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Review

Clathrin-Mediated Endocytosis at Synapses

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Neurons are communication specialists that convert electrical into chemical signals at specialized cell–cell junctions termed synapses. Arrival of an action potential triggers calcium-regulated exocytosis of neurotransmitter (NT) from small synaptic vesicles (SVs), which then diffuses across the synaptic cleft and binds to postsynaptic receptors to elicit specific changes within the postsynaptic cell. Endocytosis of pre- and postsynaptic membrane proteins including SV components and postsynaptic NT receptors is essential for the proper functioning of the synapse. During the past several years, we have witnessed enormous progress in our understanding of the mechanics of clathrin-mediated endocytosis (CME) and its role in regulating exo-endocytic vesicle cycling at synapses. Here we summarize the molecular machinery used for recognition of synaptic membrane protein cargo and its clathrin-dependent internalization, and describe the inventory of tools that can be used to monitor vesicle cycling at synapses or to inhibit CME in a stage-specific manner.

Key words: adaptors, cargo recognition, clathrin, postsynaptic receptors, stage-specific inhibition, synaptic vesicle recycling

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Clathrin-mediated endocytosis (CME) is a ubiquitous process operating in all cell types that provides a pathway for internalization of extracellular hormones and signaling factors, and of transmembrane receptors involved in nutrition uptake or transduction of extracellular signals to intracellular targets (1). One of the major neuronal functions for CME is synaptic vesicle (SV) recycling within the presynaptic compartment. It is estimated that ~90% of all clathrin-coated vesicles (CCVs) in neurons are involved in SV retrieval (2). Clathrin-mediated endocytosis also regulates the activity-dependent exo-endocytic trafficking of postsynaptic receptors, thus allowing for the fine-tuning of signal strength during neurotransmission and plasticity-associated changes thereof (3), uptake of neurotrophins in the presynapse, and sculpting of synaptic structure (4).

We will describe first the role of CME in recycling of presynaptic vesicles and in regulating the number of surface-active neurotransmitter (NT) receptors. Then, we will elaborate on the molecular mechanisms by which clathrin-associated adaptor proteins recognize and internalize synaptic cargo destined for endocytosis. Based on these insights, an arsenal of molecular weapons has been used to monitor clathrin-dependent vesicle cycling at synapses or to arrest CME at morphologically and physiologically discernible stages.

Pathways of Synaptic Vesicle Recycling: The Tortoise and the Hare?

To sustain high-frequency NT release and to prevent expansion of the presynaptic plasmalemma, SVs must undergo local endocytic recycling within the nerve terminal. An important role for CME in the SV recycling pathway was first suggested more than 30 years ago on the basis of electron microscopic studies at the frog neuromuscular junction. Heuser and Reese (5) showed that SV recycling at the periactive zone coincides with the appearance of cisternae and clathrin-coated pits (CCPs), presumably reflecting the internalization of previously exocytosed SV membranes from the plasmalemma. A plethora of genetic, morphological, physiological and biochemical studies since have underscored the importance of CME in SV retrieval (discussed in detail below). Whether under certain, perhaps mild stimulation conditions SVs may recycle by means of an alternative, so-called kiss-and-run pathway of fast retrieval, first suggested by Ceccarelli et al. (6), remains a matter of controversial debates. According to the 'kiss-and-run' mechanism, vesicles only fuse transiently and incompletely with the plasma membrane by means of a narrow fusion pore and are then retrieved almost instantaneously as intact entities from the active zone [reviewed in Harata et al. (7)]. SV endocytosis has been observed to follow two kinetically separable components (7): a fast and a slow component. Whether these correspond to molecularly different mechanisms of SV retrieval, i.e. CME and kiss-and-run cycling, or reflect depletion of rate-limiting components of a single pathway remains uncertain.

Clathrin-mediated endocytosis of SV membranes can occur directly from the plasma membrane of the periactive zone or by bulk endocytosis of large membrane infoldings from which CCVs bud (8,9). Additional sorting steps may involve early endosomal intermediates (10,11). The vesicular

transporters for glutamate [vesicular glutamate receptor 1 (VGLUT1)] (12) and GABA (VGAT1) (13) have been observed to at least in part recycle by means of an endosomal pathway involving the neuronal isoform of the AP3 adaptor complex (AP3B) but not clathrin itself. So far it is unclear whether a fraction of SVs under certain conditions follows an endosomal AP3B pathway or whether AP3B-dependent sorting is required as a 'proofreading' mechanism to retrieve escaped SV proteins such as vesicular NT transporters.

Clathrin-Mediated Endocytosis is a Major Pathway of SV Recycling

Clathrin-mediated endocytosis presumably represents the major pathway for compensatory endocytosis and retrieval of SV membranes following their complete fusion with the presynaptic plasmalemma during exocytosis. Over the years, we have witnessed the identification and characterization of a number of adaptor and accessory proteins that cooperate with clathrin in deforming the membrane, assembling the clathrin scaffold, or assisting dynamin–GTPase-mediated CCV fission. Many of these proteins are enriched at synapses, some as neuron-specific isoforms. Deletion of clathrin adaptor (14,15), accessory proteins (16,17) or enzymes involved in regulating the phosphorylation state of phosphoinositides (18), essential cofactors in CME, severely reduce the ability of neurons to functionally recycle SVs in a variety of model organisms. A similar impairment of SV cycling that correlates with the accumulation of morphologically distinct endocytic intermediates is seen after perturbation of the clathrin-based endocytic protein interaction network following application of dominant interfering peptides or specific antibodies at the giant synapses of squid and lamprey (8). A complete block of SV endocytosis was also observed in recent experiments in central nervous system synapses based on overexpression of a dominant-negative clathrin/AP2-binding fragment of the neuronal clathrin adaptor AP180, RNAi-mediated clathrin knock down (19) or acute chemical inhibition of dynamin–GTP-mediated vesicle fission (20) (see also Figure 1B). A major role for CME in SV recycling is also supported by proteomic analysis of CCVs isolated from synaptic terminals (21).

