Measurements of Vesicle Recycling in Central Neurons

Timothy A. Ryan and Harald Reuter

Neurotransmitter-containing vesicles in presynaptic nerve terminals are essential for synaptic transmission. The vesicles undergo a cycle that leads to transmitter release by exocytosis followed by endocytosis and refilling of the vesicles. Here we discuss new optical methods that have helped researchers study this cycle at functional and molecular levels, which is essential for our understanding of the regulation of synaptic transmission.

Synaptic transmission is a key element of intercellular communication of neurons in the central nervous system (CNS). The release of neurotransmitters from presynaptic nerve terminals (boutons) and their subsequent binding to specific postsynaptic receptors are the fundamental events leading to excitatory or inhibitory functions of the CNS. These events can be modulated in a number of ways. For example, they can be potentiated or depressed by coincident activation of pre- and postsynaptic structures in axons and dendrites. Modulations of synaptic transmission are thought to be important for cognitive functions such as memory and learning. To understand mechanisms of neurotransmitter release from axonal boutons and subsequent signal transduction in dendritic spines, we not only need detailed knowledge of the molecular machineries involved but also quantitative measurements of the evoked electrical signals and their structural correlates. As a matter of fact, postsynaptic electrophysiological recordings were the first successful attempts to unravel synaptic transmission. However, they provide only indirect information about presynaptic structural changes, such as docking and fusion of transmitter-containing vesicles in boutons. Therefore, it is not surprising that in the literature considerable uncertainty exists about the quantal nature of transmitter release in CNS synapses. In recent years, a number of new methods have been developed for direct measurement of exocytosis and endocytosis of vesicles in presynaptic nerve terminals. Membrane capacitance and optical measurements have proven to be extremely useful for the study of presynaptic vesicle recycling (see Fig. 1). In this brief review, we will focus on experimental evidence obtained with optical methods on the kinetics and mechanisms of vesicle-cycling presynaptic terminals of central neurons.

Exocytosis of presynaptic vesicles

The beautiful demonstration by Katz and colleagues (cited in Ref. 7) of quantal acetylcholine release in frog neuromuscular junctions by simple electrophysiological recordings and most insightful interpretation has been the guideline of our thinking about synaptic transmission in chemical synapses. The sequence of events in synaptic transmission consists of several discrete steps. Action potentials generated in the soma of a neuron travel down the axon and invade boutons. The ensuing depolarization opens Ca2+ channels in the presynaptic membrane, and Ca2+ influx into the terminal leads to the fusion of docked vesicles that pour their transmitter content into the synaptic cleft. Binding of transmitter molecules to closely apposed specific receptors in dendritic spines causes either excitation or inhibition of electric activity in postsynaptic dendrites. The number of fused presynaptic vesicles is crucial for the amount of transmitter released and thus for the strength of the postsynaptic response. After exocytosis, clathrin-coated vesicular membranes are retrieved and the endocytosed vesicles are newly filled with neurotransmitter and prepared for the next cycle of release. This seemingly simple picture of transmitter release requires an incredibly complicated array of synaptic proteins, which include the soluble N-ethylmaleimide-sensitive factor (NSF) attachment receptors (SNAREs) present in vesicular and presynaptic target membranes. The SNARE proteins form a complex during vesicle docking and fusion, a reaction that is only partially understood. After fusion of a vesicle, the complex has to be dissociated again by the ATPase NSF before endocytosis of the vesicular membrane can proceed.

