Synaptic Vesicle Dynamics sans Dynamin

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The neuron-specific guanosine triphosphatase dynamin 1 has been hypothesized to be critically required for pinching off synaptic vesicles during endocytosis. In a recent publication in *Science*, Ferguson et al. describe a series of experiments demonstrating an unexpectedly selective requirement of dynamin 1 in synaptic vesicle endocytosis only during high frequency (<10 Hz) stimulation, but not after cessation of the stimulus train.

During synaptic transmission, small synaptic vesicles filled with neurotransmitter fuse with the plasma membrane to release their content. For maintaining synaptic transmission, the exocytosed vesicle proteins have to be retrieved thereafter by compensatory endocytosis. Different mechanisms for synaptic vesicle protein retrieval and recycling have been suggested more than three decades ago. While clathrin-mediated reclustering, resorting, and endocytosis of vesicle proteins is thought to be the major pathway (Heuser and Reese, 1973; Cremona and De Camilli, 1997), alternatively, vesicles might connect only briefly to the plasma membrane without full collapse and loss of protein content ("kiss-and-run") (Ceccarelli et al., 1973; Fesce et al., 1994). Clathrin-mediated endocytosis proceeds in a series of spatially distinct steps: the recruitment of clathrin from the cytosol by adaptor proteins, binding to the plasma membrane, subsequent invagination, and finally fission of clathrin-coated vesicles from the membrane by the GTPase dynamin (Cremona and De Camilli, 1997). While full collapse fusion and subsequent clathrin-mediated endocytosis highlight the irreversibility of reactions during exo-endocytosis, in contrast, kiss-and-run is envisioned as a transient, i.e., reversible fusion pore formation (Fesce et al., 1994). Thus, both modes of retrieval might be distinguishable molecularly by their different need for the fission-promoting protein dynamin.

To delineate the physiological role of dynamin in synaptic vesicle recycling, De Camilli and colleagues from several labs have now genetically ablated the major isoform in the CNS, dynamin 1, and analyzed ultrastructurally and physiologically the effects on synaptic vesicle recycling. Besides that normal mice developed—which nobody else would have dared to predict for knocking out such a fundamentally important protein—a number of surprises awaited the scientists and the scientific community. First, despite the predicted essential role of dynamin 1 in synaptic vesicle recycling, a functional nervous system developed in the KO mice, showing that dynamin 1, by far the most abundant isoform in the nervous system, is largely dispensable for synapse development. Furthermore, even synaptic vesicle recycling was intact. Overall ultrastructure of synapses looked normal, but more clathrin-coated vesicles were visible, synaptic vesicles had slightly larger diameters, and more abnormally large vesicles were encountered.

Its essential role in synaptic vesicle recycling, however, is highlighted under intense stimulation: in electron-microscopic pictures, large extended branched tubular networks, still connected to the plasma membrane and capped by clathrin-coated pits, are observed, clearly underscoring the dynamin's essential function in fission.

The most astonishing physiological finding, however, comes from experiments using synaptopHluorin, a fusion construct of the vesicle protein synaptobrevin/VAMP and a pH-sensitive form of GFP that emits green fluorescence at neutral pH but is fully quenched at acidic pH. The GFP-moiety points into the acidic vesicle lumen, thus rendering it the perfect indicator for exo-endocytosis (Miesenbock et al., 1998; Sankaranarayanan and Ryan, 2000). When stimulating cultured hippocampal neurons from dynamin 1 KO mice with 300 action potentials, Ferguson et al. (2007) to their surprise observed normal fluorescence signals; i.e., fluorescence in the synaptic boutons rose during the stimulus train due to dequenching of released synaptopHluorin and after the stimulus slowly recovered back to baseline, as the synaptopHluorin was endocytosed and its fluorescence quenched by reacidification of endocytosed vesicles. Comparison to fluorescence signals during block of vesicle reacidification by the proton pump antagonist bafilomycin, however, led to a remarkable observation. During the stimulus train, endocytosis was completely blocked, but after cessation of stimulation, endocytosis and fission of synaptic vesicles proceeded with almost identical kinetics as in control cultures.