In agreement with these molecular studies, SV proteins exocytosed and subsequently retrieved by endocytosis are nonidentical (22,23), suggesting that SV recycling involves complete fusion followed by an endocytic sorting process, presumably requiring the activity of adaptor proteins.

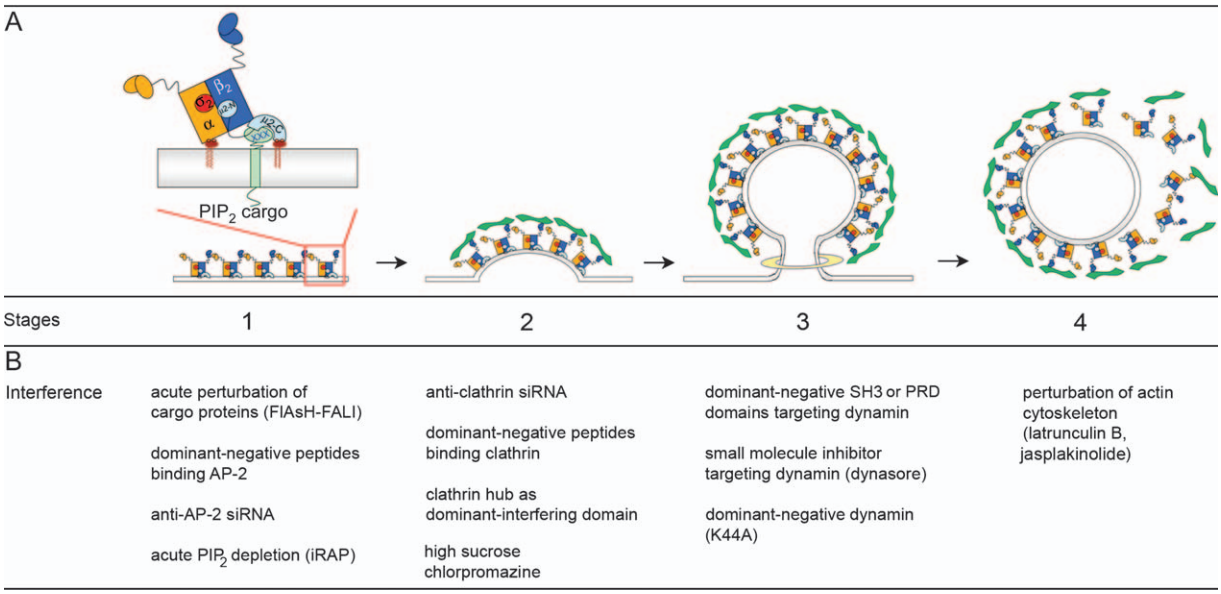

Figure 1: Stage-specific inhibition of CME. (A) Stages of CME. CME is initiated by the recruitment of AP2 and perhaps other adaptors to PIP_2 and cargo-enriched membrane sites (stage 1). Clathrin lattice assembly and concomitant membrane bending by accessory factors (stage 2) prepares the nascent vesicle for dynamin-mediated fission (stage 3). Finally, CCVs undergo uncoating (stage 4) and are trafficked to downstream endosomes. (B) Select inhibitors of CME. RNAi-mediated depletion of AP2 and clathrin or dominant-negative AP2- or clathrin-binding peptides or protein fragments can be used to inhibit early stages of CCP formation (stages 1 and 2). Ill-characterized drugs such as chlorpromazine or high sucrose may yield similar effects, albeit of uncertain specificity. SH3 domains or dynamin-derived proline-rich (PRD) peptides as well as inactive dynamin mutants interfere with CCV fission (stage 3). Acute inhibition of dynamin-mediated fission can also be achieved by application of the small molecule inhibitor dynasore. Perturbation of the actin cytoskeleton (i.e. by drugs such as latrunculin B or jasplakinolide) affects late stages of trafficking such as returning presynaptic endocytic vesicles back to the SV cluster (stage 4).

Calming Down the Excitement: CME and Its Role in Regulating the Number of Surface-Active Postsynaptic Receptors

Over the past few years, it has become clear that the strength of synaptic connections, in particular with respect to postsynaptic responses, is subject to plastic changes. At excitatory synapses, activation of glutamate receptors, such as AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type glutamate-gated ion channels, provides the primary depolarization in excitatory neurotransmission. AMPA receptors, heterotetramers composed of related GluR1-4 subunits, undergo constitutive as well as stimulation-dependent internalization through CME (3) and this correlates physiologically with activity-dependent long-term depression (LTD). Conversely, during long-term potentiation, a cellular model for learning and memory, an increase in the number of functional cell-surface-exposed AMPA receptors at the postsynaptic membrane is observed (3,24). This observation is consistent with the predominant localization of endocytic proteins including clathrin, AP2 and dynamin lateral to the postsynaptic density (25). The exact molecular mechanisms of the constitutive and regulated pathways for AMPA receptor internalization are not yet completely understood. Although all pathways are dependent on the GTPase dynamin and its SH3-domain-containing binding partners, they seem to be spatially segregated and differentially influenced by protein kinases, phosphatases and calcium ions (3,24). Activated AMPA receptors colocalize with the clathrin adaptor complex AP2 and the accessory protein eps15 in CCPs. Direct binding of the basic stretch within the cytoplasmic tail of the AMPA receptor subunits GluR2 and GluR3 to the μ-subunit of AP2 (26) (discussed in detail below) is required for NMDA (N-methyl D-aspartate)-induced AMPA receptor endocytosis (27). Additional factors, such as the AMPA receptor auxiliary subunits TARPs (28), the activity-regulated gene product Arc/Arg3.1 (29) and the BAR-domain-containing protein PICK1 (30), may exhibit further modulatory functions in controlling CME of AMPA receptors. In nematodes, GluR is subject to multiubiquitination, which may target glutamate receptors for CME and late endosomal/lysosomal degradation (31). Similar endocytic mechanisms may operate in the case of $GABA_A$ (g-aminobutyric acid type A) receptors at inhibitory synapses, where phosphoregulation controls the association of receptors with the clathrin adaptor complex AP2 (32).

