

Catching synaptic vesicles in action

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and

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The synaptic vesicle (SV) cycle was initially discovered at the neuromuscular junction using electron microscopy (EM) analysis.¹ With the introduction of fluorescent probes that are able to monitor real-time cellular events in live cells, EM analysis was pushed to the side lines because it could not provide meaningful kinetic analyses of the various steps in the synaptic vesicle cycle.

First attempts to overcome this shortfall were made early on by capturing SV exocytosis and endocytosis using fast fixation techniques at defined time points^{2,3} and then analyzing the SVs by EM. These early studies successfully used rapid freezing onto a supercooled copper block to accurately stop SV cycling at the neuromuscular junction, and the results obtained from these studies still provide a foundation of our understanding of SV release.

This technique could not be applied to brains because brain tissue would have undergone severe anoxia during dissection. Another impasse was the lack of a stimulation technique allowing for precise neuron activation that would not interfere with the freezing process. It took until 2013 before a successful approach of using high-pressure freezing to capture exo- and endocytotic events at hippocampal synapses was found. Jorgensen and coworkers^{4,5} used optogenetic techniques to solve the stimulation problem and modified the specimen holder of a high-pressure freezer so that light can reach the specimen. Cultured hippocampal neurons expressing light-sensitive channel-rhodopsin-2 were directly grown on the specimen holder. These modifications

cleared the way for ultrafast, high-pressure freezing to capture the synaptic morphology of mammalian neurons just milliseconds after stimulation. Fixation was so rapid, in these experiments, that exocytic vesicles were captured at the active zone only 2 ms after stimulation, proving that neurotransmitter is released at active zones. But the real big surprise in this study was the discovery of endocytic events just 100 ms later.

A second approach designed to capture SV retrieval after neurotransmitter release, using a different technique, was published at approximately the same time.⁶ Instead of using ultrafast high-pressure freezing, superfast microwave-assisted aldehyde fixation was used to stop the SV cycle at precise times. Microwave exposure has been shown to stabilize tissue in tens of milliseconds and to accelerate chemical reactions.⁷ Endocytic vesicles were labeled with the fluorescent dye FM1-43, and after photoconversion (a process that translates the fluorescence into an electron dense polymer that darkens the vesicle lumen in the electron microscope), the fluorescence was visualized using EM analysis. In addition, a quantifiable stimulus was used; that is, the release of the readily releasable pool (RRP). Since the number of vesicles in the RRP coincides with the number of SVs docked at the active zone,⁸ this technique allowed for measuring the number of released vesicles and the number of retrieved vesicles at each time point by comparing the number of docked SVs with the number of labeled vesicles, respectively. In this study, the first retrieved vesicles were found just

above the active zone, suggesting retrieval at the active zone instantly after release. This mechanism is called 'kiss and run'. In contrast to current hypotheses about "kiss and run," however, these vesicles did not maintain their identity but fused with unknown membrane compartments to form large vesicles 10–20 s after the release of the RRP. These large vesicles disappeared in favor of typical SVs that randomly mixed with the SV cluster.

Despite some differences in the time course of SV endocytosis, which may be explained by differences in temperature, both studies reported essentially similar results. One point on which these studies unequivocally agree is that SV endocytosis is not mediated by coated pits and coated vesicles. This structural property is a characteristic of the clathrin-dependent pathway, which has been the hallmark of SV endocytosis. The discovery of the lack of clathrin-coated vesicles was highly unexpected and undoubtedly will shift research into new directions.

Super- and ultrafast fixation have already revealed surprising aspects of the SV cycle at hippocampal synapses and are likely to be the basis for further revolutionary insights. Each technique has its own unique strengths. Ultrafast high-pressure freezing may be able to capture the structural organization of the release machinery before and immediately after (perhaps even during) vesicle

fusion. High-pressure freezing also guarantees exceptional structural preservation. Together, these attributes provide the basis for building realistic 3D models of the release machinery and its conformational changes during release with a time resolution of possibly only 1–2 ms. Superfast fixation with microwaves is not capable of such a high time resolution, but it still can capture protein assemblies before and after release. The real strength of superfast fixation lies in its compatibility with other labeling techniques. Superfast fixation has already been used together with an endocytic marker, and combinations with immunogold protein localization and genetically encoded labels (like horseradish peroxidase⁹) are feasible. With this rich set of tools, labeled vesicles from genetically manipulated cells can be tracked, and the molecular composition can be probed using immunogold labeling. Such approaches are likely to answer fundamental questions about clathrin-independent endocytic mechanisms, and subsequently, clear the way for a thorough investigation of the molecular mechanisms.

With these exiting tools we are well positioned to go through another cycle of retrieving knowledge about the shape-shifting existence of SVs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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