SYMPOSIUM REPORT

Clathrin-mediated endocytosis: the physiological mechanism of vesicle retrieval at hippocampal synapses

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The maintenance of synaptic transmission requires that vesicles are recycled after releasing neurotransmitter. Several modes of retrieval have been proposed to operate at small synaptic terminals of central neurons, but the relative importance of these has been controversial. It is established that synaptic vesicles can collapse on fusion and the machinery for retrieving this membrane by clathrin-mediated endocytosis (CME) is enriched in the presynaptic terminal. But it has also been suggested that the majority of vesicles released by physiological stimulation are recycled by a second, faster mechanism called 'kiss-and-run', which operates in 1 s or less to retrieve a vesicle *before* it has collapsed. The most recent evidence argues against the occurrence of 'kiss-and-run' in hippocampal synapses. First, an improved fluorescent reporter of exocytosis (sypHy), indicates that only a slow mode of endocytosis ($\tau = 15$ s) operates when vesicle fusion is triggered by a single nerve impulse or short burst. Second, this retrieval mechanism is blocked by overexpressing the C-terminal fragment of AP180 or by knockdown of clathrin using RNAi. Third, vesicle fusion is associated with the movement of clathrin and vesicle proteins out of the synapse into the neighbouring axon. These observations indicate that clathrin-mediated endocytosis is the major, if not exclusive, mechanism of retrieval in small hippocampal synapses.

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Endocytosis in hippocampal synapses

Neurons transmit information at chemical synapses by the release of neurotransmitter contained within small vesicles (Katz, 1969). Most synaptic terminals in the brain typically contains only 100–200 of these vesicles, so their fusion with the cell surface must be followed by efficient retrieval to maintain synaptic communication during ongoing activity. These endocytic mechanisms remain controversial, despite intensive study (Marte, 2002; Rizzoli & Betz, 2003; Royle & Lagnado, 2003; Fernandez-Alfonso & Ryan, 2004; Tyler & Murthy, 2004; Dickman *et al.* 2005; Li *et al.* 2005; Harata *et al.* 2006).

Optical techniques applied to hippocampal neurons in culture have been interpreted as indicating that both fast and slow modes of endocytosis occur at synaptic boutons, but the molecular basis of these kinetically distinct mechanisms has been difficult to establish (Klingauf *et al.* 1998; Pyle *et al.* 2000; Aravanis *et al.* 2003*b*; Gandhi & Stevens, 2003; Royle & Lagnado, 2003). It is known that synaptic vesicles can collapse on fusion and the machinery for retrieving this collapsed membrane by clathrin-mediated endocytosis (CME) is enriched at many synapses (Takei *et al.* 1996). What has been less clear is the speed at which CME operates and its importance compared to other mechanisms of vesicle retrieval. In fact, it has been suggested that the large majority of vesicles released by physiological stimulation are recycled by a second, faster mechanism called 'kiss-and-run', which operates in 1 s or less (Aravanis *et al.* 2003*b*; Harata *et al.* 2006). A key feature of the 'kiss-and-run' model is that the vesicle is retrieved at the site of fusion *before* it has collapsed into the surface membrane (Fesce *et al.* 1994; Klingauf *et al.* 1998; Klyachko & Jackson, 2002; Aravanis *et al.* 2003*b*; Gandhi & Stevens, 2003; Richards *et al.* 2005; Harata *et al.* 2006).

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The strongest evidence for the 'kiss-and-run' mode of recycling in hippocampal boutons derives from experiments investigating the behaviour of single vesicles, either by observing the loss of a membrane dye from the synaptic bouton (Aravanis *et al.* 2003*b*; Richards *et al.* 2005), or by using synaptopHluorin, a fluorescent protein that reports fusion and retrieval of vesicles (Gandhi & Stevens, 2003). The most commonly used membrane dye is FM1-43, which enters recycled vesicles from the extracellular medium when the synapse is stimulated. After washing the dye off, applying single action potential stimuli occasionally triggers the release of a small amount of FM1-43 from the synapse, but this amount varies from stimulus to stimulus (Aravanis *et al.* 2003*a*,*b*), as does the speed of loss (Richards *et al.* 2005). The larger events have been attributed to full collapse of the vesicle and the smaller events to partial loss of the dye through a fusion pore, envisioned to be less than 1 nm in diameter, which closes before all the dye has a chance to diffuse out of the vesicle (Aravanis *et al.* 2003*a*). Of course, another interpretation might be that all vesicles collapse but the amplitude of the signal varies because different vesicles contain different amounts of dye.

