PDZ domain	ELKS / CAST	CAZ scaffolding
	C2A	Piccolo	CAZ scaffolding
		N-type calcium channels	Channel anchoring and/or clustering
	Pro-rich motif btw. C2A & B	RIM-BPs	CAZ scaffolding
	C2B	α-Liprin,	CAZ scaffolding,
		N-type calcium channels	Anchoring and/or clustering of Ca channels
		synaptotagmin	CAZ scaffolding, organization of Ca sensing
RIM-binding proteins	SH3 domains 2 and/or 3	RIMs	CAZ Scaffolding
RIM-BP1 RIM-BP2		N-type ($Ca_v2.2$) and L-type ($Ca_v1.3$) Ca channels	Channel anchoring and/or clustering
Piccolo–Bassoon family	Zn fingers	PRA1 (prenylated Rab3 acceptor)	Link to SV?
Bassoon and Piccolo	Between CC1 and CC2	CtBP/BARS	Membrane trafficking?
		Ribeye/CtBP2	Scaffolding of synaptic ribbons
	CC3	ELKS / CAST	CAZ scaffolding
Piccolo/Aczonin only	N-terminal Q domain	Abp1 (actin-binding protein 1)	Link to actin cytoskeleton, endocytosis
	Between CC1 and CC2	GIT (ARF-GAP)	Membrane trafficking
	Pro-rich region btw CC1 and 2	Profilin	Actin regulation
	PDZ domain	cAMP-GEFII/Epac2	Insulin secretion in pancreatic β-cells
	C2A	RIMs	CAZ scaffolding, organization of SV cycle
		Piccolo	Homophilic interaction, scaffolding
	C2A and C2B	L-type Ca channel	Channel anchoring in β-cells

Continued

Table 2 Continued

Protein family *Relevant members and synonymous names*	*Domains and motifs*	*Interacting protein*	*Proposed function(s)*
UNC13 proteins	M13-1 and ubM13-2 N-term.	α-RIMs	CAZ scaffolding,
Munc13-1		Calmodulin	Ca^{2+}-dependent plasticity
bMunc13-2	Conserved homology region	Spectrin β-spIIIΣ	Anchoring to actin/spectrin cytoskeleton
ubMunc13-2	(MUN)	msec7-1 ARF-GEF	Regulation of actin cytoskeleton
Munc13-3		DOC2α (double C2 domain	?
Munc13-4		protein)	
UNC13 (C.e.)	C-terminus	Syntaxin (in debate)	SV priming, control of SNARE complex formation
Dunc13 (D.m.)			
ELKS/CAST proteins	CC regions	Bassoon, Piccolo	CAZ scaffolding
ELKS1A/ERC1A		α-Liprins	CAZ scaffolding
ELKS1B/ERC1B	C-terminus	RIMs	CAZ scaffolding
ELKS2/CAST/ERC2		Syntenin	Active zone organization?
α-Type Liprins	N-term CC region	RIMs	CAZ scaffolding
Liprin-α1–α4		ELKS/CAST	CAZ scaffolding
SYD-2 (C.e.)		GIT (ARF-GEF)	Regulation of membrane trafficking and actin cytoskeleton
		Kif1A (kinesin motor)	Transport
	SAM domains	LAR receptor protein tyrosine Phosphatase	Regulation of membrane protein anchoring
		β-Liprin	
		CASK (MAGuK)	Regulation of transsynaptic adhesion?
		MALS/Veli	Regulation of transsynaptic adhesion?
	C-terminal PDZ binding site	GRIP	Receptor transport and clustering/postsynaptic

to the actin-spectrin cytoskeleton via binding to a β-spectrin isoform and to presynaptic membrane-trafficking processes via the ARF guanine nucleotide exchange factor msec7-1.

