

## Vesicular Release

I've already indicated that the physiologically measured quanta of transmitters are thought to correspond to the amount of transmitter contained within a synaptic vesicle (SV). That is, release of transmitter in response to an AP is believed to be caused by fusion of synaptic vesicles with the synaptic plasma membrane and diffusion out of the cells. A substantial amount of evidence indicates that this is the basic release mechanism at most, if not all, chemical synapses, and I'd like to review some of the evidence that supports this hypothesis.

Much of the evidence is morphological--i.e., based on structural analysis of the presynaptic nerve terminal, especially at the neuromuscular junction (nmj). First, if one looks at an electron micrograph of the nmj, then one sees that the vesicles in the nerve terminal are not randomly distributed, as they might be if they were merely storage containers. Rather, they are concentrated on the side of the nerve terminal nearest to the muscle cell, and particularly they're opposite high density concentrations of ACh receptors at "active zones", as shown in the cartoon in Fig. 5.8C. Moreover, one can occasionally take EM photographs of the nmj in which it appears that the vesicles have been caught in the act of fusing with the plasma membrane. But to demonstrate that this organization is functionally meaningful, what's needed is evidence that stimulating the nerve to cause ACh release causes vesicles to fuse with the synaptic membrane.

Several kinds of evidence suggest that this is so. One line of evidence is to patch clamp nerve cells in culture (they're more or less spherical), stimulate them repeatedly to release transmitter and measure the capacitance of the nerve cell. It's known that capacitance is roughly proportional to the area of a membrane; that is, a large cell has larger capacitance than a small cell. What Wolf Almers (who's now at OHSU) and others found in these capacitance measurement experiments is that the capacitance of nerve cells increases when the cells are stimulated repeatedly to release transmitter. What is your interpretation of that result? They assumed that fusion of SVs with the nerve plasma membrane caused the increase in capacitance. Interestingly, this increase was transient; that is, when they stopped stimulating the neuron to release transmitter, the capacitance slowly returned to its baseline value.

A different line of evidence was provided by John Heuser and colleagues, using a combination of morphological and electrophysiological studies of the frog nmj. Heuser developed a system in which the muscle, nerve and stimulating electrode could be mounted on a plunger above a metal block that was cooled with liquid Helium. When the muscle was slammed against the cold metal block it froze instantly (in less than one msec), and could be studied by a form of electron microscopy called freeze etching. In this method, the water (ice) is sublimed away from the surface of the cell in a vacuum at low temperature, then a thin film of platinum (Pt) is sprayed over the cell surface at an oblique angle. Finally, the Pt cast of the nerve cell surface is studied in the EM. What Heuser did was to treat the nerve-muscle preparation in various ways to alter the amount of transmitter release. This included stimulating or not stimulating the nerve to release transmitter, just before the muscle slammed into the metal block, and altering the concentration of a K<sup>+</sup> channel blocker (4-aminopyridine), which prolongs the AP in the nerve terminal ; this will have what kind of effect on release? (Prolongs Ca<sup>2+</sup> influx and increases transmitter release). The left side of Fig. 5.8A in Purves et al. shows what he saw at the surface of an unstimulated nerve terminal. If he then stimulated the nerve while the muscle was headed toward the freezing block, he saw what's shown on the right side of Fig. 5.8A. The "dimples" are believed to be the SVs fusing with the presynaptic nerve terminal, and the conclusion they drew was that stimulation causes a substantial increase in the number of vesicles that fuse with the n.t. But Heuser and his colleagues went even further. The physiologists Michael Dennis and Lily and Yuh Nung Jan conducted parallel experiments in which they counted the total number of quanta released at various concentrations of 4-AP, while Heuser counted the total number of vesicle profiles that he saw on a nerve terminal. They then plotted the relationship between the number of quanta released and the number of vesicles fusing, and got the result in Fig. 5.8B. As you can see, there's a virtually perfect correspondence, indicating what?