A second approach for investigating endocytosis in hippocampal synapses is to use synaptopHluorin, a genetically encoded reporter made by fusing pH-sensitive GFP to the synaptic vesicle protein synaptobrevin (Miesenbock *et al.* 1998). The fluorescence of synaptopHluorin increases when vesicles fuse, and then declines when they are re-acidified after endocytosis. SynaptopHluorin signals triggered by long trains of action potentials recover relatively slowly, with time constants of 15 s or more, but the responses to single action potentials are more variable (Atluri & Ryan, 2006; Granseth *et al.* 2006). A consistent feature of the fluorescence signal triggered by an action potential reported by Gandhi & Stevens (2003) was a fast decline occurring in less than 1 s, which they attributed to vesicle retrieval by 'kiss-and-run'. This interpretation does not easily square with the speed of vesicle reacidification, which occurs with a time constant of 3–4 s (Atluri & Ryan, 2006; Granseth *et al.* 2006), and recent work using pHluorin-based reporters indicates that the fast decline in the signal is not attributable to endocytosis, but to the movement of the reporter out of the vesicle on fusion (Li & Murthy, 2001; Granseth *et al.* 2006).

SypHy, an improved reporter of exocytosis and endocytosis, only detects a slow mode of endocytosis

Our own work on endocytosis in hippocampal synapses began with the aim of answering the following questions. Is slow retrieval of synaptic vesicles synonymous with clathrin-mediated endocytosis? Does fast retrieval really reflect reversal of a transient connection through a fusion pore, or might it also be dependent on clathrin? And what is the relative importance of these different endocytic mechanisms during normal synaptic activity? To address these questions we began by using synaptopHluorin, but quickly became aware of two problems with this reporter. First, a lot of synaptopHluorin is expressed on the cell surface, generating a high background signal that makes it difficult to detect the fusion of individual vesicles. Second, synaptopHluorin rapidly diffuses out of the synaptic bouton into the neighbouring axon when exocytosis is stimulated, so one has to be careful to measure intensity changes over large regions of interest that contain the reporter. To overcome these problems we designed a new reporter of exocytosis and endocytosis which was better localized to synaptic vesicles. This reporter used the same superecliptic pHluorin, but fused to the second intravesicular loop of synaptophysin and was named sypHy (Granseth *et al.* 2006; see also Zhu *et al.* 2004). We suspected that sypHy might localize to synaptic vesicles better than synaptopHluorin because antibodies to synaptophysin provide excellent markers of synapses while barely staining the surface membrane (Valtorta *et al.* 2004).

We used sypHy to measure synaptic vesicle retrieval after single action potentials (APs), because this is the condition when fast kiss-and-run has been reported to be the predominant mode of fusion. To our surprise, we found that the fluorescence signal triggered by an action potential recovered in just one phase, which occurred with a time constant of 15–20 s at room temperature (Fig. 1; Granseth *et al.* 2006). Further, the speed of endocytosis was the same at synapses of high and low release probability and constant for a range of stimulus strengths up to 40 APs at 20 Hz, indicating the existence of just one, relatively slow, mode of vesicle retrieval over a range of conditions spanning the physiological activity of hippocampal synapses.

