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The Synaptic Vesicle Cycle: Is Kissing Overrated?

In this issue of *Neuron*, Granseth et al. re-examine the mechanism of endocytosis at hippocampal synapses using a new optical reporter, sypHy. They conclude that only a single slow mode of endocytosis operates at this synapse and that retrieval after physiological stimuli is largely, if not solely, dominated by the clathrin-mediated pathway. These conclusions dispute previous assertions that "kiss-and-run" is a major mechanism of vesicle recycling at hippocampal synapses.

The cycling of synaptic vesicles through repetitive episodes of exocytosis and endocytosis is fundamental to synaptic transmission, but competing views of the underlying mechanisms are still hotly debated. On the one hand, considerable evidence suggests that synaptic vesicles fully incorporate into the plasma membrane as they release their neurotransmitter cargo (full fusion), followed by retrieval through clathrin-mediated endocytosis (see Figure 1, cycle A). On the other hand, other evidence supports a "kiss-and-run" cycle, in which fusing vesicles release their contents through a transient pore and then pinch off without collapsing into the plasma membrane (see Figure 1, cycle B). It seems likely that both can occur, but the question is which is prevalent and under what conditions (reviewed by Matthews, 2004). In this issue of *Neuron*, Granseth et al. (2006) present evidence challenging the prevailing view that kiss-and-run is the dominant cycle at small synaptic boutons of cultured hippocampal neurons. Instead, they found that endocytosis was almost exclusively mediated by a relatively slow ($\tau \approx 15$ s at 23°C) clathrin-dependent pathway, consistent with full fusion.

To monitor exocytosis and subsequent endocytosis, Granseth et al. made use of pHluorin, which is a pH-sensitive GFP variant that can be targeted to synaptic vesicles by fusing it to the intravesicular domain of vesicle membrane proteins. Because the interior of synaptic vesicles is acidic, pHluorin facing the vesicle lumen is protonated and its fluorescence is low in the resting state (Figure 1). Upon exocytosis, vesicles lose their protons, and the fluorescence of pHluorin increases. The fluorescence decreases again when vesicles reacidify after being retrieved by endocytosis. When fused to the lumenal end of the vesicle SNARE protein synaptobrevin/VAMP, pHluorin forms the widely used reporter synaptopHluorin (Miesenbock et al., 1998). For instance, synaptopHluorin was used by Gandhi and Stevens (2003) to conclude that an exocytic event at hippocampal synapses may be followed by one of three distinct modes of vesicle retrieval, whose relative prominence depends on the release probability of the synapse. Their measurements indicated a fast kiss-and-run mode lasting <900 ms, a slower "compensatory" mode lasting 8–21 s, and a "stranded" mode where vesicles are caught at the plasma membrane to await retrieval triggered by subsequent stimuli.

Measurements using synaptopHluorin are complicated by the fact that it is expressed substantially in the plasma membrane as well as in synaptic vesicles, which introduces considerable background fluorescence. Also, synaptopHluorin molecules that appear in the plasma membrane after vesicle fusion are mobile and can move out of synaptic active zones into the surrounding axon after exocytosis (Sankaranarayanan and Ryan, 2000). Indeed, Granseth et al. (2006) concluded that a fast component of "endocytosis" reported by synaptopHluorin in their experiments is most likely an artifact produced by such lateral diffusion, leaving only a relatively slow decline in fluorescence ($\tau \approx 20$ s) attributable to true endocytosis. To overcome the problems introduced by diffusion of synaptopHluorin and to lessen background fluorescence, Granseth et al. designed an improved pHluorin fusion protein. Abbreviated sypHy, the new optical reporter was generated by fusing pH-sensitive GFP to the synaptic vesicle protein synaptophysin, and it proved to be more specific to synaptic vesicles and more confined to active zones after exocytosis than synaptopHluorin. Using sypHy, only a slow component of fluorescence decrease was detected after a single action potential, yielding an estimated time constant of ~15 s for endocytosis after correction for the measured time course of reacidification ($\tau \approx 4$ s; similar to the rate of vesicle acidification estimated previously by Atluri and Ryan, 2006). There was no indication of a rapid component of internalization of sypHy like that expected for kiss-and-run, in agreement with previous findings based on acid-quenching of synaptopHluorin fluorescence in hippocampal synapses (Atluri and Ryan, 2006). The results were identical at synapses with the lowest and highest release probability and were not dependent on the amount of stimulation, in contrast to previously reported evidence suggesting dependence of the prevalence of kiss-and-run endocytosis on release probability (Gandhi and Stevens, 2003) or stimulus frequency (Harata et al., 2006).