Fences, Garrottes and Mr Tickle: The Molecular Machinery of CME in Neurons

We will now turn to the molecular machinery involved in CME of pre- and postsynaptic proteins. The clathrin machinery required for compensatory endocytosis at the synapse is very similar to that used for constitutive or ligand-stimulated endocytosis in other cell types (1,9). From a somewhat simplified perspective, the process of CME can be divided into several steps: (i) clathrin coat nucleation and assembly, (ii) CCP maturation, (iii) CCV fission and (iv) uncoating (Figure 1A). These morphologically distinguishable steps presumably represent metastable intermediates of a complex process whose spatiotemporal dynamics are controlled by a network of protein–protein and protein–lipid interactions. This clathrin-based endocytic network is organized around a limited number of factors central to the pathway that show an unusually high number of binding partners. These include clathrin itself, the heterotetrameric AP2 adaptor complex and the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2), which recruits AP2 and some of its accessory protein binding partners to the plasmalemma (18,33). The structure of AP2 has been solved by X-ray crystallography. It is comprised of a compact solenoid core that is attached by means of long flexible arms to two globular appendage domains that may grasp accessory proteins from the cytosol for transient coassembly, reminiscent to the appearance of Mr Tickle (a popular figure from animated cartoons in the UK; see http://www.mrsneeze.com/mrmen/meetmrmen.html for further information) (34). Spatiotemporal control is achieved as the network progresses from a largely AP2-adaptor-based assembly process to one that is driven by polymerization of the clathrin scaffold into a lattice comprised of a mixed hexa-/pentagonal array of assembled clathrin triskelia (35). The clathrin lattice appears to act as a molecular fence that serves to organize and concentrate endocytic proteins and lipids into a two-dimensional interaction network. Along this pathway, AP2 and other alternative adaptors, also referred to as CLASPs (for clathrin-associated sorting proteins), sort and concentrate cargo prior to or concomitant with the progressive deformation of the membrane by curvature-inducing accessory proteins such as epsin, amphiphysin and others (36,37). Recruitment and assembly of dynamin by means of SH3-domain-containing factors including amphiphysin, endophilin, intersectin and syndapin to the neck of the forming vesicle finally leads to CCV fission. Vesicle scission critically depends on the ability of dynamin ring-like polymers to constrict upon GTP hydrolysis, similar to a 'garrotte'. This activity may be paired *in vivo* with actin-dependent creation of longitudinal tension along the neck of CCVs [see Roux et al. (38) and references therein].

Although the basic mechanism of CME is similar in neuronal and nonneuronal cells, a number of differences have been noted. Clathrin-mediated endocytosis within the presynaptic compartment appears to depend on intracellular calcium levels (39), perhaps reflecting the close coupling between calcium-triggered SV fusion and endocytosis. Clathrin-coated vesicles isolated from synapses show a very narrow size distribution peaking at about 70–75 nm with a coat comprising 12 clathrin pentagons and about 24 hexagons (40), consistent with a primary role for CME in direct recycling of SVs within the nerve terminal. How such a precise control of CCV

