Comment on "The Dynamic Control of Kiss-and-Run and Vesicular Reuse Probed with Single Nanoparticles"

Björn Granseth,[1] Benjamin Odermatt,[2] Stephen J. Royle,[3] Leon Lagnado[2]*

Zhang *et al*. (Research Articles, 13 March 2009, p. 1448) reported that synaptic vesicles usually release neurotransmitter through a kiss-and-run mechanism occurring within 1 second but that full collapse of the vesicles becomes more prevalent with repeated stimuli. We report that the kinetics of vesicle retrieval do not change during a stimulus train, with endocytosis occurring in 10 to 15 seconds.

The process by which synaptic vesicles interact with the surface membrane to release neurotransmitter has been debated since Katz described his quantal theory of neurotransmission (*1*). Do vesicles collapse into the surface membrane? Or can neurotransmitter release occur through a short-lived fusion pore by a process of kiss-and-run? This fundamental issue has been difficult to resolve because the techniques used to investigate it provide measurements open to different interpretations (*2*). One of the most direct approaches for assaying vesicle fusion and retrieval is expression of pH-sensitive green fluorescent protein molecules (pHluorins) in the lumen of synaptic vesicles by fusion to membrane proteins (*3*, *4*). When exocytosis is triggered, the loss of protons from the vesicle interior unquenches the pHluorin to generate a fluorescence increase, and this signal decays when the vesicle is reacidified after retrieval. Recent studies using pHluorins report only full collapse of synaptic vesicles, with retrieval occurring in 10 to 15 s through a clathrin-dependent mechanism (*5*–*7*). In contrast, Zhang *et al*. (*8*) reported the dominance of kiss-and-run under certain conditions by using quantum dots to assay fusion.

The fluorescence of quantum dots is also pH-dependent and increased by 15% on vesicle fusion. In Zhang *et al*. (*8*), recovery of the signal followed two patterns: A rapid return to baseline was interpreted as retention of the particle by kiss-and-run, while a much larger fall below baseline was interpreted as escape of the particle from the synaptic cleft after vesicle collapse. On this basis, the authors concluded that kiss-and-run (0.5 to 1 s) occurs with a probability of ~60% if the synapse is activated after a period of rest, falling to ~10% after the sixth stimulus delivered at 0.1 Hz. The observation that transient signals becomes less prevalent at 0.1 Hz might lead one to expect that their steady-state probability will be even lower when the frequency of stimulation is increased further, but the opposite was observed: a 3- to 4-fold increase in the probability of transient signals at 10 Hz [figure 4C in (*8*)]. Zhang *et al*. suggest that this biphasic modulation of the fusion process by stimulation frequency makes the protocol we used to investigate endocytosis inappropriate for detecting kiss-and-run using synaptophysin-pHluorin (sypHy) (*5*). In order to measure the pHluorin signal generated by a single action potential (AP) while avoiding bias introduced by selection of events from a noisy background, we delivered single APs at very low frequency (0.02 Hz) and then simply averaged traces to obtain the behavior of a population of vesicles. Using simulations, Zhang *et al*. (*8*) concluded that this approach will not have the sensitivity to detect fast kiss-and-run unless the response to the first AP after a period of rest is separated from responses to later stimuli. We have examined this prediction experimentally (*9*, *10*) and find that it does not hold.

