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Review

Studies of Synaptic Vesicle Endocytosis in the Nematode *C. elegans*

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After synaptic vesicle exocytosis, synaptic vesicle proteins must be retrieved from the plasma membrane, sorted away from other membrane proteins, and reconstituted into a functional synaptic vesicle. The nematode *Caenorhabditis elegans* is an organism well suited for a genetic analysis of this process. In particular, three types of genetic studies have contributed to our understanding of synaptic vesicle endocytosis. First, screens for mutants defective in synaptic vesicle recycling have identified new proteins that function specifically in neurons. Second, RNA interference has been used to quickly confirm the roles of known proteins in endocytosis. Third, gene targeting techniques have elucidated the roles of genes thought to play modulatory or subtle roles in synaptic vesicle recycling. We describe a molecular model for synaptic vesicle recycling and discuss how protein disruption experiments in *C. elegans* have contributed to this model.

Key words: *C. elegans*, neurotransmission, synaptic vesicle, endocytosis

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Neurons communicate with each other by secreting neurotransmitters at synapses between cells (Figure 1). Classical neurotransmitters, such as GABA, glutamate, and acetylcholine are small molecules, which are synthesized in the cytoplasm and packaged into synaptic vesicles. Synaptic vesicles require a large repertoire of proteins to function (1). Synaptic vesicle proteins are required for transport of the vesicle to the nerve terminal, loading of neurotransmitter into the synaptic vesicle, association of the vesicle with the cytoskeleton, and docking the vesicle at the plasma membrane in preparation for release. Of particular importance is the vesicle-associated SNARE protein synaptobrevin (VAMP), which is required for fusion, and synaptotagmin, which is the calcium sensor required for regulated release. Fusion results in the dispersion of synaptic vesicle proteins into the plasma membrane. These proteins must be retrieved to regenerate a functional synaptic vesicle. Although a direct route for the recovery of synaptic vesicles, called 'kiss and run' exocytosis, may exist (2), the primary mechanism for membrane and protein retrieval is through clathrin-mediated endocytosis.

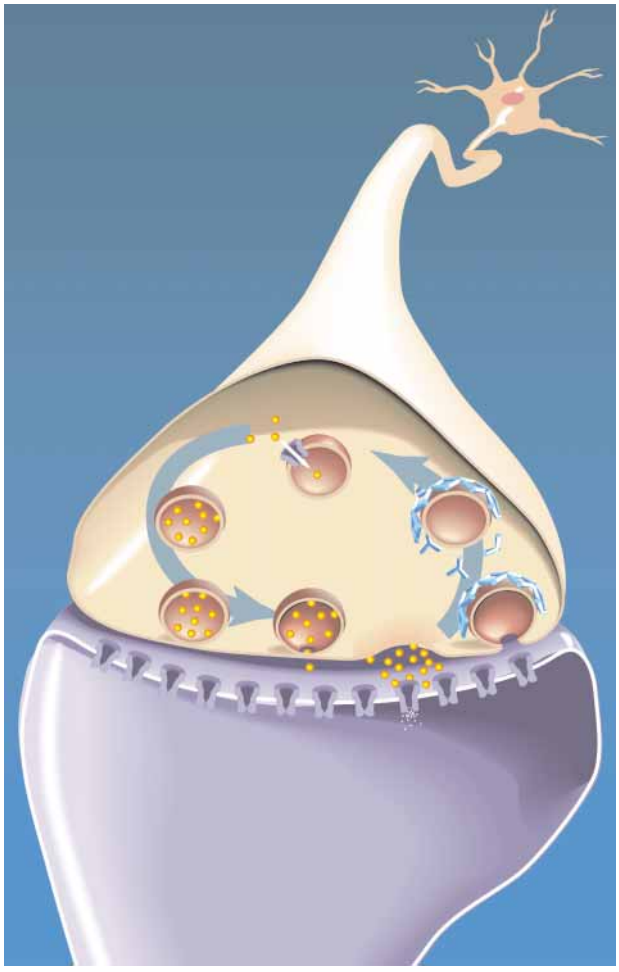

Figure 1: The synaptic vesicle cycle. Synaptic vesicles in the reserve pool are held near release sites by the cytoskeleton. They can be mobilized to dock with the plasma membrane, but only primed vesicles are readily releasable when the neuron is depolarized. Calcium influx causes the vesicle membrane to fuse with the plasma membrane and neurotransmitter is released into the synaptic cleft. The synaptic vesicle proteins and membrane must be recovered via endocytosis. Finally, the regenerated vesicles need to be loaded with neurotransmitter to make a mature synaptic vesicle.