ELKS/CAST proteins display a very high content of coiled-coil structures and are considered as a major structural component of the CAZ. They can physically interact with RIMs as well as with Bassoon and Piccolo and thus might interconnect the major scaffolding proteins of the CAZ. Bruchpilot, an ELKS/CAST-related protein in *Drosophila*, is responsible for the proper anchoring of particular specializations of the CAZ, so-called T-bars, to the active zone membrane. Via syntenin, ELKS/CAST proteins also connect to neurexins, which are specific cell adhesion molecules of the presynaptic membrane occurring as long α- and short β-forms involved in Ca^{2+} channel localization and linkage to postsynaptic neuroligins, respectively. Neurexins are additionally anchored to the presynaptic cytomatrix via a trimeric protein complex of CASK, Mint, and MALS/Veli, which in turn links to Ca^{2+} channels and α-liprins (Figure 3).

Bassoon and Piccolo are considered as very large multidomain scaffolding molecules of the CAZ. Most of their interaction partners have still to be discovered. They both bind ELKS/CAST and the small prenylated Rab3 acceptor (PRA1), which potentially links into the SV cycle. In addition, Bassoon has been reported to interact with CtBP/BARS and its homolog RIBEYE, a specific component of synaptic ribbons in retinal photoreceptors and inner ear hair cells. The RIBEYE–Bassoon interaction was suggested to be essential for anchoring of ribbons to the presynaptic plasmalemma. The interaction of Bassoon (and potentially also Piccolo) with CtBP/BARS is of interest, as this protein has been implicated in vesicular fission from the *trans*-Golgi complex. By analogy, a similar function might be envisioned in the presynapse.

Links of the CAZ to the Endocytic Machinery and the Actin-Based Cytoskeleton

While multiple interactions of the previously described CAZ proteins underscore the role of the presynaptic cytomatrix in organizing the apparatus for regulated exocytosis, a link to clathrin-mediated endocytosis is less clear. Specific interaction partners for Piccolo might be of particular interest in this context, as they can provide the basis for the physical linkage of exocytic and endocytic presynaptic processes. On the one hand, Piccolo can bind directly to Ca^{2+} channels and the guanine nucleotide exchange factor cAMP-GEFII/Epac2, as revealed from studies on pancreatic β-cells. Moreover, the C2A domain of Piccolo is discussed as a candidate low-affinity Ca^{2+} sensor. These characteristics argue for a role in active zone organization and exocytosis. On the other hand, Piccolo can bind to the ARF-GTPase-activating protein GIT, which has been implicated in endocytic processes such as receptor internalization. The N-terminus of Piccolo specifically interacts with Abp1, an actin-binding factor directly regulating the GTPase dynamin. Indeed, the N-terminal Q-domain of Piccolo can interfere with endocytic processes in heterologous systems. Yet another link between Piccolo and the actin cytoskeleton is profilin, a small G-actin- and phosphoinositide-binding protein that is involved in local remodeling of the actin cytoskeleton. Thus, Piccolo might indeed be an important mediator between the neurotransmitter release apparatus and the neighboring endocytic machinery.

Regulation of the SV Cycle by Membrane Lipids

Phosphoinositide Regulation of SV Cycling

Cycling of presynaptic vesicles requires the precise spatial and temporal regulation of protein–lipid interactions. Many synaptic proteins – including synaptotagmin1, calcium-dependent activator protein for secretion (CAPS), the Munc18-interacting proteins Mint-1 and Mint-2, voltage-gated P/Q-type calcium channels, and a variety of endocytic proteins such as AP-2, AP180, epsin, and dynamin (Table 1) – directly bind to and are regulated by [$PI(4,5)P_2$]. SV cycling thus appears to be nested into a local cycle of phosphoinositide phosphorylation and hydrolysis. Accordingly, $PI(4,5)P_2$ acts at multiple stages of the vesicle cycle. Knockout mice lacking PIP kinase type Iγ, the major $PI(4,5)P_2$-synthesizing enzyme at synapses, display defects in neurotransmitter release and endocytic recycling that cause synaptic depression. The activity of PIPK Iγ is regulated by a variety of factors, including phosphatidic acid (PA), the small GTPases Arf6 and Rac1, and the actin cytoskeleton-associated adhesion protein talin. Association of PIPK Iγ with these factors is dependent on their phosphorylation status, providing a means for the temporal and spatial regulation of phosphoinositide metabolism. $PI(4,5)P_2$ is eventually consumed by synaptojanin-mediated dephosphorylation, resulting either in formation of PI(4)P and perhaps PI, cleavage via phospholipase C, or PI3 kinase-dependent synthesis of $PI(3,4,5)P_3$. At least in neuroendocrine PC12 cells, $PI(4,5)P_2$ appears to be concentrated within cholesterol-enriched microdomains near release sites, where it may aid vesicle docking and/or fusion. The recent observation that SV proteins remain clustered during their exocytic–endocytic journey, together