The direct cause of the decline in the pHluorin signal after exocytosis is reacidification of the synaptic vesicle by the action of the proton pump, which occurs only *after* vesicle scission from the surface membrane. The rate of vesicle reacidification must therefore be taken into account to obtain a proper estimate of the rate of endocytosis. Recent measurements using synaptopHluorin and sypHy indicate that the time constant of reacidification (τ_r) is 3–4 s (10 mM Hepes; Atluri & Ryan, 2006). This information allowed us to compare our measurements with a model in which quenching of sypHy fluorescence was controlled by two consecutive and irreversible reactions with first-order kinetics: endocytosis ($k_e = 1/\tau_e$) followed by reacidification ($k_r = 1/\tau_r$). The black trace in Fig. 1*B* is a best-fit to this model with $\tau_r = 4$ s, which yields an estimate of $\tau_e = 15.5$ s.

Endocytosis is clathrin dependent

What is the mechanism that retrieves synaptic vesicles with a time constant of ~15 s? The slow mode of endocytosis in retinal bipolar cells operates at a similar speed and has been shown to be dependent on clathrin and its accessory proteins (Jockusch *et al.* 2005). Is retrieval in hippocampal synapses also clathrin dependent? To investigate this question we inhibited CME using two different methods. The first was a dominant-negative approach entailing over-expression of the C-terminal fragment of AP180 – an accessory protein involved in CME (Ford *et al.* 2001). The second method was to deplete clathrin heavy chain (CHC) using RNA interference (Royle *et al.* 2005). Both manouveres strongly inhibited endocytosis of synaptic vesicles released by four APs (Granseth *et al.* 2006). The picture that emerges from these experiments is therefore relatively simple: after physiological levels of activity, CME with a time constant of about 15 s retrieves most, if not all, vesicles at small hippocampal synapses.

Exocytosis is associated with the movement of vesicle proteins out of the synapse

A second series of observations indicate that synaptic vesicles normally collapse on fusion: the diffusional loss of proteins from the vesicle membrane into the surrounding axon, which is difficult to explain on the basis of a connection through a proteinaceous fusion pore. This phenomenon was first described in detail by Li & Murthy, (2001), and has since been observed by several other groups (Fig. 2; Sankaranarayanan & Ryan, 2000; Fernandez-Alfonso *et al.* 2006; Granseth *et al.* 2006; Wienisch & Klingauf, 2006). Two examples of proteins that diffuse out of vesicles are synaptobrevin and synaptotagmin, which are key players in calcium-triggered fusion. But their loss from the vesicle is not a problem, because these proteins are also present on the surface membrane, from where they can be retrieved along with the vesicle (Fernandez-Alfonso *et al.* 2006; Wienisch & Klingauf, 2006). It therefore seems that some vesicle proteins are exchanging with a pool on the surface membrane during repeated cycles of exocytosis and endocytosis.

Endocytosis is associated with the movement of clathrin out of the synapse

If proteins associated with synaptic vesicles can leak out of the synapse into the neighbouring axon after fusion, where does endocytosis occur? Exclusively in the synaptic bouton, or also in the neighbouring axon? The possibility that