How can we measure the turnover of vesicles in small presynaptic boutons of central neurons? These boutons contain between 100 and 200 vesicles that have to recycle. The boutons are too small to record changes in the membrane capacitance during vesicle fusion and retrieval (Fig. 1A). However, Betz and colleagues (2) have developed an optical method to measure vesicle recycling in presynaptic terminals of neuro-
FIGURE 1. Biophysical techniques for studying exocytosis and endocytosis in neurons. Top: membrane capacitance can be used to monitor net surface area of the whole cell or total membrane under a patch pipette at very high time resolution (ms). This works best in chromaffin cells, in which fusion of individual secretory granules (100–200 nm diameter) can be measured (1). In giant synaptic terminals, simultaneous fusion and retrieval of dozens of small clear synaptic vesicles can be measured (15). Middle: assays of presynaptic function using FM 1–43 are done in 3 steps. Action potential stimuli are evoked in nerve terminals (1) either during application of FM 1–43 or before application by a time delay (2). FM 1–43 has a permanent charge of +2, which prevents the molecule from passively diffusing across cell membranes. In the loading phase (2), the dye is taken up by endocytosis of vesicle membranes that ensues after exocytosis. The newly captured membrane, with the dye, is then refashioned into a releasable synaptic vesicle. On subsequent stimulation, the dye is released into the medium during exocytosis (3). Quantitative assays of the amount of dye taken up during 1 or 2 is performed using fluorescence imaging of the labeled nerve terminals during this 2nd round of electric stimulation. To reduce background, terminals are rinsed extensively between loading and unloading. The time scale of endocytosis can be determined by measuring total dye uptake as a function of delay time Δt. Bottom: synaptic vesicle membrane protein recycling. A genetically encoded reporter of vesicle lumen pH (ecliptic pHluorin) is used to determine the recycling of VAMP from synaptic vesicles to the cell surface and back. Exocytosis relieves the proton-dependent quenching of pHluorin fluorescence. The pHluorin molecule is attached to the luminal aspect of VAMP. At the resting pH (5.6), the fluorescence signal from pHluorin is completely quenched. During firing of action potentials, the vesicles undergo fusion with the plasma membrane, leading to the externalization of pHluorin (pH 7.4). This relieves the proton-dependent quenching and causes an increase in fluorescence. The fluorescence signal then recovers after endocytosis by reacidification of vesicles. The reacidification is very rapid (11), and the kinetics of fluorescence decay reflect the time course of endocytosis of VAMP.
muscular junctions. They introduced the styryl dye FM 1–43, which is taken up into membranes and there becomes strongly fluorescent. The dye-labeled vesicular membranes remain fluorescent during endocytosis. After washout of the dye from surface membranes, the boutons containing labeled vesicles are clearly visible under a confocal microscope. Fluorescence intensity of boutons depends on the number of labeled vesicles. The dye can be released again during electric stimulation of the cells when the vesicles are exocytosed. Uptake of FM 1–43 does not inhibit refilling of the vesicles with neurotransmitters, as judged by measurements of spontaneous post synaptic currents. We first used this technique to measure vesicle turnover in cultured hippocampal cells (Fig. 1B). The sensitivity of fluorescence measurements has been refined to such an extent that endo- and exocytosis of single vesicles could be determined in individual boutons. The results clearly showed the quantal nature of vesicle uptake and release (9). After endocytosis, vesicles do not seem to be processed in an endosomal compartment in these cells, but after loading with neurotransmitter they are ready for the next cycle (6).

A crucial step in the fusion of vesicles with the plasma membrane is the opening of nearby Ca$^{2+}$ channels. Specific voltage-gated Ca$^{2+}$ channels (P/Q- and N-type channels) are localized in high densities in presynaptic membranes and seem to interact directly with proteins of the SNARE complex, notably with syntaxin. Thus these channels are closely linked to the site of vesicle docking. On opening of the channels, the Ca$^{2+}$ concentration rises to high levels near these sites and allows the vesicular membrane to fuse with the plasma membrane. However, how exactly this final fusion step takes place is still unclear. Possible additional insight may become available thanks to a new optical technique, called “evanescent-wave video microscopy,” in combination with molecular-biological tools. Evanescent-wave microscopy has so far been used in chromaffin cells (14), but it should also be useful for studying exocytosis in certain neuronal preparations. This technique makes use of the total internal reflection of a laser beam at the glass-water interface where cells are plated. In addition to the reflected light into the glass, a small amount of light penetrates into the water. This evanescent wave decays exponentially over a distance of as little as 50–100 nm and therefore excites the fluorescence of only a few labeled vesicles in a pool localized in close proximity to the surface membrane.