There are four steps to synaptic vesicle recycling: recruitment, budding, fission, and uncoating (Figure 3) (3). First, during recruitment, the clathrin adaptor complex AP2 and synaptic vesicle proteins are recruited to the site of endocytosis. AP2 then facilitates the recruitment of clathrin to the presumptive vesicle. This suggests that a region of the plasma membrane is marked for endocytosis and that the AP2 complex and synaptic vesicle proteins are specifically recruited to this region. These observations introduce two questions: What proteins are required for the targeting of AP2 to the plasma membrane? What proteins are required to recruit synaptic vesicle proteins to the site of endocytosis?

In the second step of synaptic vesicle recycling, assembly of the clathrin matrix pulls membrane into the cell to form a budding vesicle. Synaptic vesicles are uniform in size; however, in *in vitro* experiments, clathrin self-assembles into cages that are of variable sizes (4). This suggests that, although clathrin is sufficient for the formation of vesicles, it is insufficient to regulate the size of the vesicle. What components assist clathrin in forming the vesicle and in regulating its size?

Third, during the fission step, the GTPase dynamin is required to cleave the clathrin-coated vesicle from the plasma membrane. Dynamin interacts with a variety of proteins and lipids of the synaptic vesicle (5). These interactions may serve to position dynamin at the neck of the vesicle prior to cleavage. Is the primary function of these dynamin-interacting proteins to localize dynamin, or do they play additional roles as well?

In the fourth step, the clathrin coat is removed from the recycled vesicle. This step occurs very rapidly, suggesting that it is tightly regulated. One protein with a well-documented *in vitro* role in clathrin coat removal is the ATPase chaperone Hsc70. How is Hsc70 action regulated *in vivo*? What proteins remove the clathrin coat from the vesicle? What proteins remove the clathrin adaptor complexes after endocytosis?

Perturbation Studies of Synaptic Vesicle Endocytosis

A large number of proteins have already been described that are thought to play a role in endocytosis [Table 1, and reviewed in (6)]. Most of these were identified based on interactions with other endocytic proteins. Subsequent disruption experiments, such as antibody or peptide injections, have confirmed the requirement for many of these proteins in synaptic vesicle endocytosis. Injected antibodies bind the target protein and block its function by occluding other protein interactions. Injections of specific antibodies into neurons, such as the lamprey reticulospinal neurons, have been shown to block synaptic vesicle endocytosis [for examples, see (7,8)]. Peptides can also be injected into cells (9,10). These peptides usually comprise an interaction domain of a protein, and disrupt specific protein–protein interactions by competing with the endogenous interaction. Both types of disruption experiments have limitations. First, the disrupted protein may still have residual function resulting in a phenotype that is less severe than expected for a null mutation. Second, the disruption may affect other proteins, and the phenotype may be more severe than expected for a loss of the protein. An alternative to these types of perturbation experiments is to study mutations in the genes encoding these proteins in a genetic model organism.

The nematode *C. elegans* is well suited for genetic studies of endocytosis for several reasons (11). First, *C. elegans* is easily maintained in the laboratory and has a rapid generation time of only 3 days. Second, the molecular mechanisms of endocytosis are conserved in *C. elegans*. The worm genome encodes all of the proteins that have been implicated in endocytosis from previous biochemical and protein disruption experiments (Table 1). Third, functional redundancy has proven to be less of a problem in *C. elegans* than in vertebrate systems. The genome of *C. elegans* frequently has single copies of genes that are found in multiple copies in vertebrates. Fourth, the greatest benefit of studying a biological process in *C. elegans* is that gene disruptions can be easily generated. For this reason, the next part of this review will discuss the forward and reverse genetic techniques that are available in *C. elegans*.

The goal of forward genetics is to identify the molecules involved in a biological process. Forward genetic screens identify mutants that are defective in that process. Here, we discuss screens for mutants with defects in synaptic vesicle recycling; these mutants usually exhibit an uncoordinated phenotype. The starting point for reverse genetics is an interesting molecule. The goal of the geneticist is to identify the biological process in which the molecule functions. We discuss two approaches, RNA interference (RNAi) and targeted deletion, which are used to disrupt proteins implicated in endocytosis.

Forward genetic screens

The main strength of *C. elegans* is its use in forward genetic screens. Several unusual attributes of this organism have facilitated the identification of genes that function in synaptic vesicle recycling. First, the *C. elegans* nervous system is largely nonessential in the laboratory. Feeding is mediated by a muscular pump that requires almost no neuronal innervation (12,13). Moreover, reproduction does not require a functional nervous system, since *C. elegans* are self-fertilizing hermaphrodites. Thus, even mutations that severely disrupt nervous system function are likely to be viable, and recoverable in a genetic screen.