Now, the capacitance experiments and these experiments of Heuser indicate that the nerve terminal membrane enlarges when the SVs fuse with it. This suggests that there would over time be an increase in N.T. membrane area and a decrease in SV concentration, leading to eventual inactivation of the synapse, unless something is going on to oppose this process. What could that be? In a classic series of experiments in the 1970s Heuser and Tom Reese studied the frog

sartorius nmj to answer this question. They stimulated the nerve repeatedly for 15 minutes, then examined the nerve terminal in the EM. What they saw was a decrease in the number of SVs, but an increase in the area of large membrane enclosed compartments that they called cisternae (which are now called endosomes), an increased number of structures called coated vesicles, and somewhat more nerve terminal membrane area. If they allowed the nerve to rest after stimulation and then studied it, it looked more or less normal--more SVs, fewer cisternae and coated vesicles and less nerve terminal membrane than the stimulated nerve terminal. What's going on?

What they did to figure this out was to use a large molecule that couldn't cross the plasma membrane as a marker of the inside and outside of the cell--they used horseradish peroxidase (HRP), a protein that catalyzes a reaction whose product can be detected in the electron microscope. When they soaked a resting muscle in Ringer's solution that contained HRP, they found all the HRP was in the extracellular fluid and none in the cytoplasm of nerve or muscle; i.e., the HRP did not normally cross the cell's membrane. Then they stimulated the nerve briefly while it was soaked in HRP, and saw a few vesicles that contained HRP. When they stimulated repeatedly in the presence of HRP, they saw many vesicles that contain HRP. (And lots of clathrin-coated vesicles and labeled endosomes). If they stimulated the nmj in HRP, washed it out, and then stimulated some more, they found that the number of HRP-labeled vesicles decreased.

How would you interpret these findings? Fig. 5.9 shows a drawing of their model of the process of vesicle membrane retrieval and reformation of vesicles. Thus, two processes are going on at chemical synapses. One is a  $\text{Ca}^{2+}$  dependent fusion of SVs with the plasma membrane in the nerve terminal, leading to release of transmitter. This is the process called exocytosis. But a second process, endocytosis, is necessary to retrieve the synaptic vesicle membrane and recycle it for reformation of SVs and refilling with transmitter in the nerve terminal.

If one grants the validity of this model, then it raises several questions. In particular, neurobiologists would like to know more about the details of the processes of exocytosis and endocytosis. What proteins in the synaptic vesicle, nerve terminal cytoplasm, and plasma membrane are involved, and how does the concentration of  $\text{Ca}^{2+}$  in the nerve terminal regulate the probability of fusion of the vesicles with the synaptic plasma membrane? The answers still aren't

completely in hand, but people have used three different, but complementary, strategies to address these questions, including:

1. Purifying SVs to identify the proteins that are uniquely associated with them.
2. Trying to "reconstitute" the vesicle fusion process *in vitro* (in a test tube) by adding SVs, plasma membrane, and other substances (proteins, ATP, etc.) necessary for fusion.
3. Using genetic approaches in which organisms (such as *Drosophila*, *Caenorhabditis elegans*, mice) are mutated to eliminate the function of a protein suspected to act in release of SVs and then analyzing the characteristics of the synapses of these mutant animals.

This is very much an active area of study, which means that the final picture is not yet available, and some tentative conclusions may have to be revised on the basis of further studies. Nonetheless, there are several broad conclusions that are likely to withstand further study, even if some of the details will be altered by new evidence.

First. The synaptic plasma membrane and synaptic vesicle membrane contain a number of unique and complementary proteins that appear important in aligning the vesicle at the release site. These proteins are identified in Fig. 5.13, and their normal location is indicated, if it is known. These include subunits of a proton pump that is probably required for uptake of the neurotransmitter into the vesicles, and then there is a bunch of proteins that are believed to be involved in moving the vesicle around within the nerve terminal, lining it up near the active zone, and then catalyzing fusion with the plasma membrane in the presence of  $\text{Ca}^{2+}$ .