How is the Ca$^{2+}$ concentration near the release sites controlled? In addition to the opening of Ca$^{2+}$ channels, which allows Ca$^{2+}$ to flow into the bouton, the subsequent activation of a Na$^+$/Ca$^{2+}$ exchanger and of a Ca$^{2+}$-ATPase leads to an extrusion of the ions. The exchanger is electrogenic, and, depending on the membrane potential and the intracellular Na$^+$ concentration, it can reverse its mode of operation and can even lead to an uptake of Ca$^{2+}$ into the small presynaptic compartment, sufficient to cause facilitated neurotransmitter release. Mitochondria participate in intracellular Ca$^{2+}$ buffering to a variable extent. As shown by quantitative electron microscopy and three-dimensional reconstruction of axons (13), ~50% of the boutons lacked these organelles. This morphological finding is consistent with a rather heterogeneous response of the Ca$^{2+}$ concentration in boutons to electric stimulation under conditions in which the Na$^+/Ca^{2+}$ exchanger was inhibited. In many boutons, the Ca$^{2+}$ concentration decreased very slowly, whereas in others it returned to basal levels rather rapidly. When mitochondria were also reversibly inhibited, the Ca$^{2+}$ concentration remained high in all boutons until Na$^+/Ca^{2+}$ exchange was reactivated. Simultaneously, the frequency of miniature postsynaptic currents, as a measure of transmitter release, was greatly increased. Thus the tight interplay between specific channels, transporters, and mitochondria is essential for a fine-tuned regulation of Ca$^{2+}$ concentrations and transmitter release in individual boutons (12).

Exocytosis of vesicles and transmitter release are not a simple function of the rise in the intracellular Ca$^{2+}$...
A new technique was introduced that follows the fate of specific synaptic vesicle membrane proteins to be measured. Depending on size and lifetime of the fusion pore, the vesicle releases somewhere between none and all of its neurotransmitter. This mode implies that no continuity between the two lipid bilayers exists, but rather a transient protonation bridge forms during fusion providing an aqueous channel connecting the vesicle lumen to the synaptic cleft. If this picture is correct, then vesicle membrane components should not intermix with the plasma membrane during the fusion event. It is interesting to note that such fusion events could not possibly have been seen in freeze-fracture electron microscopy. Recall that the pits or dimples seen there were at least 25 nm in diameter, about half of the cross-section of a synaptic vesicle.

Convincing evidence of the existence of such transient fusions comes from membrane capacitance measurements in chromaffin cells. In those cells, it is possible to simultaneously measure the transient addition of membrane to the cell surface while measuring the conductance of a transient fusion pore. When cells are exposed to 5 mM extracellular Ca\textsuperscript{2+} concentrations, transient fusion events account for ~7% of all fusion events and have a mean fusion time of ~800 ms. At very high external Ca\textsuperscript{2+} levels (90 mM), the frequency of occurrence increases 10-fold and the mean fusion time decreases to ~41 ms (1). Unfortunately, no such direct observations of transient fusion events have been possible at synaptic terminals, where secretory vesicles are typically 5–6 times smaller in diameter than in chromaffin cells.

Endocytosis timescales have also been measured in small synaptic terminals using a pulse-chase protocol similar to that depicted in Fig. 1B. By bath applying the fluorescent tracer FM 1–43 at different times after exocytosis and subsequently measuring the extent of loading, we (10) and others (reviewed in Ref. 2) have estimated the time scale of endocytosis to be at least several seconds and to be even longer with heavy stimulation. Indirect estimates of endocytosis times have also been obtained using measurements of the destaining efficiency of membrane probes such as FM 1–43 and related analogs during exocytosis. This approach has been used to argue both for (4) and against (10) the existence of a kiss-and-run pathway. The crux of this experiment is to take advantage of the relatively slow dissociation times of FM 1–43 and related probes from the plasma membrane (~3 s for FM 1–43 and ~2-fold slower or faster for different analogs; see Ref. 10). If no intermixing of vesicle membrane and plasma membrane occurs, then FM 1–43 should be greatly compromised in its ability to escape during fusion events, with lifetimes much shorter than this time scale of dissociation. The main difficulty with this approach is that it does not distinguish between a fast clathrin-mediated endocytosis pathway and a true kiss-and-run pathway since even dye that has diffused laterally beyond the site of fusion might be reendocytosed before dissociating from the membrane.