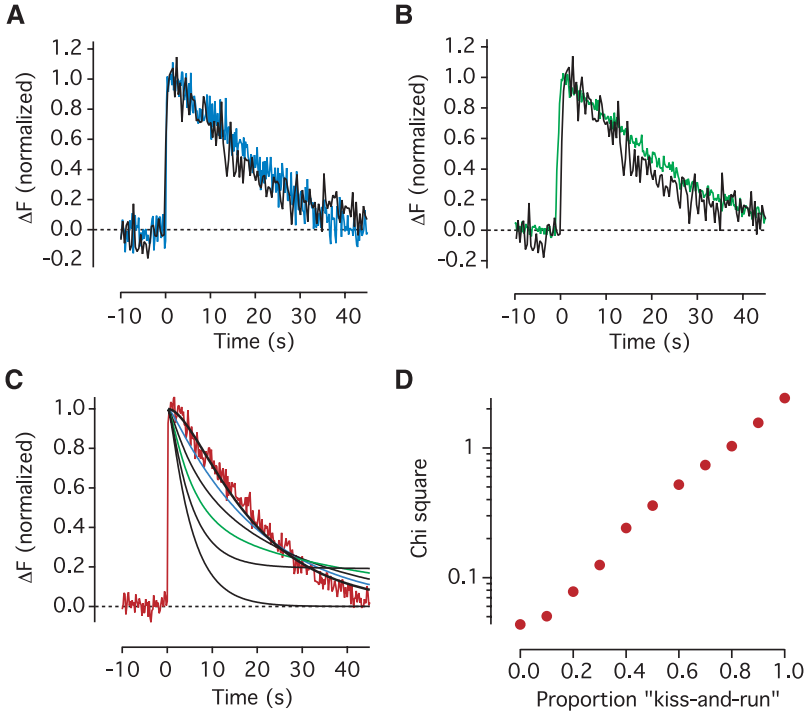

Fig. 1. Analysis of endocytosis using synaptophysin-pHluorin (sypHy). (**A**) Averaged sypHy response to the first AP in a train (black trace, $n = 139$) compared to the 8th to 10th APs (blue trace, $n = 443$) when APs are repeated at 0.02 Hz. The average for the first AP has been decimated to a frequency of 2.5 Hz from an original acquisition rate of 5 Hz. (**B**) Averaged sypHy response to the first AP in a train (black trace, $n = 139$) compared to a burst of 20 APs at 20 Hz (green trace, $n = 79$). There was no obvious change in the sypHy signal under these different conditions. (**C**) The decay of sypHy fluorescence modeled with different proportions of fast and slow endocytosis ($\tau_{KR} = 0.95$ s; τ_{FF} = open parameter) followed by reacidification ($\tau_r = 5$ s). The model predictions were fit with a least sum of squares method to the average fluorescence trace from 1480 single APs (red). The model with 100% slow endocytosis was most similar to the data (heavy black line). Colored lines are models with 20% (blue) and 60% (green) kiss-and-run, and both clearly deviate from measurements. The expected time course of decline in the sypHy signal is similar for any value of $\tau_{KR} < 2$ s. The experimental trace was collected at a sampling frequency of 5 Hz, which might not be sufficient for precisely quantifying the speed of any fast component of endocytosis occurring in a fraction of a second but is quite sufficient for the detection of any significant amount of endocytosis occurring with a time constant of 2 s or less. (**D**) Graph relating chi-square values of the fit to the model (log scale) to the proportion of kiss-and-run. Assuming just 10 to 20% kiss-and-run caused an increase in chi-square value (i.e., a deterioration in the quality of the fit).

[1]Department of Clinical and Experimental Medicine, Division of Cell Biology, Linköping University, SE 581 83 Linköping, Sweden. [2]Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, UK. [3]The Physiological Laboratory, School of Biomedical Sciences, Crown Street, University of Liverpool, Liverpool L69 3BX, UK.

*To whom correspondence should be addressed. E-mail: LL1@mrc-lmb.cam.ac.uk

First, we averaged responses to the first AP delivered after ~5 min rest, when Zhang *et al*. (*8*) predict ~60% of fusion events to occur by kiss-and-run (Fig. 1A, black trace). We then made a separate average of the responses to the 8th to 10th AP during stimulation at 0.02 Hz, when ~20% of events are predicted to be kiss-and-run (Fig. 1A, blue trace). However, the time-course of recovery was indistinguishable in the two cases, indicating that the history of stimulation did not alter the kinetics of endocytosis at this low frequency. Recovery was best accounted for by a single mode of endocytosis with a time constant of 14 s (Fig. 1C). As a further test, we averaged the sypHy signal observed in response to a burst of 20 APs at 20 Hz, when more vesicles are released (Fig. 1B, green trace). The prediction of Zhang *et al*. is that ~80% of events should be kiss-and-run (*8*), but again, a fast mode of endocytosis could not be detected.

Zhang *et al*. (*8*) also suggested that pHluorins may not be suited to detection of fast endocytosis because the fall in fluorescence lags after endocytosis by the time required to reacidify the vesicle—about 5 s at room temperature. However, reacidification occurs in ~1 s at 35°C, when it is still not possible to detect a fast mode of endocytosis (*11*). We also analyzed pHluorin responses using a model in which there are two competing modes of endocytosis, as suggested by Zhang *et al*. (*8*): fast (τ_{KR} = 0.95 s) and slow (τ_{FF} = open parameter). The equation describing fluorescence recovery is