It's thought that many of the vesicle proteins interact specifically with other proteins that are in the nerve terminal membrane and soluble in the cytoplasm of the nerve terminal. so that the vesicle "docks" at the active zone of the presynaptic membrane. That is, it's believed that in a resting nerve terminal there are a bunch of vesicles sitting right up against the plasma membrane and held there by protein-protein interactions.

It turns out that a similar process is believed to be involved in movement of other kinds of vesicles in cells. So the second important point is that the secretory process for synaptic vesicles seems to be a modified form of the movement and fusion of other vesicles in eukaryotic cells. The proteins involved at the synapse are similar to those found in other vesicle transport/fusion

processes such as vesicle trafficking between endoplasmic reticulum, Golgi apparatus, and plasma membrane. The general model that describes these vesicle-mediated docking and fusion activities is called the SNARE hypothesis by James Rothman and his colleagues who developed it, based on an elegant series of reconstitution experiments conducted *in vitro*.

I should say that the terminology here is somewhat confusing. Rothman et al. call the protein in the vesicle membrane that is required for docking the v-SNARE; the complementary protein in the target membrane is the t-SNARE. Other proteins that are necessary for the budding, movement, and fusion of vesicles are called NSF, SNAP, and a GTP binding group of proteins called Rabs. What I want you to get from this information is not the specific names of the players, but the general features that seem to characterize these membrane fusion processes.

Fig. 5.14 shows you a current model derived on how the SV fusion complex is organized. As you see, it is believed that at many different proteins are required. One is the v-SNARE class, an integral membrane protein of the vesicle--in synaptic vesicles it seems to be the protein called either VAMP (vesicle-associated membrane protein) or synaptobrevin, as in the book. Another is the t-SNARE, an integral membrane protein of the target plasma membrane, which is probably syntaxin, a non-vesicle protein that's concentrated in the nerve terminal. In addition there are several soluble proteins that bind to these membrane associated proteins and presumably help to hold them together. These include NSF, and the SNAPs. (See Figs. 5.14 B1 and B2). Finally all the fusion pathways are stimulated by a small GTP binding protein of the Rab family (over 30 different Rab proteins have been identified) that is embedded by a lipid tail in the vesicle membrane for the duration of the fusion event, but that is recycled back to the origin and reused in formation and fusion of new vesicles. Rab 3A appears to be associated with synaptic vesicle fusion, and there is a complementary protein called rabphilin 3A that interacts with Rab. It's believed that the Rab protein hydrolyzes GTP, which provides the driving force to propel the SV into its docking site on the nerve terminal membrane. (Rabs are omitted from Fig. 5.14, presumably to avoid scaring you to death).

Third. Although these docking steps do not require calcium, the final step in vesicle release is  $\text{Ca}^{2+}$  dependent. The  $\text{Ca}^{2+}$  sensor on the vesicle is probably a protein called synaptotagmin.

That is, the model is that the SV docks at the appropriate t-SNARE (syntaxin) and just sits there until  $\text{Ca}^{2=}$  levels rise. By a mechanism not well understood, synaptotagmin, which binds two  $\text{Ca}^{2=}$  ions is thought to undergo a conformational change in the presence of  $\text{Ca}^{2=}$  and thereby assist or allow the two membranes to fuse and the transmitter to be released by the nerve terminal. (Recent results suggest that synaptotagmin may also function in vesicle endocytosis). Mice that have mutant forms of synaptotagmin have serious defects in transmitter release. (Fig 5.14 B3 and B4)

Reclamation (recycling) of the vesicular membrane appears to involve a protein called dynamin, the clathrin proteins that form coats around the vesicles, and likely other proteins as well, but again, the details are not yet well worked out (see Fig. 5.14 C and D).

Here the specific model I'm presenting is somewhat tentative. It's more like a best guess based on available evidence rather than a well-supported hypothesis. It's likely to change over time, as new evidence becomes available. However, the general features are probably essentially correct, and the identification of several important molecular components of the fusion process, the ability to create mutant *Drosophila* or "knock-out" mice to investigate the function of these proteins, coupled with classical electrophysiological analysis of release should allow rapid progress in this area.

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