Does this specific block of endocytosis during stimulation reveal the existence of two alternative endocytosis pathways that are molecularly distinct—a faster one during stimulation that depends critically on dynamin 1 and a slower, presumably clathrin-dependent, one that does less critically? In the latter case, the fission by dynamin 1 may not be rate limiting for the speed of endocytosis, and its function may thus easier be substituted for by another isoform, perhaps dynamin 3, that is much less abundant but quite well rescued endocytosis in dynamin

KO neurons, when overexpressed. The faster form that is absent in dynamin 1 KO mice, however, may be the long sought for kiss-and-run mechanism, where fusion pore opening is not followed by full collapse. If dynamin 1 were immediately available for fast fission, the curvature of the fused vesicle maybe could be maintained for this short time—in the absence of dynamin, however, it will eventually collapse. Unlike in its original formulation, however, such "noncanonical" kiss-and-run then would not be a reversible fusion pore opening, but instead an irreversible opening would be followed by a dynamin-dependent fission step—very much reminiscent to what has been reported previously for endocytosis in chromaffin cells. In chromaffin cells of the adrenal gland, injection of antibodies against dynamin 1 blocked a strictly calcium-dependent fast endocytosis that ensues after mild stimulation, while antibodies against dynamin 2 blocked a slow calcium-independent clathrin-mediated endocytosis pathway (Artalejo et al., 2002). This scenario, which is also not considered by the authors, however, is a very unlikely explanation for the dynamin 1 KO phenotype observed in CNS synapses for several reasons. In electronmicrographs, and even more so in the electron tomography pictures, numerous omega profiles not bearing a clathrin coat should be visible at the plasma membrane, which, however, was not the case. A second powerful argument comes from the detailed morphometric analysis of the ultrastructure. If dynamin 1 specifically supported a "noncanonical" form of kiss-and-run, while slow clathrin-mediated endocytosis after stimulation utilized a different isoform, e.g., dynamin 2, the vesicle size distribution in the KO synapses, i.e., in the complete absence of such kiss-and-run mechanism should be unchanged. Instead, Ferguson et al. (2007) observed a very heterogeneous size distribution of vesicles and many abnormally large vesicles. If anything, complete block of one pathway of retrieval should result in a more homogeneous size distribution.

But if it is not another mechanism of retrieval, what else may be the cause of this distinct absence of endocytosis during stimulation? A clue comes from experiments where Ferguson et al. (2007) tested different frequencies of stimulation. For frequencies below 10 Hz, there is indeed also an endocytic component found during stimulation; its contribution, however, progressively diminishes with increasing frequency reaching zero at about 10 Hz. In wild-type, actually a similar phenomenon is observed, but here the point, where exocytosis by far exceeds endocytosis such that no significant relative endocytosis can be measured during stimulation, is reached at 20 Hz. Likewise, when reducing the external calcium concentration, 2- to 3-fold robust endocytosis is also observed in synapses from KO neurons. These data point to a continuous or gradual rather than abrupt transition between both modes of retrieval and thus rather to one single molecular mechanism, which however is differentially modulated during the two phases of ongoing and ceased stimulation.

Two other dynamin isoforms are found in neurons, albeit at much lower concentrations. Ferguson et al. (2007) found that dynamin 3, which is expressed in brain and testis, can fully rescue endocytosis during stimulation when overexpressed, while dynamin 2, ubiquitously expressed in all cell types, cannot. What distinguishes both neuronal forms from dynamin 2 is that they both share conserved phosphorylation sites. For dynamin 1, it is well established that during stimulation it undergoes, along with other accessory proteins of endocytosis, swift Ca^{2+}-dependent dephosphorylation by calcineurin for activation, a process that is quickly reversed after stimulus cessation (Cousin and Robinson, 2001). Thus, it is conceivable that both dynamin 1 and 3 can support fission during stimulation, while the ubiquitous dynamin 2 is perfectly capable to support fission after stimulation—and in the absence of dynamin 1 in the knockout synapses. Because dynamin 3 is expressed only at low concentration, however, it can compensate for the lack of dynamin 1 in knockout synapses only partially at low-frequency stimulation (<10 Hz) or at reduced external calcium, but not during stronger stimulation.

In summary, the data are less in line with the notion of at least two different pathways mediating synaptic vesicle endocytosis (Robinson, 2007), but rather point to one mechanism, namely clathrin-mediated endocytosis, which by different accessory proteins, however, is tailored to work equally well under different conditions—in the absence of stimulation as well as in the presence, where the endocytosis machinery has to cope with an extra internal calcium load, for which it originally may not have been evolved. Actually, very high internal calcium has been even reported to block synaptic vesicle endocytosis (von Gersdorff and Matthews, 1994). Thus, the isoform dynamin 1, which is exclusively expressed in the brain, may have formed late together with the evolution of a nervous system as a synaptic "pinchase" that works efficiently even during stimulation and increased internal calcium.

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