Second, not only are mutants defective for nervous system function viable, but such mutants can be selected in mutagenic screens, specifically, by using pharmacological agents such as acetylcholinesterase inhibitors. At neuromuscular junctions acetylcholine is released into the synaptic cleft by exocytosis. Acetylcholine in the cleft is degraded by acetylcholinesterase. Blocking acetylcholinesterase activity by

Table 1: *C. elegans* homologs of proteins implicated in endocytosis

Protein	Gene	Mutant phenotype	References
Amphiphysin	F58G6.1	–	–
Auxilin	W07A8.3	RNAi – larval arrest, clathrin mislocalization, defects in receptor-mediated endocytosis	(29)
Clathrin heavy chain	T20G5.1	RNAi – dead embryos	(25)
Clathrin light chain	T05B11.3	–	–
	W05H9.1	–	–
Clathrin adaptors			
α-adaptin	T20B5.1	RNAi – dead embryos	(25)
β-adaptin	Y71H2B.10	RNAi – dead embryos	(25)
σ2 (AP17)	F02E8.3	RNAi – dumpy morphology	(25)
μ2 (AP50)	*dpy-23*	Dumpy morphology	(P. Baum and G. Garriga, personal communication)
AP180/CALM	*unc-11*	Uncoordinated, mislocalization of synaptobrevin; increased diameter of synaptic vesicles	(21)
Dap160/intersectin	Y116A8C.36	–	–
Dynamin	*dyn-1*	Temperature-sensitive allele: uncoordinated phenotype in adults and lethal in embryos at restrictive temperature. Null allele: lethal at all temperatures	(18) (A. van der Bliek, personal communication)
Endophilin	T04D1.3	–	–
EPS15	*ehs-1*	Temperature-sensitive defect in endocytosis in null mutant	(28)
Epsin	T04C10.2	–	–
Hsc70	F26D10.3	RNAi – embryonic lethal	(30)
Stoned B	C27H6.1	–	–
Synaptojanin	*unc-26*	Defects throughout recycling pathway	(19)
Synaptotagmin	*snt-1*	Defect in early stage of endocytosis	(31)
Syndapin/pacsin	F45E1.7	WT RNAi phenotype	(32)

nerve gas in vertebrates or by specific pesticides in nematodes causes acetylcholine to accumulate in the synaptic cleft (14). This accumulation will eventually kill a worm due to chronic activation of acetylcholine receptors. Mutants that cannot generate synaptic vesicles due to defects in vesicle recycling, or mutants that cannot release vesicles due to defects in exocytosis cannot release acetylcholine into the synaptic cleft. Such mutants are resistant to acetylcholinesterase inhibitors. Genetic screens have led to the identification of 22 genes in this pathway (15–17). It should eventually be possible using such drug resistance assays to conduct a saturation mutagenesis, identifying all the genes capable of being mutated to the resistant phenotype. Five mutants resistant to acetylcholinesterase inhibitors disrupt synaptic vesicle recycling. These mutants, *dyn-1* (dynamin), *snt-1* (synaptotagmin), *unc-11* (AP180), *unc-26* (synaptojanin), and *unc-41* also exhibit similar behavioral phenotypes; they are small, slow growing, and move in a jerky uncoordinated phenotype (18–21).

Despite the success of forward genetic screens in *C. elegans*, certain classes of genes will not be isolated in these screens. In particular, essential genes that mutate to lethality will be lost in most forward screens. Furthermore, genes that play a modulatory role will likely lead to extremely subtle phenotypes that are easily overlooked in a genetic screen. To address these problems, two reverse genetic approaches can be employed – RNA interference of gene function and the generation of targeted deletions.

RNA interference

RNA interference (RNAi) can perturb protein function without requiring the generation of a mutant. Using this method, double-stranded RNA, which is identical in sequence to the gene to be disrupted, is introduced into the worm by injection or feeding (22,23). The double-stranded RNA targets the endogenous mRNA for degradation and thereby blocks synthesis of the protein (24). RNAi has been used to study proteins that are required for endocytosis in all tissues. Mutations in genes such as this are difficult to isolate in standard forward genetic screens, and would be absent altogether in a screen based on behavioral phenotypes of the adult. For example, RNAi has been used to study the importance of clathrin and the clathrin adaptor complex in *C. elegans* (25). This study demonstrated that these proteins are essential for viability of the organism. Although RNAi is an effective method for disrupting genes required early during worm development, it also possesses certain limitations. The method is less effective for proteins required post-embryonically, and is specifically ineffective against proteins expressed in neurons (22,26). To study genes thought to play more subtle roles in endocytosis in the nervous system, conventional mutations are required.