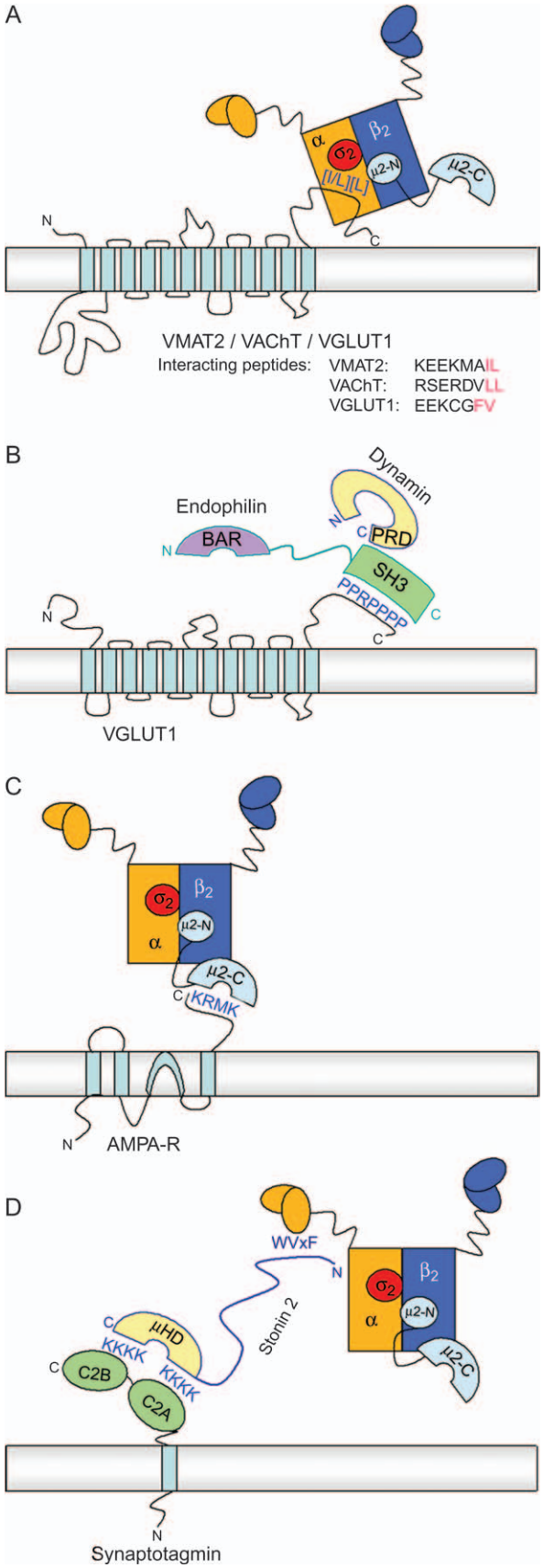

Figure 2: Molecular mechanisms of cargo sorting during CME at synapses. (A) Recognition of D/ExxxL[LI]-type motifs within vesicular NT transporters (VMAT2, VAChT, VGLUT1) by AP2α/σ. (B) Presynaptic cycling of VGLUT1 is affected by interaction of a proline-rich peptide within its cytoplasmic tail with the SH3 domain of the BAR-containing accessory protein endophilin. This mechanism acts in conjunction with D/ExxxL[LI]-type motif recognition as in (A). (C) AMPA receptors undergo endoctyosis at the postsynaptic side by interacting by means of a basic motif within its cytoplasmic tail with AP2μ. (D) The SV calcium-sensing protein synaptotagmin is sorted by the specialized endocytic AP2-binding adaptor stonin 2, which by means of its μ-homology domain (μHD) recognizes basic determinants in its C2 domains.

size is achieved is uncertain, but mutant studies have suggested that the accessory protein AP180 might be involved (8). Given that sustained neurotransmission relies on effective SV endocytosis, the time scale for CME at the presynapse has to function in the order of seconds (9). The fast kinetics of CME may relate to the presence of a number of neuron-specific splice variants or isoforms of proteins participating in CME in the brain. These include neuronal splice variants of clathrin light chains and AP2α, as well as neuron-specific isogenes encoding the accessory proteins AP180 (a homolog of ubiquitously expressed clathrin assembly lymphoid myeloid leukemia protein), endophilin 1, amphiphysin 1, intersectin 1-l, NECAP 1, dynamin 1 [further discussed below (41)] and auxilin 1 (42).

Mr Tickle and Family: Adaptable Adaptors for Cargo Recognition

As clathrin does not bind to lipid bilayers or cargo proteins itself, CCV formation requires the participation of adaptor proteins linking clathrin with membranes and internalizing cargo. More than a dozen clathrin adaptors have been identified, all sharing a common design principle (43). These are composed of structurally compact folded domains with which they bind to proteins or phospholipids, and unstructured linkers that associate with the β-propeller terminal domain of clathrin heavy chain and/or AP2. Adaptor proteins sort membrane cargo by recognizing short-peptide motifs within their cytoplasmic domains (43). Yxxø-type tyrosine-based signals (where ø denotes a large hydrophobic residue) perhaps represent the most common type of endocytic motif and are recognized directly by the C-terminal domain of AP2μ following a phosphorylation and PIP_2-induced conformational change within the complex (44). Internalization of Yxxø-motif-containing cargo is sensitive to RNAi-mediated depletion of AP2 or clathrin- or peptide-mediated sequestration of these components (Figure 1B, stages 1 and 2). Another major class of internalization signals are acidic cluster dileucine motifs. Recent data indicate that these motifs directly associate with AP2 by means of an interface between its α and σ2 subunits (45). D/ExxxL[LI]-type

endocytic signals have been identified in the vesicular transporters for monamines, acetylcholine and glutamate (VGLUT1, further discussed below) (12,46,47) (Figure 2A); synaptobrevin 2; and $GABA_A$ receptor-β2 (48). Apart from these and a few other examples, Yxxø or D/ExxxL[LI] motifs appear to be absent from most other presynaptic vesicle proteins or postsynaptic ion channels. One possible interpretation could be that nonconventional, perhaps synapse-specific, recognition mechanisms are being used, which may reflect the necessity to sort SV proteins or postsynaptic NT transporters away from other constitutively internalized cargo. One such mechanism has recently been described for VGLUT1. Vesicular glutamate receptor 1 associates by means of a proline-rich sequence in its cytoplasmic tail with the accessory protein endophilin that directs the transporter toward an AP2- and D/ExxxL[LI]-motif-dependent fast recycling pathway (12) (Figure 2B).

Alternative endocytosis and AP2-binding signals have also been identified in postsynaptic AMPA-type glutamate and $GABA_A$ receptors. Atypical basic motifs within the cytoplasmic domains of AMPA receptor subunits GluR2 and 3 (Figure 2C) as well as in $GABA_A$ receptor β3 and γ2 subunits associate with a site within AP2μ distinct from that involved in recognition of conventional Yxxø-motif-containing cargo (26,32). In the case of $GABA_A$ receptor-β3, interaction with AP2 and corresponding surface receptor removal are subject to phosphoregulation (32). Moreover, phosphoinositides appear to compete with receptor cytoplasmic domains for AP2μ association, thus providing additional means for modulation, perhaps uncoupling of CME of postsynaptic ion channels from constitutive internalization processes. A similar atypical AP2-binding site is found within the cytoplasmic C2 domains of synaptotagmin family members (49), important regulators of calcium-triggered exo- and endocytosis (50). Converging genetic (51–53) and biochemical studies (49) support a role for synaptotagmin in coupling SV fusion and retrieval. Proteomics data indicate that SVs indeed comprise a very distinct set of proteins present in defined molecular stoichiometries that need to be maintained during repetitive rounds of recycling (54). How this is accomplished at the molecular level is not understood, but direct interactions between synaptotagmin and AP2μ as well as with stonin 2, a specialized endocytic sorting adaptor dedicated to SV retrieval, are likely to be involved (55). Mechanistically, stonin 2 appears to couple synaptotagmin 1 to the clathrin machinery by direct interactions with AP2 (Figure 2D) and other endocytic proteins including eps15, thereby facilitating retrieval of surface-stranded synaptotagmin molecules. Whether all SV proteins indeed must contain their own endocytosis and/or sorting signal remains an open question. Recent high-resolution imaging data suggest that SV components could remain clustered (56) or undergo rapid reclustering (22) during their exo-endocytic itinerary, perhaps through diffusional control involving cholesterol-enriched microdomains (57).

