Vesicle reuse revisited

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Neurons communicate at the synapse by releasing neurotransmitter from synaptic vesicles. After exocytosis, synaptic vesicle membrane and protein are locally recycled for reuse in the presynaptic terminal. The mechanisms involved in the recycling of synaptic vesicle material have yet to be fully resolved (reviewed in ref. 1). The traditional model of vesicle recycling posits that vesicles merge with the plasma membrane and then are later retrieved in perisynaptic regions by endocytosis (2–4). A second model, often referred to as "kiss-and-run" (5), suggests that neurotransmitter is released through a fusion pore that allows the vesicle to release neurotransmitter while retaining its protein and/or lipid (6). By refilling with neurotransmitter, vesicles formed in this manner could potentially become rapidly available for reuse. Evidence for rapid reuse of synaptic vesicles has been found in rat hippocampal neurons (7, 8), yet the contribution of this mechanism to pool recycling remains debated. The work by Li *et al.* (9) in a recent issue of PNAS introduced new tools and new data to this debate.

thy-1 SynaptopHluorin Mice: A New Tool for Visualizing Synaptic Transmission

Li *et al.* (9) develop a new tool for neurobiologists to exploit in their study of synaptic transmission. The authors make use of synaptopHluorin (10), a synaptic vesicle protein (synaptobrevin) that has been concatenated with a pH-sensitive GFP on its luminal side. pHluorin exhibits minimal fluorescence under acidic conditions and because secretory vesicles maintain an acidic interior, exocytosis of vesicles carrying synaptopHluorin is indicated by an abrupt brightening in fluorescence, and the retrieval of vesicle constituents is indicated by a dimming in the emission signal as newly formed organelles acidify (11). This technique for visualizing exocytosis, first developed by Miesenbock and colleagues (10), has been used by neurobiologists to study properties of exocytosis and endocytosis in cultured neurons (11, 12). Previous studies have transiently overexpressed synaptopHluorins in primary cell cultures; however, the method is cumbersome and offers little or no opportunity for selective label of a given cell type. The methodological advance by Li *et al.* is the generation of 12 lines of transgenic mice in which synaptopHluorin expression in the brain is driven by the *thy-1* promoter. The *thy-1* promoter has previously been shown to drive expression of GFP variants into different subsets of neurons. Despite the variability in expression pattern from line to line, the pattern itself apparently depends on either the transgene chromosomal insertion site and/or transgene copy number and thus is heritable (13). Previous characterization of the behavior of the *thy-1* promoter by Feng *et al.* (13) indicates that each of the 12 mouse lines is expected to express synaptopHluorin in a unique subset of neurons.

The mice generated by Li *et al.* (9) should prove useful for a variety of applications for neuroscientists interested in various aspects of synaptic transmission and neuronal signaling. For example, by selecting mice with synaptopHluorin expression in particular neuronal cell types of interest, one now has an easier way to distinguish and visualize exocytosis in particular classes of neurons in culture. The authors take advantage of this feature using two of their lines, one which expresses synaptopHluorin mostly in excitatory neurons of the hippocampus and another that specifically targets inhibitory neurons for expression. Because inhibitory and excitatory neurons are not readily distinguishable in tissue culture, the authors were more easily able to study vesicle reuse independently in inhibitory and excitatory neurons.

The utility of labeling a subset of neurons is not limited to studies in tissue culture. Li *et al.* (9) demonstrate this by visualizing stimulus-evoked exocytosis in inhibitory neurons in hippocampal slice cultures. These mice may also prove useful for *in vivo* imaging of synaptic transmission. Recently, mice expressing synaptopHluorin in olfactory receptor neurons were used to visualize odorant-evoked synaptic release *in vivo* (14). The thy1-synaptopHluorin mice should enable researchers to extend *in vivo* imaging to neurons, for which specific promoters have not been found. Moreover, given advances in imaging technologies, it is not unreasonable to speculate that these mice could be used to visualize synaptic activity near the surface of the brain not only in anesthetized living mice but also in awake behaving mice (15) or even at synapses deep within the brain (16).

Revisiting Rapid Vesicle Reuse in Hippocampal Neurons

The work of Li *et al.* (9) also adds new information to the debate over what happens to newly recycled vesicles after exocytosis. After exocytosis, a vesicle's membrane and protein are retrieved for reuse at the synapse. After retrieval, synaptic vesicles are reacidified by a V-type ATPase, which can be blocked by bafilomycin. By adding bafilomycin, vesicles containing synaptopHluorin are prevented from acidifying and thus remain bright even after endocytosis, enabling the measurement of exocytosis independently of endocytosis. The authors used this technique, first developed by Sankaranarayanan and Ryan (17), to determine whether vesicle protein (and by extension vesicles themselves) used during one stimulus are preferentially reused when the neuron is challenged by a second stimulus. Because synaptopHluorin molecules exposed to the surface during the first stimulus are prevented from reacidifying, a second stimulus in the presence of bafilomycin would be expected to produce a smaller increase in fluorescence during the second depolarization if vesicles are reused. Instead, Li *et al.* find that the second response is exactly the same size as the first, indicating that different vesicles undergo exocytosis during the second stimulus. The results were the same for both inhibitory synapses and excitatory synapses.

No evidence for preferential recycling of recently endocytosed vesicles was found.