Deletion mutants

Proteins that play a modulatory role are likely to lead to subtle phenotypes, and would be difficult to recover in forward genetic screens. The function of such proteins can still be determined in *C. elegans* by searching for small deletions that specifically remove the gene (27). Using this method, worms are exposed to mutagens which introduce double-strand breaks. A deletion of a particular gene in a strain is identified by PCR. Using this methodology, a deletion knockout was isolated and used to characterize the requirement for the *C. elegans* homolog of Eps15 (EHS-1) in synaptic vesicle recycling (28).

Both forward and reverse genetic approaches have their inherent biases. Forward genetics is good for identifying previously unknown proteins that act in a specific process. However, if a gene is mutated to lethality or plays a subtle modulatory role, it will be difficult to recover in such screens. On the other hand, reverse genetics is useful for determining the function of a previously identified protein, but the protein must be implicated in that process before it will be targeted. Using both forward and reverse genetic approaches will enable a thorough analysis of endocytosis in *C. elegans*.

A Molecular Model for Synaptic Vesicle Recycling

In the introduction, we described the four steps in the recycling of a synaptic vesicle: recruitment, budding, fission, and uncoating. We also described some of the remaining questions at each of these steps. In the remainder of this review we will revisit these issues, describing the contributions of studies in *C. elegans* to the resolution of these mysteries in relation to a model for synaptic vesicle endocytosis (Figure 2).

Synaptic vesicle protein and clathrin recruitment

The initial stages of vesicle recycling can be divided into three related steps: marking a region of the plasma membrane for internalization, recruitment of vesicle proteins to the site of endocytosis, followed by the recruitment of clathrin. The signals that designate the region of membrane to be recycled are not known, but are likely to depend on both specific lipid and protein interactions. Targeting of the clathrin adaptor complex AP2 to the membrane may be sufficient to demarcate a region for endocytosis. Studies of receptor-mediated endocytosis reveal that interactions between the μ2 subunit of AP2 and the receptor assist in receptor internalization (33). Thus, there is a direct link between the protein targeted for endocytosis and clathrin assembly. A candidate for the AP2 receptor during the endocytosis of synaptic vesicles is synaptotagmin, the putative calcium sensor for synaptic vesicle exocytosis. Following exocytosis, this synaptic vesicle protein will be located in the plasma membrane and could signal to the endocytic machinery that there are proteins that need to be recycled. Because synaptotagmin is located on the vesicle but resides on the plasma membrane following vesicle fusion, it is in the appropriate position to recruit the