The Many Little Helpers: Accessory Factors in CME

Accessory proteins can be assigned to the following functional classes: (i) CLASPs involved in cargo selection and coat initiation (discussed above); (ii) factors regulating coat maturation and membrane invagination, and proteins regulating; (iii) CCV scission; and (iv) uncoating (Figure 1A). Among these are regulatory enzymes, such as GTPases (Arf6, dynamin), lipid and protein kinases (phosphatidylinositol 4-phosphate 5-kinase PIPKIγ, adaptor-associated kinase AAK1), phosphatases (synaptojanin 1, calcineurin), membrane-bending accessory proteins (amphiphysins, endophilin, epsins), actin-associated factors (eps15, syndapin, intersectin), and proteins involved in vesicle scission (dynamin) and uncoating (auxilin, synaptojanin, hsc70) (42). In general, most of these factors show a modular character with one or more folded domains linked to a natively unfolded region harboring peptide motifs that target the protein to the endocytic network (35,42) centered around clathrin, AP2 and PIP_2 as major hubs. Structural studies have identified distinct sites within the appendage domains of AP2α and β2 that serve as platforms for recruitment of accessory proteins harboring FxDxF, DP[FW], WXX[FW]x[DE], [FL]xxGFxDF or [ED]xxFxx[FL]xxxR motifs (35,58). Likewise, at least two types of clathrin-binding motifs have been identified that associate with the β-propeller terminal domain of its heavy chains (42). Peptides or protein fragments showing one or more of these motifs have been used as dominant-negative inhibitors to interfere with early steps in CCP formation (Figure 1B, stages 1 and 2).

Membrane deformation is thought to be a team effort between accessory proteins that are able to insert amphipathic helices into one leaflet of the lipid bilayer (36,37) and scaffolding proteins that stabilize curved membrane domains, most notably clathrin itself (59). Membrane-bending proteins include the large family of BAR-domain-containing proteins (i.e. amphiphysin, endophilin, syndapin/pacsin, Toca) and ENTH-domain-containing proteins such as epsin (36,37). Amphiphysin, endophilin and syndapin also contain SH3 domains, which by associating with distinct proline-rich peptides aid in recruiting dynamin to the neck of the forming vesicle, thereby coupling membrane deformation to CCV fission (as discussed earlier in this review). Acute chemical (20) or dominant-negative inhibition of dynamin function by SH3 domains, dynamin-derived proline-rich peptides or GTPase-deficient dynamin mutants (Figure 1B, stage 3) results in a complete block of compensatory SV recycling and an increase in the number of surface-active postsynaptic NT receptors (32). Surprisingly, it appears that the brain-specific isoform dynamin 1 is selectively required for the activity-dependent retrieval of SVs during phases of increased calcium influx into the nerve terminal, suggesting a mechanism to plastically adapt CME to high neuronal activity (41).

Fine-Tuning: Regulation of CME

A number of regulatory mechanisms have been described that contribute to adapting CME to the specific physiological requirements of fast chemical neurotransmission. These include a regulated cycle of phosphoinositide turnover nested into the exo-endocytic cycling of SVs [reviewed in Di Paolo and De Camilli (18)] and the calcium-dependent dephosphorylation of a subset of endocytic proteins, termed dephosphins by calcineurin. Among the dephosphins are dynamin 1, amphiphysin, AP180, synaptojanin, epsin and eps15. The calcium-dependent activation of clathrin endocytic proteins allows for the coupling of SV exocytosis with retrieval. Conversely, Cdk5-mediated phosphorylation of dynamin has been shown to inhibit its interaction with the SH3-domain-containing F-BAR protein syndapin, thereby downregulating the efficiency of SV retrieval (60). These data identify an activity-dependent cycle of protein phosphorylation–dephosphorylation that regulates SV internalization. Similar mechanisms operate within the postsynaptic compartment where calcium influx through activated NMDA receptors regulates the number of surface AMPA receptors during cellular models of LTD (3,24).

A regulatory role analogous to phosphorylation might be exerted by the activity-dependent control of protein ubiquitination at synapses (61). The mechanisms are still somewhat unclear but may involve the direct ubiquitination of internalizing cargo membrane proteins (31) or their associated ligands (62) and their recognition by ubiquitin-interacting motif-containing endocytic accessory proteins.

Through the Looking Glass: Tools to Study CME Endocytosis at Synapses

Clathrin-mediated endocytosis at synapses is a fast process that in the case of the presynaptic compartment occurs on a time scale of seconds. Moreover, synapses in most experimental model systems are relatively small structures, often requiring spatial resolution beyond the limits of conventional light microscopy. Thus, historically, electron microscopy has played an important role in delineating endocytic pathways at synapses. The recent advent of fluorescent proteins with new optical or molecular properties combined with novel imaging technologies have allowed unprecedented insights into synaptic CME at work. Most importantly, among these are pHluorins, fusion proteins between a pH-sensitive ecliptic green fluorescent protein moiety and a synaptic cargo of interest. PHluorin fluorescence is quenched at acidic endosomal pH, thus allowing investigators to follow exo-endocytic vesicle trafficking in real time. This technology has been successfully used to tag SV proteins including synaptobrevin 2 (synaptopHluorin), synaptophysin (syPhy) (19), synaptotagmin 1 (sytpHluorin) (22), the VGLUT1 (12), as well as postsynaptic $GABA_A$- and AMPA-type glutamate receptors (54). Amphiphilic fluorescent FM dyes have often been used in conjunction with pHluorins, i.e. to determine cycling SV pool sizes as well as kinetic data. Live-cell fluorescent imaging may be also combined with new technologies to improve spatial resolution such as stimulated emission depletion microscopy (55), providing nanoscale pictures of the synaptic trafficking world.

