The fidelity of neurotransmission at synapses depends on the maintained availability of transmitter-filled synaptic vesicles (SVs) in the presynaptic terminal. The availability of SVs is in turn governed by the number of releasable vesicles in the presynaptic terminal and the rate at which these vesicles are recycled within the terminal. Thus, elucidating these two rate-limiting determinants of the SV cycle is essential for understanding the constraints on information transfer at synapses. Recently, we developed a new functional method for counting releasable SVs in cultured hippocampal autaptic neurons. Extending this method, we show here that, in addition to a larger than previously-thought population of releasable vesicles, presynaptic terminals are endowed with the ability to utilize a rapid local recycling mechanism, thus boosting the efficiency of the SV cycle under high-frequency stimulus conditions.

The rate of information transfer at a synapse is restricted in large part by the biophysical properties of the presynaptic terminal. Specifically, the number of functionally available vesicles, as well as the kinetics with which spent vesicles are replenished within the terminal, represent presynaptic rate determinants for synaptic transmission. At small central synapses, these presynaptic determinants have traditionally been probed using electron microscopy (EM) and, more recently, real-time optical approaches (i.e., FM dyes, SynaptopHluorin).

EM studies have been invaluable in providing the spatial resolution necessary to identify the total number of SVs present within a presynaptic terminal [100–200 at small cortical synapses]. However, these observations only offer a static snapshot; thus, the functional availability of these morphologically-identified vesicles has remained unclear. Although SVs all look alike in EM images, the existence of functionally heterogenous pools of vesicles has, in fact, been noted for some time. Current models recognize three functionally distinct pools of vesicles: the readily releasable pool (RRP), the recycling pool, and the reserve pool. The RRP vesicles are immediately available for release, and are thought to correspond to those which are morphologically docked on the presynaptic membrane. The recycling pool of vesicles comprises those which maintain release under moderate stimulation. Finally, the reserve pool consists of vesicles that are thought to be refractory to all but the strongest (possibly non-physiological) stimuli.

This three-pool model has, in part, been established with the aid of the newer optical methods mentioned above (FM dyes, SynaptopHluorin, etc.). These methods have lead to the conclusion that a surprisingly small percentage of the total number of vesicles in a nerve terminal, only about 20–30%, is functionally available for release. That is, ~70–80% of SVs in hippocampal terminals are thought to reside in the reserve pool, where they may be inaccessible for release under normal conditions. These findings seem inconsistent with the need for small synaptic terminals (which contain relatively few SVs) to make optimal use of their limited synaptic resources.

A New Method of Counting SVs Reveals that More Vesicles are Available for Release

To address this paradox, we recently introduced a new electrophysiological assay for studying the SV cycle. In the past, two
main considerations have complicated the use of electrophysiological methods for studying the SV cycle at small central synapses. First, the small size of most central presynaptic terminals prevents electrode access for electrophysiological techniques such as amperometry and capacitance measurement. Second, the constant replenishment of used SVs makes it difficult to track individual vesicles through the SV cycle. Our new method has overcome both of these limitations. It involved recording excitatory postsynaptic currents (EPSCs) originating in the self-synapses (autapses) that are formed by a single hippocampal pyramidal neuron when it is grown in isolation in cell culture.8

We counted SVs by measuring the postsynaptic electrical response (the miniature EPSC) that is produced by the release of the contents of a single vesicle. Thus, we were able to bypass the need for direct access to presynaptic terminals. To eliminate the confounding factor of vesicle replenishment, we applied Baflomycin-A1 (Baf), a potent inhibitor of the SV proton pump. Baf prevents the reacidification of spent vesicles. Baf thereby inhibits the activity of the proton-dependent vesicular transporter that is responsible for refilling recycled SVs with neurotransmitter. Thus, in the presence of Baf, a given vesicle will contribute to the postsynaptic response only once before becoming functionally invisible, because it is unable to reload.

We used this new method of tracking individual SVs to count the number of releasable vesicles within a terminal. We found that the majority of morphologically identified SVs within a hippocampal nerve terminal can be released under mild stimulus conditions, given sufficient time.7 This contrasts with previous suggestions that most SVs are locked away in the reserve pool. That is, our results show that many more SVs are functionally accessible than previously thought, with benefits for the fidelity of information flow at small central synapses. It should be noted, however, that the additional vesicles in the reserve pool, although accessible with mild stimulation, are likely to be only slowly accessible. Thus, they may not be useful under demanding conditions of high-frequency synaptic transmission. This brings us to the second determinant of synaptic fidelity, the kinetics of vesicle replenishment.