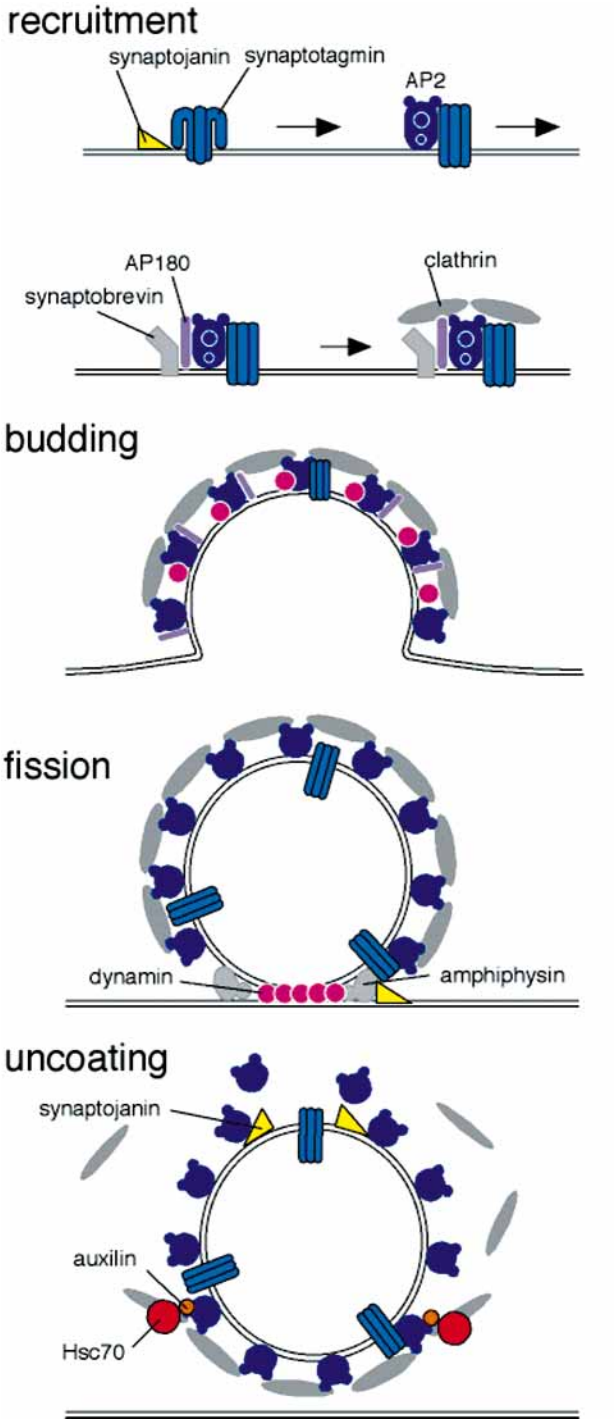

Figure 2: A model for synaptic vesicle endocytosis. 1. Recruitment of adaptors and clathrin. Synaptojanin releases synaptotagmin C2B domain from the membrane. Synaptotagmin is the receptor for the clathrin adaptor complex. 2. Budding. The clathrin coat is converted from a flat hexagonal array to a sphere by the actions of AP2 and AP180. 3. Fission. The neck of the vesicle is severed by dynamin. 4. Uncoating. Clathrin is removed by the action of auxilin and the ATPase Hsc70. The adaptors are removed by synaptojanin.

endocytic complex to the plasma membrane. The first evidence of a role for synaptotagmin in endocytosis came from biochemical experiments which demonstrated that the C2B domain of synaptotagmin binds to both μ2 and α-adaptin subunits of the AP2 clathrin adaptor complex (8,34–36).

In vivo support for a role of synaptotagmin in synaptic vesicle recycling came from studies of the *C. elegans snt-1* mutant (31). Animals defective for synaptotagmin exhibit a depletion of synaptic vesicles at the neuromuscular junction, as revealed in electron micrographs (Figure 3). A block in vesicle recovery from the membrane causes this depletion, rather than a failure in the *de novo* production of synaptic vesicles. If axonal transport of vesicles is blocked, vesicles accumulate in the neuron cell body, demonstrating that vesicles are still synthesized in the absence of synaptotagmin. Moreover, other synaptic vesicle proteins are transported to the synapse in synaptotagmin mutants. However, these proteins are diffusely distributed in the plasma membrane at the terminal, because they are not recycled and sequestered into synaptic vesicles.

Thus, it is likely that the adaptor complex is directed to a site of endocytosis by interactions with a specific synaptic vesicle protein. Other data from *C. elegans* mutants indicate that lipids may also play a role in AP2 recruitment. The *unc-26* gene encodes the *C. elegans* homolog of the synaptojanin protein (19). Synaptojanin is a polyphosphoinositide phosphatase implicated in vesicle recycling by its interactions with amphiphysin (37). Synaptojanin contains two distinct phosphatase domains that cleave phosphates at the 3-, 4-, and 5-positions from polyphosphoinositides (37,38).

Synaptic vesicles are depleted in *unc-26* mutants (Figure 3) (19). This depletion is partly due to a defect in fission of endocytic pits. However, the number of synaptic vesicles trapped as endocytic intermediates is not equal to the number of synaptic vesicles normally found at a synapse. These data suggest that there may be a defect in the recruitment of clathrin or clathrin adaptors to the sites of endocytosis. One potential target of synaptojanin function may be synaptotagmin. The C2B domain of synaptotagmin binds PIP_2 (39); this association may occlude interactions of the C2B domain with the AP2 clathrin adaptor proteins (36). Accumulation of PIP_2 in the absence of synaptojanin may result in a sequestration of synaptotagmin C2B domains by membrane interactions and hence block clathrin recruitment.

The AP2 adaptor complex is another potential target for synaptojanin function. AP2 binding to phosphoinositide-3,4,5-