What is the molecular mechanism underlying the single component of endocytosis detected at hippocampal synapses by Granseth et al. (2006)? It is known that the machinery required for clathrin-mediated endocytosis is enriched at CNS synapses. To explore the role of clathrin at hippocampal synapses, Granseth et al. employed two complementary methods: overexpression of a dominant-negative form of the clathrin-adaptor protein AP180, and RNAi knockdown of the clathrin heavy chain. Both approaches led to a complete block of endocytosis in response to a weak stimulus. The results provide strong evidence that the principal mode of retrieval in hippocampal boutons is clathrin-dependent. Therefore, Granseth et al. concluded that cycle A of Figure 1 dominates at synapses of cultured hippocampal

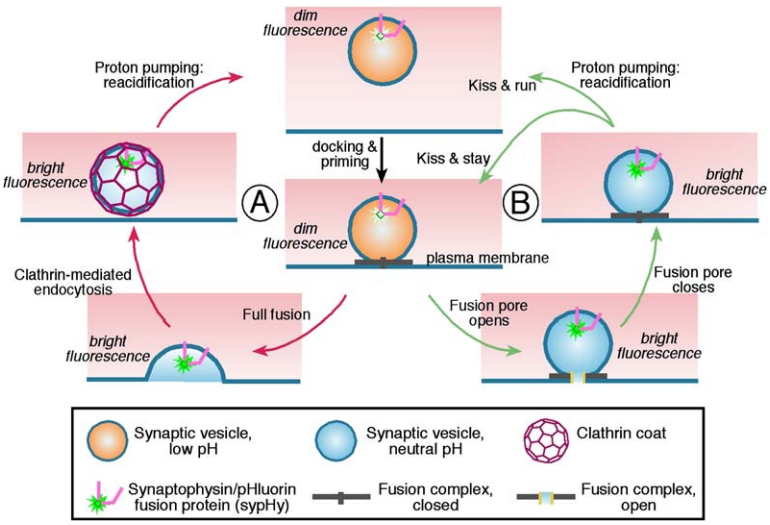

Figure 1. Two Alternative Schemes for the Synaptic Vesicle Cycle at Synapses of Cultured Hippocampal Neurons

In scheme A, the vesicle membrane fully fuses with the plasma membrane, and vesicles are then retrieved by clathrin-mediated endocytosis. In scheme B, vesicle contents are released by a transient opening of a fusion pore, and the vesicle membrane remains separate from the plasma membrane. In either case, vesicles undergoing exocytosis lose their high concentration of protons, which is generated by vesicular H^+-ATPase and is used to support neurotransmitter uptake. Exocytosis and subsequent reacidification after endocytosis can therefore be tracked using an improved sensor called sypHy, which is formed by fusing pH-sensitive GFP (pHluorin) to the intralumenal portion of the synaptic vesicle protein synaptophysin. Unlike the previously used sensor synaptopHluorin, sypHy localizes primarily in synaptic vesicles under resting conditions and tends to remain near the active zone after exocytosis. In the low-pH environment inside synaptic vesicles, pHluorin is protonated and its fluorescence is largely quenched. Upon exposure to the pH-neutral extracellular space during exocytosis, pHluorin increases its fluorescence ~20-fold, and this state is maintained until the intravesicular H^+ gradient is restored by proton pumping after endocytosis.

neurons, whereas evidence for kiss-and-run exocytosis (cycle B, Figure 1) was not found.

The conclusions of Granseth et al. (2006) are at odds with those reached recently by Harata et al. (2006), who used an external quenching agent, bromophenol blue (BPB), to assay the relative contributions of full fusion and kiss-and-run at hippocampal synapses. In those experiments, Harata et al. analyzed changes induced by BPB in the fluorescence of FM1-43 or lumenal-tagged GFP, and argued that the kinetics were more complex than expected for full fusion. What accounts for the different conclusions? In the experiments on small hippocampal boutons, fluorescence of the reporter—whether FM dye, synaptopHluorin, or sypHy—is measured from a region encompassing the entire terminal, and lateral spread of fluorescence from the site of exocytosis to other parts of the bouton should therefore influence the time course of measured signals. Lipophilic FM dyes introduced into the plasma membrane after full vesicle fusion spread laterally from the fusion site on a millisecond time scale (Zenisek et al., 2002), but remain much longer in the surrounding plasma membrane, where they contribute to fluorescence and fluorescence quenching. SynaptopHluorin is also laterally mobile in the terminal (Granseth et al., 2006). Harata et al. (2006) and others have interpreted complexities of these fluorescence signals to indicate kiss-and-run exocytosis, which indeed seems a reasonable interpretation. However, the results of Granseth et al. should prompt us to ask whether complex changes in fluorescence accompanying stimulation must necessarily be attributed to the diffusional effect of a fusion pore, such as the pore thought to mediate kiss-and-run exocytosis. For instance, geometrical intricacies introduced by interactions between synaptic terminals and other neurons and glia in the culture may complicate both aqueous and lipid diffusion and affect the kinetics of fluorescence signals. Determining whether such reinterpretations of the primary data can account for the disparate conclusions must await further experiments, which would ideally include more direct indicators that do not depend so strongly on interpretations of derived measures.

In the long-running debate over kiss-and-run, seemingly persuasive arguments on both sides have been made, but shortly thereafter refuted. Still, we think it fair to say that the kiss-and-run mode of retrieval remains elusive. The data of Granseth et al. highlight the importance of clathrin-mediated endocytosis in the vesicle cycle at small CNS synapses and place the ball squarely in the court of the kiss-and-run advocates. Spectators await the return volley with interest.

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