The detailed structural insights into the architecture of the clathrin-based endocytic machinery described above have provided us with a growing arsenal of molecular weapons to study the role of CME in synaptic physiology. Among the approaches most successfully used has been the microinjection of dominant-interfering peptides or protein domains targeting distinct stages in CME into giant synapses of lamprey (8) or squid. Some of these tools and their stage-specific modes of action are summarized in Figure 1B. Dominant-interfering approaches have also been applied in combination with capacitance measurements or pHluorin-based live-cell imaging in goldfish retinal bipolar terminals or small central synapses, respectively (19).

Virtually, instantaneous perturbation of target proteins has been achieved by small molecule inhibitors or acute photoinactivation. Many early studies had relied on the use of somewhat non-specific inhibitors of CME such as high sucrose or chlorpromazine. The advent of high-throughput chemical biology screening platforms has provided us with a novel acute chemical inhibitor of dynamin, termed dynasore, as the latest addition to the pharmacological tool box (63) (Figure 1B, stage 3). When applied to hippocampal synapses, dynasore reversibly blocks SV retrieval, confirming an essential role for dynamin in this process (20). It should be noted that dynamin is known to also act in clathrin-independent internalization pathways and thus dynasore effects should not be taken as a sole criterion for the involvement of CME in a given physiological process. Fluorescence-assisted light inactivation (FlAsH-FALI) has been suggested as an alternative, genetically directed approach for immediate perturbation of synaptic target proteins by light-induced generation of free radicals. For example, an essential role for synaptotagmin 1 in SV retrieval in *Drosophila* has been inferred from FlAsH-FALI studies using a variant tagged with a tetracysteine motif that allows for binding of the membrane-permeable fluorescein derivative 4′,5′-bis(1,3,2-dithioarsolan-2-yl)-fluorescein (FlAsH) (52) (Figure 1B, stage 1). Whether this tool is generally applicable to inactivate a protein of interest *in vivo* remains to be determined.

Outlook

More than 30 years of research on the mechanism of CME in general and at synapses in particular have provided us

with a detailed molecular framework of the clathrin-based endocytic machinery. The molecular players of the endocytic network are largely known, whereas the details of its spatiotemporal regulation may need an additional decade of intense studies. We still know surprisingly little about the mechanisms that ensure the correct stoichiometry of SV proteins (64) and how the endocytic process within the periactive zone is coupled to the exocytic fusion process. We know that calcium levels and calcium-sensing proteins, such as synaptotagmin, play important roles in this regard. However, we do not understand the actual trigger of endocytosis nor the temporal sequence of events in detail.

Cargo-specific internalization pathways are likely to act at both sides of the synapse, but their molecular details have remained largely obscure. How these pathways are coupled to downstream endosomal sorting events and how this relates to various signaling inputs are additional questions that will need and presumably get answers in the near future.