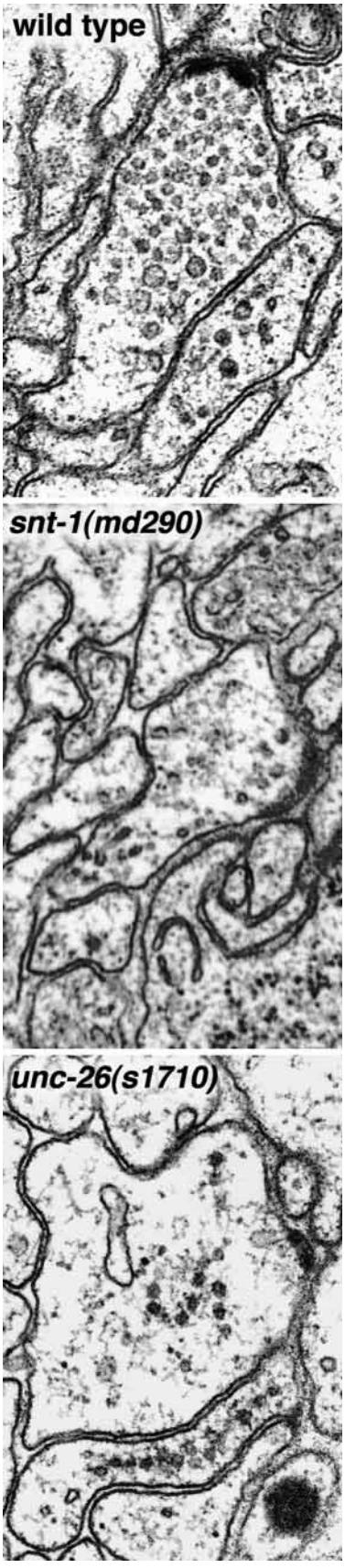

Figure 3: The *C. elegans* neuromuscular junction. Synaptic vesicles are clustered around the presynaptic specialization at a *C. elegans* neuromuscular junction in the wild type. In synaptotagmin mutants (*snt-1*), synaptic vesicles are partially depleted. In synaptojanin mutants (*unc-26*), synaptic vesicles are severely depleted and those that are present are bound to a cytoskeletal filament.

trisphosphate (PIP_3) inhibits binding of the complex to clathrin (40); accumulation of PIP_3 in synaptojanin mutants may inhibit clathrin assembly at the membrane. Alternatively, coated pits may be rare in synaptojanin mutants because clathrin abundance is limiting for pit formation (41). In this case synaptojanin may not play a specific role at the recruitment stage of endocytosis, but because it is required to liberate clathrin at later steps, clathrin is depleted and as a consequence an early defect in endocytosis is observed.

It is not clear that the recruitment defect in *unc-26* mutants is due to a loss of phosphatase activity. Synaptojanin binds to other members of the endocytic complex, such as amphiphysin, endophilin, syndapin, and intersectin (37,42–44). Therefore, the recruitment defect observed in *unc-26* mutants could be due to a failure in forming interactions with other proteins required for endocytosis. However, one of the severest synaptojanin mutations in *C. elegans* is a point mutation in the active site of the 5-phosphatase domain, suggesting that the lipid catalysis is the relevant function of synaptojanin (19). Despite the uncertainty of the role that *unc-26* plays in recruitment of clathrin, it does appear that there is a specific requirement for phospholipid composition in this process based on the large number of lipid binding proteins involved.

Once a region on the plasma membrane has been marked for endocytosis, synaptic vesicle proteins must be recruited to this site so that they can be incorporated into the recycling vesicle. It is possible that synaptic vesicle proteins bind to each other to form a raft of proteins in the plasma membrane. Alternatively, specialized adaptors may recruit particular proteins to sites of clathrin assembly. An adaptor protein appears to be required for synaptobrevin recruitment. Specifically, synaptobrevin recycling requires the clathrin assembly protein AP180 (21). In *C. elegans* AP180 (*unc-11*) mutants, GFP-tagged synaptobrevin is diffusely distributed on the plasma membrane, indicating that it is not recovered from the membrane. Other vesicle proteins, such as synaptotagmin, colocalize with synaptic vesicle clusters, indicating that these proteins are normally recovered from the plasma membrane. These data suggest that AP180 is specifically required for synaptobrevin recruitment, although a direct interaction between synaptobrevin and AP180 has not been demonstrated. Other proteins might require different proteins for recruitment to the site of endocytosis.

After synaptic vesicle proteins are localized to sites of endocytosis, clathrin triskelions, composed of three heavy chains and three light chains, assemble into a lattice on the plasma membrane at these sites (45). Clathrin recruitment and assembly is promoted by binding to the clathrin adaptor proteins AP2 and AP180, which are targeted to sites of endocytosis by interacting with specific proteins and phospholipids in the plasma membrane (40,46,47).

Vesicle budding

As the clathrin matrix assembles on the plasma membrane, the membrane begins to invaginate. Clathrin probably assembles as a flat hexagonal matrix, and curvature is caused by the introduction of pentagons into the matrix (45). As the vesicle is formed, the edge of the bud becomes more deeply invaginated. Since clathrin by itself can assemble into cages that have a variable diameter, and all synaptic vesicles are of a uniform size, specific factors must regulate the size of synaptic vesicles.

Studies on the *C. elegans* gene *unc-11* show that the clathrin adaptor protein AP180 is responsible for regulating synaptic vesicle size (21). In the wild-type, synaptic vesicles are 30 nm in diameter, whereas in *unc-11* mutants, the vesicle diameter is increased to 40 nm. These large vesicles occasionally appear to be docked at the plasma membrane, providing evidence that they are bona fide synaptic vesicles and not a recycling intermediate. Mutants in *Drosophila* AP180 also exhibit an increase in synaptic vesicle size, with a corresponding increase in quantal size (which reflects the amount of neurotransmitter in a single synaptic vesicle) indicating that these large vesicles are competent for release and are thus bona fide synaptic vesicles (48).