**Two Main Pathways for the Recycling of SVs**

Broadly speaking, there are thought to be two main ways in which SVs release their load of neurotransmitter and are recycled: (1) full-collapse fusion with the presynaptic membrane, followed by clathrin-dependent endocytosis (often called ‘classical recycling’; Fig. 1), and (2) transient fusion followed by rapid recovery of the vesicle (sometimes called ‘local recycling’ or ‘kiss-and-run’; Fig. 1). In full-collapse fusion, each vesicle loses its identity, and new vesicles must be assembled from scratch,9 a process that has been estimated to take from 30 to 60 s.10,11 Hence, if the rapidly-accessible recycling pool contains only ~30 vesicles per synapse, the kinetic restrictions imposed by the full-collapse pathway would restrict the rate of vesicle release to 0.5–1 Hz. This limit can only be exceeded by engaging a more rapid recycling mechanism.

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Figure 1. High-frequency synaptic stimulation in the presence of Baflomycin-A1 (Baf) reveals the existence of local recycling of synaptic vesicles. If classical recycling mechanisms predominate during high-frequency stimulation, the size of the functional readily-releasable pool (RRP) will be unaffected soon after the addition of Baf. In contrast, if a local recycling mechanism contributes to the synaptic vesicle cycle, the size of the functional RRP later in the train is reduced after Baf addition.
The local recycling pathway\textsuperscript{12} has been proposed to fill this kinetic gap.\textsuperscript{13-15} Recently, the use of quantum dot nanoparticles has provided more direct evidence of single vesicles undergoing multiple rounds of local recycling before committing to full-collapse fusion.\textsuperscript{16} Generally, these pieces of evidence for local recycling have relied on presynaptic optical measurements, leading to some controversy in the field. We have addressed this issue by using the functional counting method described above, allowing us to supplement optical studies with direct electrophysiological measurements.

A Functional Assay Reveals Fast Local Recycling

The basic idea behind our experiments is illustrated in Figure 1. According to the quantal model of synaptic transmission, the size of the postsynaptic response is directly proportional to the presynaptic release probability. Release probability, in turn, correlates with the size of the RRP: the larger the number of release-ready vesicles in the RRP, the higher the probability that a vesicle will be released. This chain of reasoning allows us to draw inferences about the size of the RRP from the size of the postsynaptic response.

The two kinds of SV recycling—full-collapse fusion and local recycling—have different consequences for the size of the RRP during a rapid train of stimuli (Fig. 1). For full-collapse fusion, the RRP is replenished from the adjacent pool of recycling vesicles (Fig. 1, left). All of these recycling pool vesicles are likely to be full of glutamate, because they have had time to be recycled and refilled. Thus, when the RRP is replenished via this pathway, it will always appear functionally full during the train of stimuli. This situation would be unchanged immediately after Baf application, because most SVs have not yet had the opportunity to release and to lose (permanently) their load of glutamate. Thus, most SVs in the recycling pool and the RRP are still fully-charged with glutamate, with or without Baf treatment.

The situation is very different if local recycling predominates (Fig. 1, right). Now SVs are released, recovered and reused locally within the RRP. Immediately after Baf application, used vesicles quickly find their way back into the RRP but remain empty. Hence, their presence in the RRP dilutes the number of vesicles that are functional, leading to a lower apparent release probability and a reduction in the postsynaptic response during the train of stimuli. Thus, if local recycling occurs, Baf treatment is expected to unmask a significant depression in EPSC amplitude during a train.

These predictions are tested in the experiment illustrated in Figure 2. Figure 2A shows a typical example of the autaptic EPSC recorded in response to a 20 Hz train of action potentials before (black) and immediately after (purple) acute application of Baf. Although the amplitude of the first EPSC in the train after Baf application is indistinguishable from the control trace, subsequent EPSC amplitudes in the train are smaller after Baf treatment compared to control. This would suggest that, although depression of the response is unavoidable with particularly demanding stimuli, an alternative recycling mechanism (local recycling) is available to partially compensate for this presynaptic depression.

Conclusions

Here we have described electrophysiological experiments in isolated hippocampal pyramidal neurons that support the existence of a larger functional pool size than previously thought, as well as rapid re-use of SVs in small central synapses. Our findings on re-use are consistent with...
earlier work using other approaches. A strength of our approach is that the autaptic culture system provides a homogeneous population of presynaptic terminals that can be studied under well-defined conditions. Our results establish that synapses have evolved at least two mechanisms for the efficient use of synaptic vesicles in the brain, thereby ensuring the maintenance of neurotransmission for information processing.

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