The results by Li *et al.* (9) agree well with an earlier study by Fernandez-Alfonso and Ryan (12), who used similar techniques, in which they found no evidence for preferential recycling of recently endocytosed vesicles at physiological temperatures. These two studies stand in direct contrast with other work in hippocampal synapses (7, 8), which concluded that a subset of vesicles undergo kiss-and-run and are preferentially reused rather than being

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returned to the general recycling pool. It is not yet clear how to reconcile the differences between these results, but several differences in the methodologies used are notable. First, as mentioned by Li *et al.*, the assays for exocytosis are considerably different. Studies advocating a role for vesicle reuse have used styryl dyes (7, 8, 18), whereas evidence against such a mechanism comes from studies using alkaline trapping of synaptopHluorin-labeled vesicles. Styryl dyes, such as FM1–43, are environmentally sensitive fluorescent dyes, which are fluorescent in lipid but nonfluorescent in aqueous solution, and which reversibly intercalate into exposed membrane but cannot cross the bilayer. Hence, synaptic vesicles, whose membrane inner leaflets can be stained with the dyes by endocytosis, release the dyes during exocytosis when the inner surface becomes exposed to the outside of the cell and the dye desorbs from the membrane into the surrounding aqueous solution (19). Thus, synaptopHluorin fluorescence reports deacidification through the release of protons from the vesicle, whereas FM1–43 destaining results from a combination of desorption and diffusion. Moreover, in addition to being found in organelles, synaptopHluorin and styryl dyes are likely found in other organelles. FM1–43 will label any endocytic membrane, not just synaptic vesicle membrane. Conversely, synaptopHluorin localizes to peptide containing secretory granules (20) and endosomes (21) in some cell lines and could get localized to organelles other than synaptic vesicles in neurons. If any of these organelles labeled by FM1–43 or synaptopHluorin undergo stimulus-dependent exocytosis, it could confound the results.

Second, the stimuli used to release neurotransmitter differ between studies. As mentioned by Li *et al.* (9), much of the evidence for reuse comes from exocytosis driven by sucrose release, which works by a mechanism that is not completely understood. Nonetheless, results consistent with vesicle reuse have also been shown for electrical stimulation (7, 8). It should be noted, however, that the stimulus frequencies and durations used differ somewhat in the different studies.

Lastly, by necessity, alkaline trapping experiments are performed in the presence of bafilomycin. Although there are no apparent effects of bafilomycin on FM1–43 release kinetics in hippocampal neurons when stimulated at 10 Hz (17, 22), it remains possible that bafilomycin may have subtle effects at other stimulus frequencies. Could any of these differences account for the disparate results or could there be some other explanation? It appears that more work will have to be done before this debate can be resolved.

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1. An, S. & Zenisek, D. (2004) *Curr. Opin. Neurobiol.* **14,** 522–530.
2. Heuser, J. E. & Reese, T. S. (1973) *J. Cell Biol.* **57,** 315–344.
3. Heuser, J. (1989) *Cell Biol. Int. Rep.* **13,** 1063–1076.
4. Cremona, O. & De Camilli, P. (1997) *Curr. Opin. Neurobiol.* **7,** 323–330.
5. Fesce, R., Grohovaz, F., Valtorta, F. & Meldolesi, J. (1994) *Trends Cell Biol.* **4,** 1–4.
6. Meldolesi, J. & Ceccarelli, B. (1981) *Philos. Trans. R. Soc. London B* **296,** 55–65.
7. Pyle, J. L., Kavalali, E. T., Piedras-Renteria, E. S. & Tsien, R. W. (2000) *Neuron* **28,** 221–231.
8. Aravanis, A. M., Pyle, J. L. & Tsien, R. W. (2003) *Nature* **423,** 643–647.
9. Li, Z., Burrone, J., Tyler, W. J., Hartman, K. N., Albeanu, D. F. & Murthy, V. N. (2005) *Proc. Natl. Acad. Sci. USA* **102,** 6131–6136.
10. Miesenbock, G., De Angelis, D. A. & Rothman, J. E. (1998) *Nature* **394,** 192–195.
11. Sankaranarayanan, S. & Ryan, T. A. (2000) *Nat. Cell Biol.* **2,** 197–204.
12. Fernandez-Alfonso, T. & Ryan, T. A. (2004) *Neuron* **41,** 943–953.
13. Feng, G., Mellor, R. H., Bernstein, M., Keller-Peck, C., Nguyen, Q. T., Wallace, M., Nerbonne, J. M., Lichtman, J. W. & Sanes, J. R. (2000) *Neuron* **28,** 41–51.
14. Bozza, T., McGann, J. P., Mombaerts, P. & Wachowiak, M. (2004) *Neuron* **42,** 9–21.
15. Helmchen, F., Fee, M. S., Tank, D. W. & Denk, W. (2001) *Neuron* **31,** 903–912.
16. Levene, M. J., Dombeck, D. A., Kasischke, K. A., Molloy, R. P. & Webb, W. W. (2004) *J. Neurophysiol.* **91,** 1908–1912.
17. Sankaranarayanan, S. & Ryan, T. A. (2001) *Nat. Neurosci.* **4,** 129–136.
18. Richards, D. A., Guatimosim, C. & Betz, W. J. (2000) *Neuron* **27,** 551–559.
19. Betz, W. J., Mao, F. & Smith, C. B. (1996) *Curr. Opin. Neurobiol.* **6,** 365–371.
20. Tsuboi, T. & Rutter, G. A. (2003) *Curr. Biol.* **13,** 563–567.
21. Machen, T. E., Leigh, M. J., Taylor, C., Kimura, T., Asano, S. & Moore, H. P. (2003) *Am. J. Physiol.* **285,** C205–C214.
22. Zhou, Q., Petersen, C. C. & Nicoll, R. A. (2000) *J. Physiol.* **525,** 195–206.