The requirement for AP180 in regulating vesicle size is somewhat surprising, since a more critical role in clathrin-mediated endocytosis was expected. AP180 can recruit clathrin to liposome membranes, and promote assembly of clathrin cages in the absence of AP2 and under conditions that do not support clathrin self-assembly. These clathrin cages show a tight size distribution of 60–90 nm (49). Furthermore, AP180 binds to AP2, and together they increase the rate at which a clathrin lattice forms on a membrane (50). However, the phenotype observed in *unc-11* mutants indicates that animals defective for AP180 are still capable of endocytosing plasma membrane. This suggests that AP180 does not play an essential role in clathrin recruitment in *C. elegans*, but may instead regulate the angle between clathrin triskelions at the vertices. Alternatively, AP180 may be required for AP2-mediated endocytosis. In the absence of AP180, alternative pathways such as kiss and run (2) or AP3-mediated endocytosis (51) may predominate, and these mechanisms yield vesicles of different size. However, the experiments described below indicate that bypass mechanisms for AP2-mediated endocytosis cannot function in all tissues.

The role of clathrin and the adaptor complex have been probed in *C. elegans* using RNAi (25). Elimination of clathrin heavy chain resulted in lethality, as might be expected, since AP1, AP2 and AP3-mediated budding would all be eliminated. In addition, interference of the AP2 complex subunits, α-adaptin, and β-adaptin, also caused lethality and blocked receptor-mediated endocytosis (25). Interestingly, elimination of the other subunits of the AP2 complex, μ2 and σ2, did not disrupt receptor-mediated endocytosis. In addition, these animals are viable, suggesting one of two things: either μ2 and σ2 may not be ubiquitously required for clathrin-mediated endocytosis in *C. elegans*, or alternatively, μ2 and σ2 may be functionally redundant with other μ and σ subunits, such as those in AP1 or AP3 complexes.

Vesicle fission

During the fission step of synaptic vesicle recycling, the budded vesicle is cleaved from the plasma membrane. A collar can be seen around the constricted neck of the coated vesicle, composed of the large GTPase, dynamin (5). These endocytic intermediate structures are rarely observed in normal synapses, but can be observed when endocytosis is blocked prior to fission. Temperature sensitive mutations in the GTPase domain of *shibire*, the gene encoding dynamin in *Drosophila*, lead to a reversible block in vesicle recycling, with the appearance of collared endocytic intermediates in the plasma membrane (52). It has been proposed that dynamin forms a collar around the endocytosed vesicle; the collar is constricted upon GTP hydrolysis, thereby releasing the vesicle from the plasma membrane (53), although there are alternatives to the 'pinchase' model (54).

In *C. elegans*, dynamin function is critical for viability (18). Temperature-sensitive mutations in the sole *C. elegans* dynamin, *dyn-1* result in a lethal phenotype when grown at the restrictive temperature of 25°C. Moreover, animals grown at the permissive temperature but placed at the restrictive temperature for 20 min become uncoordinated, and have pharyngeal pumping, defecation, and egg-laying defects. These data suggest one of two possibilities: the dynamin protein made in *dyn-1(ky51)* mutants is functional at low temperatures but defective at high temperatures, or alternatively endocytosis in *C. elegans* is a temperature-sensitive process. From studies on the *C. elegans* Eps15 protein, it appears that the latter is at least partially true.

The Eps15 protein is encoded by the *ehs-1* gene (eps15 homologous sequence-1) in *C. elegans* (28). Null alleles of the *ehs-1* gene result in a reversible temperature-sensitive defect in synaptic vesicle recycling. At the restrictive temperature of 30°C, *ehs-1* mutants are depleted of synaptic vesicles as revealed by electron microscopy. The uncoordinated behavioral phenotype of *ehs-1* mutants is similar to the phenotype observed in the temperature-sensitive *dyn-1* mutant, suggesting that these proteins might function in the same pathway. Moreover, direct physical interactions between mammalian dynamin and Eps15 were demonstrated using pulldown experiments from cell lysates, coimmunoprecipitation in cell culture, and affinity purification from heterologously expressed proteins (28). Genetic interactions were also observed: most *dyn-1 ehs-1* double-mutant animals are dead at 18°C, which is normally a permissive temperature for both the *dyn-1* and *ehs-1* mutants. Rare surviving animals are severely uncoordinated and slow growing. Together, these results suggest that Eps15 functions with dynamin during endocytosis; in particular, Eps15 may stabilize endocytosis at high temperatures. Such stabilization may be important because lipid fluidity may be significantly altered at high temperature.