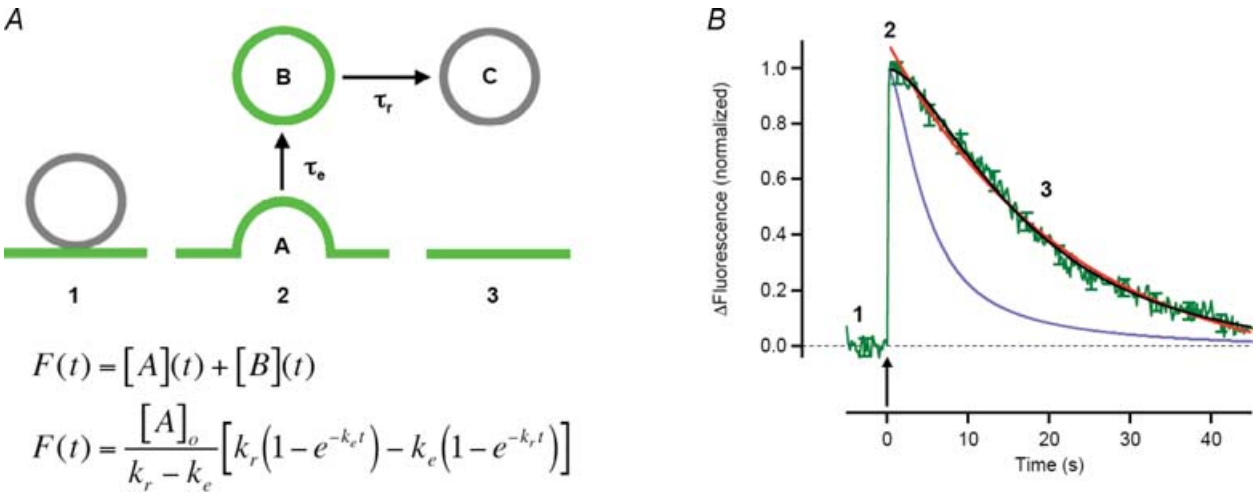

Figure 1. Calculating the rate of endocytosis from the decay in sypHy fluorescence
A, schematic illustration of the processes that regulate the time course of signals obtained using intravesicular pHluorins. 1: the pHluorin is quenched in the acidic interior of the vesicle. 2: fusion immediately causes the pHluorin to become fluorescent (state A), and it remains fluorescent after vesicle scission from the surface (state B). 3: the pHluorin is quenched as the vesicle interior becomes acidified (state C). The transitions A → B → C can be modelled as two consecutive reactions with first-order kinetics with time constants τ_e for endocytosis and τ_r for reacidification. The equations show the average time course of the fluorescence signal (F) at time *t* after vesicle fusion. *B*, a single exponential curve fit (red) with $\tau = 22$ s provided a relatively good description of the decay in sypHy fluorescence after a single AP measured with a large ROI (green). A better fit for the initial phase of the decay was obtained by the model in *A* (black) using a measured $\tau_r = 4$ s, yielding $\tau_e = 16$ s. The light blue trace models the situation in which 80% of vesicles are taken up with $\tau_{e1} = 1$ s, and the remainder with $\tau_{e2} = 16$ s, both populations being reacidified with $\tau_r = 4$ s. Reprinted from Granseth *et al.* (2006), *Neuron* 51, 773–786, with permission from Elsevier.

CME can operate at both sites is suggested by immunocytochemistry of hippocampal neurons in culture showing that clathrin, although concentrated at synapses, also distributes throughout neuronal processes, together with dynamin, and adaptor and accessory proteins (Mundigl *et al.* 1998; Yao *et al.* 2003). Given the evidence that CME was the predominant mode of vesicle retrieval during normal levels of stimulation, we investigated how the distribution of clathrin might change during synaptic activity. We used sypHy to monitor sites of exocytosis while simultaneously imaging one of synaptobrevin, synaptophysin or clathrin light chain A (LCa) tagged with mRFP. The difference images in Fig. 2*A* provide examples of the changes in the distribution of these proteins caused by a stimulus of 40 APs at 20 Hz. The concentrations of mRFP-synaptobrevin and LCa-mRFP decreased immediately over the bouton, while rising in the adjacent axon, demonstrating net movement away from the active zone.

Two observations suggested that clathrin effectively 'tracks' the vesicle proteins leaving the synapse. First, the amount of LCa-mRFP leaving the synapse was proportional to the number of vesicles released by the stimulus, as assessed by the amplitude of the sypHy signal. Second, the movement of LCa-mRFP began without a significant delay and peaked with the sypHy signal. The movement of clathrin out of the synapse together with synaptophysin and synaptobrevin is most easily explained as representing CME of vesicles at sites removed from the active zone. This interpretation is consistent with studies showing that the machinery for CME is not at the active zone, but in the surrounding regions of membrane (Heuser

