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Flowers for Synaptic Endocytosis

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DOI 10.1016/j.cell.2009.08.023

Exocytosis and endocytosis of synaptic vesicles are tightly coordinated to maintain a steady supply of new vesicles during periods of extended neuronal stimulation. Yao et al. (2009) now report that a synaptic vesicle membrane protein named Flower promotes endocytosis at neuromuscular junctions in the fruit fly *Drosophila*.

The release of neurotransmitters at synapses is mediated by the exocytotic fusion of synaptic vesicles. Because synapses are usually located far away from their respective neuronal cell bodies, local endocytosis is needed to recover and recycle synaptic vesicle components for reuse. This maintains synapse integrity and a constant supply of synaptic vesicles and guarantees that synapses can remain fully operational even when stimulated strongly and over extended periods of time. New findings by Yao et al. (2009) reveal the protein Flower as a new regulator of endocytosis at the neuromuscular junction in the fruit fly *Drosophila*. The authors provide evidence that Flower couples endocytosis to exocytosis by acting as a Ca²⁺ channel.

There are three known mechanisms of synaptic vesicle endocytosis: (1) clathrin-dependent endocytosis after full collapse of the fusing vesicle, (2) bulk endocytosis via large membrane invaginations, and (3) kiss-and-run fusion and retrieval, during which only a transient fusion pore is formed (for a recent review, see Wu et al., 2007). The relative

contributions of the different endocytosis pathways to synaptic vesicle recycling in vivo are currently unknown, but clathrin-dependent endocytosis clearly plays a major role in synapses, whereas bulk endocytosis may become important only upon intense stimulation (de Lange et al., 2003).

Clathrin-dependent endocytosis of synaptic vesicle components takes place in the plasma membrane domain that surrounds the neurotransmitter release site (Heuser and Reese, 1973). It is mediated by a complex protein machinery that controls membrane lipid composition, cargo recognition, clathrin coat assembly, membrane invagination, membrane fission, and coat disassembly (for a recent review, see Jung and Haucke, 2007). In addition, clathrin-dependent endocytosis appears to be stimulated by Ca²⁺ ions, at least in some experimental preparations (Wu et al., 2007; Hosoi et al., 2009). Ca²⁺-dependent regulation of endocytosis is intuitively plausible as it would allow the synapse to coordinate endocytosis with vesicle fusion, which is triggered by an increase in the intra-

cellular Ca²⁺ concentration. However, the exact role of Ca²⁺ in the regulation of endocytosis and the relevant Ca²⁺-entry pathways, Ca²⁺ sources, and Ca²⁺ sensors are still largely unknown, with different experimental approaches yielding divergent results. In fact, depending on the preparation used, endocytosis has been reported to be independent of Ca²⁺, dependent on bulk Ca²⁺, or dependent on Ca²⁺ microdomains near voltage-gated Ca²⁺ channels (see discussion in Hosoi et al., 2009).

In an elegant study presented in this issue of *Cell*, Yao et al. (2009) describe a previously uncharacterized protein from *Drosophila* that they name Flower (Figure 1). They show that Flower is essential for proper endocytosis of synaptic vesicles. The authors identified the *flower* gene in an unbiased forward genetic screen for proteins that affect synaptic transmission. The Flower protein resides on synaptic vesicles and the presynaptic plasma membrane. It is evolutionarily conserved from worms to humans, contains four stretches of hydrophobic sequences that may repre-

sent transmembrane domains, and is not related to any known endocytic regulatory protein. Most notably, it lacks any of the characteristic domains that are often found in endocytosis regulators, such as Bin/amphiphysin/Rvs (BAR) domains or Pleckstrin homology (PH) domains.

Loss-of-function mutations in the *flower* gene lead to phenotypic changes that are hallmarks of perturbed endocytosis. Like other mutants lacking important regulators of endocytosis (such as Endophilin, Synaptojanin, or Eps15) neuromuscular synapses of *flower* mutants display numerous extra presynaptic boutons. Synaptic vesicles are reduced in density, heterogeneous in size, and larger than normal. In addition, numerous endocytic intermediates can be observed in mutant presynaptic terminals. These morphological changes correlate with altered functional properties, including a more rapid depletion of synaptic vesicles during high-frequency stimulation and a reduced uptake of the membrane dye FM1-43 during synaptic activity.

