Soluble membrane trafficking proteins taking a break at silent synaptic vesicles

Lennart Brodin[1], Joshua A. Gregory, and Frauke Ackermann
Department of Neuroscience, Karolinska Institutet, S-171 77, Stockholm, Sweden

Synaptic nerve terminals are distinguished by clusters of synaptic vesicles that appose active zones in the presynaptic plasma membrane (Fig. 1*A*). In response to action potentials, vesicles fuse with the plasma membrane to release neurotransmitter, which is followed by vesicle recycling via endocytosis (1). It has long been recognized that subsets of synaptic vesicles within a cluster behave differently (2). One pool, often termed the recycling pool, recycles upon moderate stimulation, and a subfraction of this pool, called the readily releasable pool, is available for immediate release. The remaining vesicles in a cluster are typically reluctant to be released. Such vesicles have been shown, at least in some model systems, to be fully release-competent (2, 3) (Fig. 1*B*), which has led to the notion that reluctant vesicles serve as a reserve pool that can be recruited during periods of intense neurotransmitter release (2). As yet, however, studies regarding the role of reluctant vesicles have been limited to different in vitro situations. A study in PNAS (4) now makes the move to in vivo conditions by investigating central and peripheral synapses in a variety of animal models, ranging from worm to rat.

A common way to detect recycled vesicles is to supply a tracer compound to the extracellular medium, stimulate, and then look for labeled vesicles. Denker et al. (4) treat their experimental animals with tracers (FM1–43 or HRP) and leave them to behave undisturbed for periods of up to several hours. Subsequent electron microscopic analysis shows that only a tiny fraction of the vesicles, 1–5%, had undergone recycling (4). This proportion is lower than most in vitro estimates of the recycling pool, although a wide range of values has been reported (2, 5–7). A similar result is obtained in experiments using an alternative method (pH-sensitive synaptophluorin) that monitors proton loss from cycling vesicles (4). Denker et al. (4) go on to test whether reluctant vesicles would be recruited in a stressful situation. Locusts are left with a predator frog until captured and half-swallowed. However, despite being subjected to extreme stress, synapses in the main escape muscle are found to contain no more than 5% recycled vesicles, thus arguing against any role of the reserve pool in boosting synaptic transmission (4).

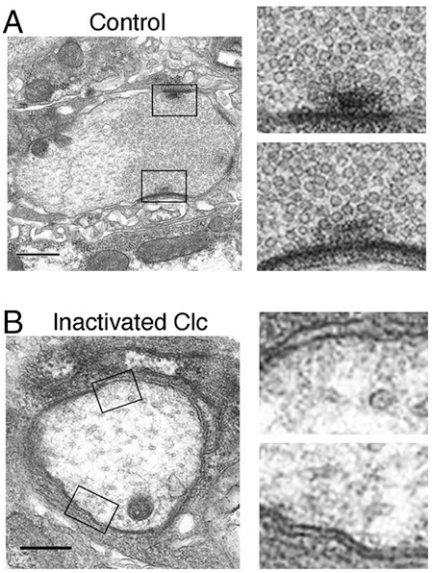

Fig. 1. All synaptic vesicles at the *Drosophila* neuromuscular junction (NMJ) can be released by action potential stimulation. (*A*) Control NMJ fixed after 6,000 stimuli at 8 Hz and 1 min of rest. The NMJ expressed the light chain of clathrin fused with Lumio, which permits photoinactivation. (*B*) Neuromuscular junction stimulated as in *A* but after endocytosis had been impaired by photoinactivation of the Lumio-labeled clathrin light chain. The synaptic bouton is now devoid of synaptic vesicles, indicating that every single vesicle is competent for release. Clc, clathrin light chain. (Scale bar: 500 nm; magnification: *Insets*, 4×.) [Reproduced from Current Biology, 18, Clathrin Dependence of Synaptic-Vesicle Formation at the *Drosophila* Neuromuscular Junction, 401–409, Copyright (2008), with permission from Elsevier.]