$$F(t) = \frac{KR}{k_r - k_{KR}}\left[k_r\left(1 - e^{-k_{KR}t}\right) - k_{KR}\left(1 - e^{-k_r t}\right)\right] + \frac{1 - KR}{k_r - k_{FF}}\left[k_r\left(1 - e^{-k_{FF}t}\right) - k_{FF}\left(1 - e^{-k_r t}\right)\right]$$

where KR is the proportion of vesicles undergoing kiss-and-run and k_r is the rate-constant of acidification (0.2 s^{-1} at room temperature). Figure 1C shows the least-squares fit of the model to the average response to a single AP for different KR. The lowest chi-square value was obtained assuming that there is no kiss-and-run, whereas assuming 10 to 20% kiss-and-run caused a significant deterioration in the quality of the fit (Fig. 1D). A very large deviation from the experimental trace and increase in the chi-square value was observed assuming 60% kiss-and-run (Fig. 1C, green trace), as estimated by Zhang *et al*. (*8*). The reacidification time of 5 s would not prevent the detection of fast endocytosis using pHluorin signals acquired at 5 Hz. Measurements made with sypHy therefore argue against a major component of kiss-and-run at hippocampal synapses, even under the very specific conditions proposed in (*8*). We conclude that the dominant mechanism of endocytosis in hippocampal synapses occurs with τ = 14 s at room temperature, a conclusion also reached by Balaji and Ryan (*6*) using VGlut1-pHluorin.

It will be important to understand why the interpretations of measurements made using pHluorins and quantum dots do not coincide. We suspect that this will become clearer with further characterization of the two techniques. The advantages of pHluorin reporters is that they are targeted to all synaptic vesicles by normal cellular processes, and they remain associated with vesicle membrane through multiple rounds of exocytosis and endocytosis. In comparison, quantum dots are large (~15 nm) relative to synaptic vesicles (~24 nm diameter lumen) and the synaptic cleft (width ~20 nm) and must be loaded by a period of concerted stimulation. It is also unknown whether quantum dots hinder collapse of the vesicle membrane after fusion, when the dense particle is sandwiched between pre- and postsynaptic membranes. In particular, we question the assumption that a transient fluorescent signal with retention of the particle always equates with kiss-and-run. If exogenously applied quantum dots are taken up into the synapse during stimulation, then why should not one that has just been released directly into the synaptic cleft?

References and Notes

1. B. Katz, *The Release of Neural Transmitter Substances* (Liverpool University Press, Liverpool, UK, 1969).
2. L. G. Wu, T. A. Ryan, L. Lagnado, *J. Neurosci.* **27**, 11793 (2007).
3. G. Miesenböck, D. A. De Angelis, J. E. Rothman, *Nature* **394**, 192 (1998).
4. S. Sankaranarayanan, D. De Angelis, J. E. Rothman, T. A. Ryan, *Biophys. J.* **79**, 2199 (2000).
5. B. Granseth, B. Odermatt, S. J. Royle, L. Lagnado, *Neuron* **51**, 773 (2006).
6. J. Balaji, T. A. Ryan, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 20576 (2007).
7. Y. Zhu, J. Xu, S. F. Heinemann, *Neuron* **61**, 397 (2009).
8. Q. Zhang, Y. Li, R. W. Tsien, *Science* **323**, 1448 (2009).
9. Dissociated neurons from hippocampi dissected from E18 rats were cocultured with glia and transfected to express a plasmid coding for sypHy using Lipofectamine2000 after 8 days in vitro (DIV). Live fluorescent imaging was carried out at 14 to 21 DIV using an inverted microscope equipped with a Photometrics Cascade 512 B camera, frame rate 5 Hz. Action potentials were evoked by field stimulation. For details, see (*10*).
10. S. J. Royle, B. Granseth, B. Odermatt, A. Derevier, L. Lagnado, *Methods Mol. Biol.* **457**, 293 (2008).
11. B. Granseth, L. Lagnado, *J. Physiol.* **586**, 5969 (2008).

12. We are grateful to A. Derevier for assistance with cell culture. B.G. is supported by Linköping Unversity and the Swedish Research Council. L.L. is supported by the Medical Research Council and a Programme Grant from the Wellcome Trust.

4 May 2009; accepted 24 August 2009
10.1126/science.1175790

Comment on "The Dynamic Control of Kiss-and-Run and Vesicular Reuse Probed with Single Nanoparticles"

Björn Granseth, Benjamin Odermatt, Stephen J. Royle and Leon Lagnado

Science **325** (5947), 1499.
DOI: 10.1126/science.1175790