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References

1. Conner SD, Schmid SL. Regulated portals of entry into the cell. Nature 2003;422:37–44.
2. Girard M, Allaire PD, McPherson PS, Blondeau F. Non-stoichiometric relationship between clathrin heavy and light chains revealed by quantitative comparative proteomics of clathrin-coated vesicles from brain and liver. Mol Cell Proteomics 2005;4:1145–1154.
3. Sheng M, Kim MJ. Postsynaptic signaling and plasticity mechanisms. Science 2002;298:776–780.
4. Dickman DK, Lu Z, Meinertzhagen IA, Schwarz TL. Altered synaptic development and active zone spacing in endocytosis mutants. Curr Biol 2006;16:591–598.
5. Heuser JE, Reese TS. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J Cell Biol 1973;57:315–344.
6. Ceccarelli B, Hurlbut WP, Mauro A. Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. J Cell Biol 1973;57:499–524.
7. Harata NC, Aravanis AM, Tsien RW. Kiss-and-run and full-collapse fusion as modes of exo-endocytosis in neurosecretion. J Neurochem 2006;97:1546–1570.
8. Brodin L, Low P, Shupliakov O. Sequential steps in clathrin-mediated synaptic vesicle endocytosis. Curr Opin Neurobiol 2000;10: 312–320.
9. Murthy VN, De Camilli P. Cell biology of the presynaptic terminal. Annu Rev Neurosci 2003;26:701–728.
10. Wucherpfennig T, Wilsch-Brauninger M, Gonzalez-Gaitan M. Role of Drosophila Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. J Cell Biol 2003;161:609–624.
11. Rizzoli SO, Bethani I, Zwilling D, Wenzel D, Siddiqui TJ, Brandhorst D, Jahn R. Evidence for early endosome-like fusion of recently endocytosed synaptic vesicles. Traffic 2006;7:1163–1176.
12. Voglmaier SM, Kam K, Yang H, Fortin DL, Hua Z, Nicoll RA, Edwards RH. Distinct endocytic pathways control the rate and extent of synaptic vesicle protein recycling. Neuron 2006;51:71–84.
13. Nakatsu F, Okada M, Mori F, Kumazawa N, Iwasa H, Zhu G, Kasagi Y, Kamiya H, Harada A, Nishimura K, Takeuchi A, Miyazaki T, Watanabe M, Yuasa S, Manabe T et al. Defective function of GABA-containing synaptic vesicles in mice lacking the AP-3B clathrin adaptor. J Cell Biol 2004;167:293–302.
14. Gonzalez-Gaitan M, Jackle H. Role of Drosophila alpha-adaptin in presynaptic vesicle recycling. Cell 1997;88:767–776.
15. Zhang B, Koh YH, Beckstead RB, Budnik V, Ganetzky B, Bellen HJ. Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. Neuron 1998;21:1465–1475.
16. Di Paolo G, Sankaranarayanan S, Wenk MR, Daniell L, Perucco E, Caldarone BJ, Flavell R, Picciotto MR, Ryan TA, Cremona O, De Camilli P. Decreased synaptic vesicle recycling efficiency and cognitive deficits in amphiphysin 1 knockout mice. Neuron 2002;33:789–804.
17. Dickman DK, Horne JA, Meinertzhagen IA, Schwarz TL. A slowed classical pathway rather than kiss-and-run mediates endocytosis at synapses lacking synaptojanin and endophilin. Cell 2005;123:521–533.
18. Di Paolo G, De Camilli P. Phosphoinositides in cell regulation and membrane dynamics. Nature 2006;443:651–657.
19. Granseth B, Odermatt B, Royle SJ, Lagnado L. Clathrin-mediated endocytosis is the dominant mechanism of vesicle retrieval at hippocampal synapses. Neuron 2006;51:773–786.
20. Newton AJ, Kirchhausen T, Murthy VN. Inhibition of dynamin completely blocks compensatory synaptic vesicle endocytosis. Proc Natl Acad Sci U S A 2006;103:17955–17960.
21. Blondeau F, Ritter B, Allaire PD, Wasiak S, Girard M, Hussain NK, Angers A, Legendre-Guillemin V, Roy L, Boismenu D, Kearney RE, Bell AW, Bergeron JJ, McPherson PS. Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling. Proc Natl Acad Sci U S A 2004;101:3833–3838.
22. Wienisch M, Klingauf J. Vesicular proteins exocytosed and subsequently retrieved by compensatory endocytosis are nonidentical. Nat Neurosci 2006;9:1019–1027.
23. Fernandez-Alfonso T, Kwan R, Ryan TA. Synaptic vesicles interchange their membrane proteins with a large surface reservoir during recycling. Neuron 2006;51:179–186.
24. Horton AC, Ehlers MD. Neuronal polarity and trafficking. Neuron 2003;40:277–295.
25. Racz B, Blanpied TA, Ehlers MD, Weinberg RJ. Lateral organization of endocytic machinery in dendritic spines. Nat Neurosci 2004;7:917–918.
26. Kastning K, Kukhtina V, Kittler JT, Chen G, Pechstein A, Enders S, Lee SH, Sheng M, Yan Z, Haucke V. Molecular determinants for the interaction between AMPA receptors and the clathrin adaptor complex AP-2. Proc Natl Acad Sci U S A 2007;104:2991–2996.
27. Lee SH, Liu L, Wang YT, Sheng M. Clathrin adaptor AP2 and NSF interact with overlapping sites of GluR2 and play distinct roles in AMPA receptor trafficking and hippocampal LTD. Neuron 2002;36:661–674.
28. Tomita S, Fukata M, Nicoll RA, Bredt DS. Dynamic interaction of stargazin-like TARPs with cycling AMPA receptors at synapses. Science 2004;303:1508–1511.
29. Chowdhury S, Shepherd JD, Okuno H, Lyford G, Petralia RS, Plath N, Kuhl D, Huganir RL, Worley PF. Arc/Arg3.1 interacts with the endocytic machinery to regulate AMPA receptor trafficking. Neuron 2006;52: 445–459.