Interestingly, an increase in extracellular Ca^{2+} concentration can partially rescue the functional defects in *flower* mutants. This observation, and the fact that the Flower protein contains four putative transmembrane segments, with a channel-like motif in one of them, led the authors to explore the possibility that Flower might function as a Ca^{2+} channel that controls Ca^{2+} -dependent endocytosis. Such a role for Flower would be consistent with the previously postulated notion that endocytosis at *Drosophila* neuromuscular synapses is regulated by an atypical Ca^{2+} channel (Kuromi et al., 2004).

Indeed, Yao et al. provide multiple lines of evidence that point to a Ca^{2+} channel function of Flower. In addition to the fact that Flower contains an evolutionarily conserved sequence with similarity to the selectivity filters of the ion channels TRPV5 and TRPV6, the following findings indicate a channel function: (1) Flower proteins form homo-oligomers; (2) enrichment of Flower in the presynaptic plasma membrane of the endocytic mutant *shibire* is paralleled by an increase in Ca^{2+} influx during extended

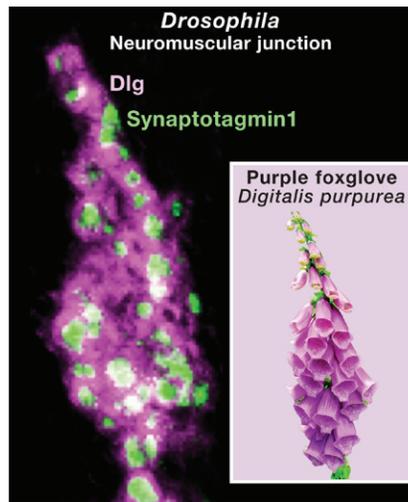


Figure 1. Flower-Shaped Presynaptic Boutons at the Neuromuscular Junction

Yao et al. (2009) named the *flower* gene of the fruit fly *Drosophila* after the unusual flower-like appearance of presynaptic boutons at neuromuscular junctions in *flower* loss-of-function mutants. Immunofluorescence staining of the neuromuscular junction in *flower* mutants shows the localization of the presynaptic marker Synaptotagmin 1 (green) and the postsynaptic marker Discs large (Dlg, magenta) (Figure modified from Yao et al., 2009). This morphology bears striking similarity to the purple foxglove (*Digitalis purpurea*, inset). Yao et al. show that the Flower protein is essential for synaptic vesicle endocytosis and probably acts as a Ca^{2+} channel that couples exocytotic fusion of synaptic vesicles to their endocytic recycling. The aberrant morphology of the presynaptic boutons in *flower* mutants is the consequence of impaired endocytosis. Image of *D. purpurea* courtesy of L. Kolb.

synaptic stimulation; (3) loss of Flower is paralleled by a decrease in the resting concentration of presynaptic Ca^{2+} ; (4) heterologous expression of Flower in salivary gland cells increases Ca^{2+} influx, an effect that is not seen with a mutant version of Flower that carries a point mutation, E79Q, in the putative ion selectivity filter; and (5) purified Flower promotes Ca^{2+} uptake into reconstituted proteoliposomes.

Based on the data presented by Yao et al., the conclusion that Flower plays an essential and hitherto unrecognized role in the regulation of presynaptic endocytosis is indisputable. In addition, the findings add Flower to a growing list of proteins that cycle between the synaptic vesicle pool, the transmitter release site, and the perisynaptic membrane at nerve endings (for a recent review, see

Shupliakov, 2009). However, the indications that Flower has a Ca^{2+} channel function remain indirect, and the clear-cut effects of the perturbation of Flower function on Ca^{2+} flux are typically seen only after several minutes. This relatively slow speed is surprising, given that any regulatory action of Ca^{2+} on endocytosis might be expected to take place rapidly. Indeed, the slow effects of Flower on Ca^{2+} flux may be due to slow Ca^{2+} leak currents, which are caused by many surface-active compounds, including transmembrane proteins.

The study by Yao et al. is fascinating and of great potential importance. The role of Ca^{2+} in the control of endocytosis continues to be a major focus of dispute despite considerable effort to understand this fundamental cell biological process. In this regard, the present study provides a genuinely new conceptual guidepost for further in-depth analyses, and the remaining questions are clear. Is the endocytic function of Flower evolutionarily conserved? Does Flower specifically support Ca^{2+} flux, or does it instead initiate a nonspecific cation leak? Does the E79Q mutation in the putative ion selectivity filter block all Flower functions described so far, including Ca^{2+} uptake into proteoliposomes and the rescue of the *flower* mutant phenotype in vivo? Lastly, can direct evidence for a Ca^{2+} channel function of Flower be obtained by electrophysiological recordings?

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