What then are all the surplus vesicles good for? In a companion paper, Denker et al. (8) suggest a simple but appealing model, that the silent vesicles are used as a spatially restricted buffer for soluble vesicle trafficking proteins. The buffered proteins are proposed to be made accessible by displacement from vesicles through an electrostatic calcium-dependent mechanism. Their model is consistent with a previous model focused on the behavior of endocytic proteins. Proteins like amphiphysin, dynamin, endophilin, and intersectin reside within the synaptic vesicle cluster at rest and relocate to the periactive zone on stimulation to participate in synaptic vesicle endocytosis (refs. 9–13; regarding synapsin, see also ref. 14). Denker et al. (8) find that a number of other soluble membrane trafficking proteins are also accumulated in the synaptic vesicle cluster. These include NSF, complexin, rab3, rabphilin, Rim2, and cysteine string protein, which serve different functions in vesicular exocytosis and protein chaperoning (8). Apart from the localization studies, three observations support the model. First, toxin-induced depletion of synaptic vesicles is shown to result in the escape of several of the exocytic proteins away from release sites, rather than binding at alternative presynaptic sites. Second, a moderate elevation of intracellular calcium, which had little effect on vesicle cycling, is shown to cause a similar escape of trafficking proteins from release sites. Third, in in vitro experiments, calcium is found to antagonize binding of many exo- and endocytic proteins to isolated synaptic vesicles (8). The model of Denker et al. (8) seems to explain how release sites can be effectively supplied with critical proteins without a need for high cytoplasmic levels. Furthermore, it agrees well with the very slow recruitment of clathrin to nerve terminals observed on repetitive stimulation (15). The two studies (4, 8), however, do not rule out additional roles of silent vesicles, such as supporting slow spontaneous neurotransmitter release (e.g., ref. 16). Nor do they rule out the possibility that a larger proportion of the vesicles is cycled at some synapses not included in the present analysis.

The work by Denker et al. (4, 8) also highlights another problem: Do distinct vesicle pools use distinct recycling pathways, and, if so, for what purpose? One model (2, 17) implies that the reserve pool vesicles are replenished by bulk endocytosis, which acts in parallel with a clathrin- and AP2-mediated endocytic pathway that replenishes the recycling pool. However, because bulk endocytosis has most commonly been observed after strong stimulation in vitro, it remains to be tested whether this mechanism operates in vivo. Another model (18) implies that reserve pool vesicles form selectively via an endosomal recycling pathway that uses the adaptor proteins AP1 and/or

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[1]To whom correspondence should be addressed. E-mail: lennart.brodin@ki.se.

AP3 as well as the small GTPase Arf1. The observation that an Arf1 inhibitor could enhance transmitter release led to the suggestion that vesicle recycling under this condition was steered away from an AP1/AP3 pathway, generating reluctant vesicles, toward an AP2 pathway that produces vesicles with higher release probability (18). The latter model was recently supported by the observation that synaptic vesicles tagged with VAMP7, a vesicular SNARE dependent on AP3 sorting (19), are far more reluctant to be released than vesicles tagged with the "common" v-SNARE VAMP2/synaptobrevin2 (16). To complicate matters, another study similarly performed in cultured hippocampal neurons but monitoring a set of endosomal SNARE proteins (syntaxin 13, syntaxin 6, and vti1a) reached a different conclusion (20). These authors found that the endosomal SNAREs recycled together with VAMP2 during brief stimulation, suggesting that readily releasable rather than reluctant vesicles recycle via endosomes. During prolonged stimulation, which is expected to attract more reluctant vesicles, recycling of VAMP2 alone dominated. The studies by Denker et al. (4, 8) have undoubtedly lifted the vesicle pool problem to the in vivo level, but detailed insights into synaptic vesicle recycling mechanisms thus far rest, to a large extent, on in vitro observations.

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