with the extremely high cholesterol content of SV membranes, suggests that cholesterol-enriched microdomains could serve to spatially organize the SV cycle, perhaps in part by locally concentrating $PI(4,5)P_2$.

At present we can only speculate about the exact mechanism of action of $PI(4,5)P_2$ during vesicle fusion, but its role in clathrin-mediated synaptic vesicle endocytosis is much better understood. $PI(4,5)P_2$ is an important factor in recruiting endocytic adaptor and accessory proteins to the membrane where these form a network of protein–protein interactions. The stability of this network critically depends on the $PI(4,5)P_2$ content of the membrane, as suggested by the observation that CCVs accumulate in nerve terminals of synaptojanin knockout mice. These observations also indicate that $PI(4,5)P_2$-hydrolysis may normally occur concomitantly with or directly after dynamin-mediated membrane fission. CCVs at presynaptic sites of synaptojanin knockout mice become trapped in a meshwork of actin filaments, consistent with the fact that $PI(4,5)P_2$ regulates actin polymerization and drives the formation of actin comet tails that may help to propel endocytic vesicles away from the plasmalemma.

Lipids and Membrane Deformation

SV fusion and the subsequent formation of endocytic clathrin-coated buds at the presynaptic peri-active zone involve radical geometric remodeling of the membrane in order to generate areas of different membrane curvature. While lipids with bulky polar headgroups and saturated or single fatty acid tails, including lysolipids and many glycolipids, promote positive curvature, lipids with compact headgroups and space-filling hydrophobic tails, such as PA and diacylglycerol (DAG), favor negative curvature. For example, lysophosphatidic acid (LPA) and PA, which are interconverted by LPA-acyl transferase and phospholipase A_2 activities, respectively, favor opposite curvatures.

Although differential distribution of distinct types of lipids between the two membrane leaflets may contribute to membrane deformation, it is generally assumed that membrane bending requires the action of proteins. During endocytic SV recycling, the forming bud must adopt a positive curvature at the bud center and negative curvatures at the edges. Endocytic proteins may act by one of several mechanisms to bend membranes. As stated previously, epsin, a PI-$(4,5)P_2$-binding clathrin accesssory protein, is able to partition into the cytoplasmic leaflet of the plasma membrane via PI $(4,5)P_2$-induced formation of an extra α-helical segment that drives acquisition of positive curvature. Dimeric BAR domain-containing proteins, including amphiphysin or endophilin (Table 1), induce curved membranes by an additional amphipathic helix at their amino-terminal end, largely via electrostatic interactions of their concave surface with negatively charged membrane phospholipids. Endophilin has originally been proposed to be an LPA-acyl transferase, but this activity has recently been called into question. Highly curved membranes may become stabilized in addition by other scaffolding proteins, including clathrin itself, which forms a rigid basket around the emanating vesicular membrane bud. Moreover, the transmembrane domains of synaptic vesicle proteins could provide a barrier that prevents local areas of high curvature from lateral diffusion and thereby contribute to maintaining vesicle identity. Finally, protein–lipid interactions and the formation of microdomains might also underlie the choice between fast and slow modes of SV cycling that would require a tight control of fusion pore expansion and constriction.

See also: Activity-Dependent Remodeling of Presynaptic Boutons; Clathrin and Clathrin-Adaptors; Dynamin; Endocytic Traffic in Spines; Endocytosis: Kiss and Run; Liprins, ELKS, and RIM-BP Proteins; Munc13 and Associated Molecules; Piccolo and Bassoon; Presynaptic Endosomes; Rab3A Interacting Molecules (RIMs); Synaptic Vesicles; Vesicle Pools.

Further Reading

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