Vesicle uncoating

The final stage of synaptic vesicle recycling, uncoating, occurs once the vesicle has been released from the plasma membrane. During uncoating, clathrin and clathrin adaptor proteins are rapidly removed from the vesicle membrane. Uncoating must occur before the synaptic vesicle can fuse to other membrane compartments. Two apparently unrelated mechanisms appear to be involved in vesicle uncoating: disruption of protein–protein interactions and release of adaptor proteins from the vesicle membrane.

First, dissolution of protein complexes is regulated by the Hsc70 heat shock cognate protein, also known as the uncoating ATPase. Hsc70 is recruited to clathrin-coated vesicles by binding to a DnaJ domain containing protein auxilin. It is thought that the interaction with auxilin serves not only to localize Hsc70 to the coated vesicle, but also to activate the ATPase activity of Hsc70, leading to the disruption of the clathrin lattice (55–58).

C. elegans in which auxilin has been disrupted by RNAi, are inviable and display an accumulation of membrane-associated clathrin (29). Receptor-mediated uptake in the gonad is also disrupted and this phenotype is similar to that of animals depleted for the AP2 adaptor complex (25). This endocytic defect is probably caused by a depletion of cytosolic clathrin as it accumulates on vesicles. In auxilin-depleted animals, there is increased fluorescence of clathrin-GFP puncta compared to the wild type. This is consistent with clathrin heavy chain accumulating on vesicles in the auxilin-defective animals. Finally, overexpression of the clathrin::GFP protein in auxilin RNAi-treated worms allows the worms to grow into adults, suggesting that in the auxilin-defective animals, the amount of clathrin is limiting.

Adaptor complexes can remain associated with endocytosed vesicles, even following removal of the clathrin coat (55,59). Lipid composition of the vesicle may play a role in removal of adaptor complexes. As described above, synaptojanin is a polyphosphoinositide phosphatase. Synaptojanin mutants in both *C. elegans* and mice accumulate clathrin-coated vesicles (19,60), whereas coated vesicles are rarely observed at wild-type synapses. This failure in uncoating may be due to stabilization in binding of the clathrin adaptor complex to lipids of the synaptic vesicle. Both the clathrin adaptor protein AP180 and the alpha subunit of the AP2 complex bind to the lipid substrates of synaptojanin (47,61). Direct hydrolysis of these lipids by synaptojanin may destabilize the association of these clathrin adaptor proteins with the vesicle membrane, causing the vesicle to shed its clathrin coat.

Future Contributions from *C. elegans*

We have provided a mere sketch of the molecular mechanisms of endocytosis at the synapse. Parts of this model are speculative, others are incomplete. What advancements do we anticipate in the near future? Predictions about the specifics of the model are foolish, particularly in light of the fact that we do not have a complete list of the actors in this

drama. However, we can outline the strategies that we believe will unravel the remaining mysteries.

In the short term, the strategies described above must be taken to their conclusion. For example, reverse strategies, either using RNAi or targeted deletions, will elucidate the function of other genes known to play a role in endocytosis (Table 1). The heart and soul of *C. elegans* is genetic elegance, that is, gene discovery through sophisticated mutant screens. Second-generation screens are already emerging. For example, clever screens using GFP as a marker for endocytosis have revealed a large number of genes required for tissue-specific endocytosis (25,62). What screens do we envision will identify genes involved in synaptic transmission? First, there are many mutants that we and others have not characterized because their phenotypes have been too subtle to follow in mapping experiments. For example, many of the synaptic transmission mutants identified in pharmacological screens are not uncoordinated and thus they lack an easily mapped phenotype. In the future, such genes can be identified using transposons as DNA tags. In particular, mobilization of *Drosophila* transposons in *C. elegans* will provide a unique tag with which to clone the mutated genes without having to genetically map the mutant phenotypes. Another useful strategy would be to screen for temperature-sensitive mutants. Such mutants will identify viable alleles of genes for which null mutations are lethal. On the other hand, some of these temperature-sensitive mutations may in fact be null mutations, since endocytosis seems to be a temperature-sensitive process, as suggested by the deletion alleles of Eps15. Finally, suppressor screens will reveal other proteins in this pathway. For example, one expects that mutations in the lipid kinase genes may suppress the defects caused by phosphoinositide accumulation observed in the synaptojanin mutants.

But what will happen when all the genes are identified, what then? At that point we will need to understand how these proteins interact to function as molecular machines. Residues that interact with other proteins will be identified by structural studies. Models will arise from these data but they will not be testable using structural data. To test these models, proteins with specific residue changes must be introduced into an organism to test their predicted effects on synaptic vesicle endocytosis. Because the introduction of recombinant molecules is so straightforward and quick in *C. elegans*, it is likely that many of these studies will be performed in this simple worm.

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