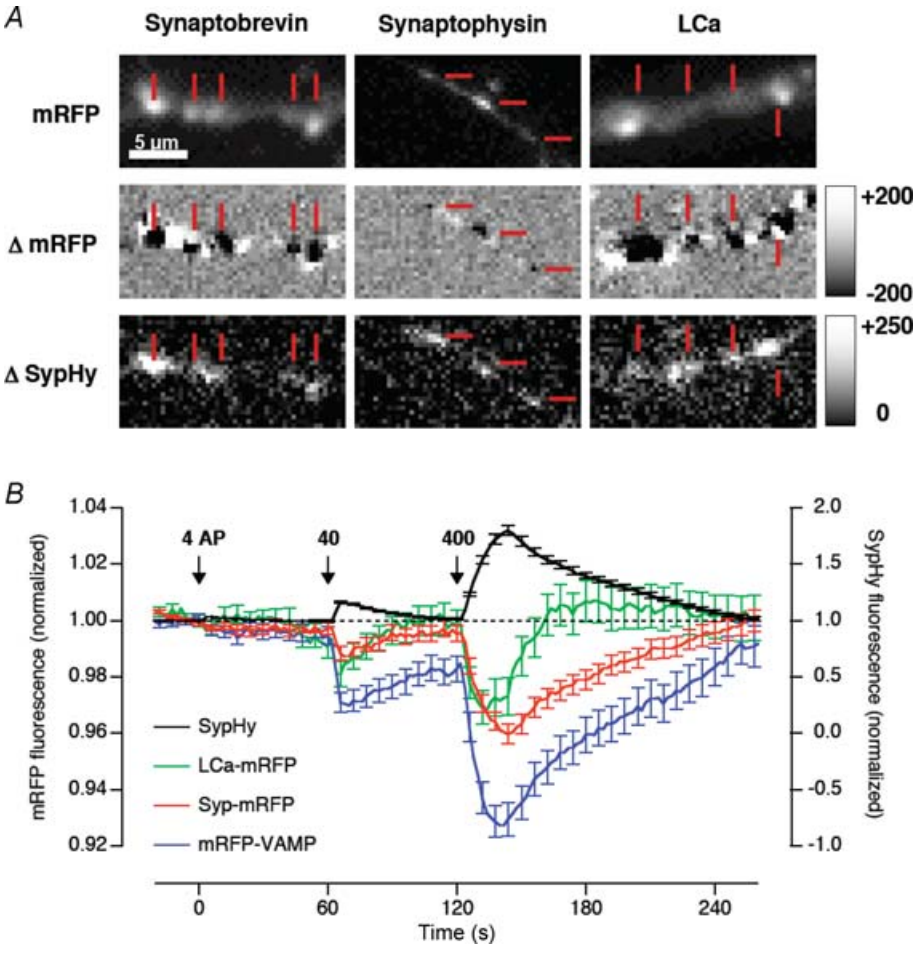

Figure 2. Clathrin, synaptobrevin and synaptophysin move out of the synapse on stimulation
A, example images obtained from neurons cotransfected with sypHy and either mRFP-synaptobrevin, synaptophysin-mRFP or LCa-mRFP (top), together with a difference image showing the change in distribution caused by a train of 40 APs (middle). Synapses were identified by sypHy responses (bottom). Synaptobrevin and clathrin leave the synapse and accumulate in neighbouring regions. Red lines indicate sites of mRFP decrease; note the colocalization of these sites with exocytic zones. *B*, average time course of the change in mRFP fluorescence in response to 4, 40 and 400 APs at 20 Hz. The sypHy response is in black. Reprinted from Granseth *et al.* (2006), *Neuron* 51, 773–786, with permission from Elsevier.

& Reese, 1973; Ringstad *et al.* 1999; Qualmann *et al.* 2000; Teng & Wilkinson, 2000).

Conclusions

The mechanism by which synaptic vesicles fuse with the surface membrane to release neurotransmitter is clearly a fundamental question, so it is perhaps surprising that it should remain controversial. Here we have emphasized the most recent evidence from optical measurements because we believe that these provide the most direct interpretations of vesicle behaviour. These measurements indicate that CME is the normal mechanism of vesicle retrieval in small hippocampal synapses and that 'kiss-and-run' does not occur to any appreciable degree.

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