Recently, a new technique was introduced that follows the fate of specific synaptic vesicle membrane proteins to be measured.

---

**Modern biophysical measurements of endocytosis**

Kiss-and-run vesicle recycling is presumed to consist of a fusion event that maintains a fusion pore open only transiently.
ured rather than just membrane during vesicle recycling. Here an optical readout based on the local pH of the luminal domain of the protein is used (see Fig. 1C). Synaptic vesicles are specialized endosomes that maintain an acidic lumen (pH ~ 5.6) due to the activity of a vacuolar H^+-ATPase. Following fusion with the plasma membrane during action potential firing, the luminal surface of the synaptic vesicle abruptly switches to the more alkaline pH of the extracellular environment (pH ~ 7.4). By attaching a pH-sensitive green fluorescent protein to the luminal domain of the synaptic vesicle protein vesicle-associated membrane protein, one can monitor the cycle of alkalization and reacidification that accompanies vesicle recycling. Once endocytosed, the reacidification of the vesicle appears to be very rapid, and therefore the kinetics of fluorescence recovery are largely dictated by the time scale of endocytosis (11). This approach provides real-time measurements of the redistribution of vesicle proteins from the intracellular vesicle pool to the cell surface and back. Analysis of the kinetics of endocytosis following different stimuli suggests that endocytosis is a rapid but saturable process: the rate of vesicle internalization appears to be up to 1 vesicle per second independent of the amount of protein that awaits endocytosis. The pathway measured in these experiments presumably does not arise from kiss-and-run-type events since the recovery times indicate that vesicles wait in queue for reinternalization. These experiments do not currently rule out another very fast endocytosis acidification pathway since such events would result in no net fluorescence change for the time resolution (perhaps seconds) of these measurements.

Modulation of endocytosis speeds

Although increasing amounts of information concerning the specific proteins involved in endocytosis have become available (see below), the framework for understanding how endocytosis is regulated is largely absent. Several lines of evidence, however, suggest that Ca^{2+} plays an important role in this regard. The precise role of Ca^{2+} is not yet clear. Experiments in retinal bipolar cells have provided very convincing evidence that when the average cytoplasmic Ca^{2+} is maintained above 1 µM, endocytosis is completely blocked (15), whereas comparisons of endocytosis times following prolonged stimuli with cytoplasmic Ca^{2+} dynamics indicate that the two are not causally related in neuromuscular junctions (see Ref. 2). In hippocampal synapses, stimulation at increasing frequency (11) or at elevated external Ca^{2+} (4) appears to accelerate the speed of endocytosis.

The molecular basis of endocytosis

Considerable progress has been made over the last decade in isolating and identifying proteins that appear to be directly involved in endocytosis at nerve terminals. Apart from the original observations of clathrin-coated pits peripheral to the active zone, the finding that dynamin plays a critical role in endocytosis has proven central to the discovery of other proteins in this pathway. The picture that has emerged is one in which synaptic vesicle proteins are retrieved by virtue of their interactions with adaptor proteins (AP-2) that in turn interact with clathrin. In addition to this, dynamin, which forms a collar around the neck of an invaginated pit, appears to interact with a number of SNARE-containing proteins via interactions with a proline-rich domain. This interaction appears capable of recruiting more proteins with a variety of functions such as covalent modifications of lipids and interactions with the actin cytoskeleton (a recent review of these proteins and pathways can be found in Ref. 5).

One of the greatest challenges for the future will be to understand how this newly emerging family of interacting proteins is coordinated to carry out the efficient retrieval of synaptic vesicle components from the cell surface. In addition, there is very little information currently available regarding whether more than one molecular pathway for this process exists. For example, it is possible that not all endocytic events occur via a clathrin-mediated pathway, as is implied by the kiss-and-run hypothesis. It is unclear, however, whether such a pathway might rely on other elements in the arsenal of endocytic proteins. Since recycling of synaptic vesicles is critical to synaptic performance, it will be especially interesting in the future to determine which molecular components are rate limiting in the catalysis of membrane and vesicle protein retrieval. Finally, determining how endocytosis is triggered, how membrane fission is achieved, and how the size of synaptic vesicles is kept constant are all questions awaiting careful experimental approaches and analyses.

References