30. Hanley JG, Henley JM. PICK1 is a calcium-sensor for NMDA-induced AMPA receptor trafficking. EMBO J 2005;24:3266–3278.
31. Burbea M, Dreier L, Dittman JS, Grunwald ME, Kaplan JM. Ubiquitin and AP180 regulate the abundance of GLR-1 glutamate receptors at postsynaptic elements in C. elegans. Neuron 2002;35:107–120.
32. Kittler JT, Chen G, Honing S, Bogdanov Y, McAinsh K, Arancibia-Carcamo IL, Jovanovic JN, Pangalos MN, Haucke V, Yan Z, Moss SJ. Phospho-dependent binding of the clathrin AP2 adaptor complex to GABAA receptors regulates the efficacy of inhibitory synaptic transmission. Proc Natl Acad Sci U S A 2005;102:14871–14876.
33. Krauss M, Haucke V. Phosphoinositide-metabolizing enzymes at the interface between membrane traffic and cell signalling. EMBO Rep 2007;8:241–246.
34. Collins BM, McCoy AJ, Kent HM, Evans PR, Owen DJ. Molecular architecture and functional model of the endocytic AP2 complex. Cell 2002;109:523–535.
35. Schmid EM, Ford MG, Burtey A, Praefcke GJ, Peak-Chew SY, Mills IG, Benmerah A, McMahon HT. Role of the AP2 beta-appendage hub in recruiting partners for clathrin-coated vesicle assembly. PLoS Biol 2006;4:e262.
36. McMahon HT, Gallop JL. Membrane curvature and mechanisms of dynamic cell membrane remodelling. Nature 2005;438:590–596.
37. Itoh T, De Camilli P. BAR, F-BAR (EFC) and ENTH/ANTH domains in the regulation of membrane-cytosol interfaces and membrane curvature. Biochim Biophys Acta 2006;1761:897–912.
38. Roux A, Uyhazi K, Frost A, De Camilli P. GTP-dependent twisting of dynamin implicates constriction and tension in membrane fission. Nature 2006;441:528–531.
39. Gad H, Low P, Zotova E, Brodin L, Shupliakov O. Dissociation between Ca2+-triggered synaptic vesicle exocytosis and clathrin-mediated endocytosis at a central synapse. Neuron 1998;21:607–616.
40. Fotin A, Cheng Y, Sliz P, Grigorieff N, Harrison SC, Kirchhausen T, Walz T. Molecular model for a complete clathrin lattice from electron cryomicroscopy. Nature 2004;432:573–579.
41. Ferguson SM, Brasnjo G, Hayashi M, Wolfel M, Collesi C, Giovedi S, Raimondi A, Gong LW, Ariel P, Paradise S, O'Toole E, Flavell R, Cremona O, Miesenbock G, Ryan TA et al. A selective activity-dependent requirement for dynamin 1 in synaptic vesicle endocytosis. Science 2007;316:570–574.
42. McPherson PS, Ritter B. Peptide motifs: building the clathrin machinery. Mol Neurobiol 2005;32:73–87.
43. Owen DJ, Collins BM, Evans PR. Adaptors for clathrin coats: structure and function. Annu Rev Cell Dev Biol 2004;20:153–191.
44. Honing S, Ricotta D, Krauss M, Spate K, Spolaore B, Motley A, Robinson M, Robinson C, Haucke V, Owen DJ. Phosphatidylinositol-(4, 5)-bisphosphate regulates sorting signal recognition by the clathrin-associated adaptor complex AP2. Mol Cell 2005;18:519–531.
45. Doray B, Lee I, Knisely J, Bu G, Kornfeld S. The {gamma}/{sigma}1 and {alpha}/{sigma}2 hemicomplexes of clathrin adaptors AP-1 and AP-2 harbor the dileucine recognition site. Mol Biol Cell 2007;18:1887–1896.
46. Krantz DE, Waites C, Oorschot V, Liu Y, Wilson RI, Tan PK, Klumperman J, Edwards RH. A phosphorylation site regulates sorting of the vesicular acetylcholine transporter to dense core vesicles. J Cell Biol 2000;149:379–396.
47. Tan PK, Waites C, Liu Y, Krantz DE, Edwards RH. A leucine-based motif mediates the endocytosis of vesicular monoamine and acetylcholine transporters. J Biol Chem 1998;273:17351–17360.
48. Herring D, Huang R, Singh M, Robinson LC, Dillon GH, Leidenheimer NJ. Constitutive GABAA receptor endocytosis is dynamin-mediated and dependent on a dileucine AP2 adaptin-binding motif within the beta 2 subunit of the receptor. J Biol Chem 2003;278:24046–24052.
49. Grass I, Thiel S, Honing S, Haucke V. Recognition of a basic AP-2 binding motif within the C2B domain of synaptotagmin is dependent on multimerization. J Biol Chem 2004;279:54872–54880.
50. Bai J, Chapman ER. The C2 domains of synaptotagmin – partners in exocytosis. Trends Biochem Sci 2004;29:143–151.
51. Jorgensen EM, Hartwieg E, Schuske K, Nonet ML, Jin Y, Horvitz HR. Defective recycling of synaptic vesicles in synaptotagmin mutants of Caenorhabditis elegans. Nature 1995;378:196–199.
52. Poskanzer KE, Marek KW, Sweeney ST, Davis GW. Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo. Nature 2003;426:559–563.
53. Nicholson-Tomishima K, Ryan TA. Kinetic efficiency of endocytosis at mammalian CNS synapses requires synaptotagmin I. Proc Natl Acad Sci U S A 2004;101:16648–16652.
54. Ashby MC, Maier SR, Nishimune A, Henley JM. Lateral diffusion drives constitutive exchange of AMPA receptors at dendritic spines and is regulated by spine morphology. J Neurosci 2006;26:7046–7055.
55. Diril MK, Wienisch M, Jung N, Klingauf J, Haucke V. Stonin 2 is an AP-2-dependent endocytic sorting adaptor for synaptotagmin internalization and recycling. Dev Cell 2006;10:233–244.
56. Willig KI, Rizzoli SO, Westphal V, Jahn R, Hell SW. STED microscopy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis. Nature 2006;440:935–939.
57. Jia J, Lamer S, Schuemann M, Schmidt M, Krause E, Haucke V. Quantitative proteomic analysis of detergent-resistant membranes from chemical synapses: evidence for cholesterol as spatial organizer of synaptic vesicle cycling. Mol Cell Proteomics 2006;5:2060–2071.
58. Edeling MA, Mishra SK, Keyel PA, Steinhauser AL, Collins BM, Roth R, Heuser JE, Owen DJ, Traub LM. Molecular switches involving the AP-2 beta2 appendage regulate endocytic cargo selection and clathrin coat assembly. Dev Cell 2006;10:329–342.
59. Hinrichsen L, Meyerholz A, Groos S, Ungewickell EJ. Bending a membrane: how clathrin affects budding. Proc Natl Acad Sci U S A 2006;103:8715–8720.
60. Anggono V, Smillie KJ, Graham ME, Valova VA, Cousin MA, Robinson PJ. Syndapin I is the phosphorylation-regulated dynamin I partner in synaptic vesicle endocytosis. Nat Neurosci 2006;9:752–760.
61. Chen H, Polo S, Di Fiore PP, De Camilli PV. Rapid Ca2+-dependent decrease of protein ubiquitination at synapses. Proc Natl Acad Sci U S A 2003;100:14908–14913.
62. Colledge M, Snyder EM, Crozier RA, Soderling JA, Jin Y, Langeberg LK, Lu H, Bear MF, Scott JD. Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression. Neuron 2003;40:595–607.
63. Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T. Dynasore, a cell-permeable inhibitor of dynamin. Dev Cell 2006;10:839–850.
64. Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, Urlaub H, Schenck S, Brugger B, Ringler P, Muller SA, Rammner B, Grater F, Hub JS, De Groot BL et al. Molecular anatomy of a trafficking organelle. Cell 2006;127:831–846.