

Figure 1. The upper part of the figure shows an apical/basal section of two interacting plasma membranes at a tight junction. The interacting membrane proteins (claudins) separate the apical (a) from the basolateral (bl) extracellular spaces. A section perpendicular to the drawing at one of the cell-cell interactions reveals that the claudins (and occludin) form a continuous linear polymer (lower part of the figure), interrupting the external leaflet of the lipid bilayer. The claudins may interact between cells to form a variety of ion-selective channels (A, B or C) joining the two extracellular spaces.

pH dependence and a diameter of ~6 Å. Exchanging the first extracellular loop between claudin-2 and claudin-4 changes the Na⁺ and Cl⁻ selectivities of the paracellular pathway in cultured epithelial cells.

Aside from forming ion channels, what else do claudins do? Claudins and occludin (also a four-pass integral membrane protein) interact laterally in the membrane circumscribing the cell to form a 'fence'. This barrier prevents the diffusion of extracellularly facing lipids between apical and basolateral plasma membrane domains. The tight junction may also be an important component of several signal transduction pathways. For example, claudins promote the activation of the

prodomain-containing form of the matrix metalloproteinase MMP-2, and have been implicated in the β-catenin-Tcf/LEF signaling pathway. Claudins may also participate in vertebrate morphogenesis, for example in embryonic left-right axial patterning.

Are they involved in disease? Claudin-3 and claudin-4 serve as *Clostridium perfringens* enterotoxin receptors. Additionally, claudin-4 is deleted in patients with Williams-Beuren syndrome, a neurodevelopmental disorder affecting multiple systems. Claudin-5 is deleted in Velo-cardio-facial/DiGeorge syndrome patients and claudin-5-deficient mice show increased leakage through the blood-brain barrier. Mutations in claudin-14 are involved in hereditary deafness, while mutations in claudin-16 are associated with hypomagnesemia. In addition, claudins may play a role in various cancers, because levels of claudin-7 and claudin-23 were found to decrease in ductal carcinoma and intestinal-type gastric cancer, respectively. Similarly, claudin-1 was downregulated in cases of hereditary breast cancer. However, increased expression of claudin-1 and claudin-4 has been observed in colorectal cancer and pancreatic and ovarian cancers, respectively.

Where can I find out more?

- Tsukita, S. and Furuse, M. (2002). Claudin-based barrier in simple and stratified cellular sheets. *Curr. Opin. Cell Biol.* **14**, 531-536.
- Colegio, O.R., C.Van Itallie, C.Rahner, and Anderson, J.M. (2003). Claudin extracellular domains determine paracellular charge selectivity and resistance but not tight junction fibril architecture. *Am. J. Physiol. Cell Physiol.* **284**, C1246-C1254.
- Tang, V.W. and Goodenough, D.A. (2003). Paracellular ion channel at the tight junction. *Biophys. J.* **84**, 1660-1673.

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Primer

Synaptic vesicles

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Chemical synapses provide the predominant form of fast functional information transfer between neurons in the brain. Synaptic transmission is initiated in a presynaptic neuron when neurotransmitter-containing vesicles release their contents into the synaptic cleft, which physically separates the presynaptic and postsynaptic neurons. The released neurotransmitter molecules then bind to their cognate receptors on the postsynaptic neuron, eliciting an array of chemical and electrical changes. Early physiological studies made profound contributions to our understanding of the discrete (quantal) nature of neurotransmitter release and its calcium-dependence. Over the past two decades, our knowledge of synapse operation has been advanced by molecular biological, genetic and biochemical analyses (Box 1).

The presynaptic terminal, located along the axon of most neurons, is a compartment where neurotransmitter-containing vesicles cluster near a highly specialized region of the plasma membrane called the 'active zone'. From there, vesicles release their contents during synaptic transmission. There are exceptions to this general architecture — for example, presynaptic specializations can occur in dendrites rather than in axons and there are synapses specialized for continuous release that do not have conventional active zones, but have 'ribbons'. Before neurotransmitter release can occur from a given release site, synaptic vesicles must be sorted, translocated to the active zone, dock and be primed for fusion. Synaptic vesicle recycling is an integral feature of presynaptic function, therefore, we have chosen the synaptic vesicle cycle as the central theme of this Primer.

Box 1

Experimental techniques for investigating the synaptic vesicle cycle.

Electron microscopy

Investigations into the synaptic vesicle cycle have their origins in a series of initial ultrastructural observations using electron microscopy. In the early 1970s, two groups made two apparently contradictory observations using the frog neuromuscular junction stimulated at different frequencies for varying lengths of time. Heuser and Reese (1973) observed clathrin-coated pits and omega-shaped regions of membrane, which suggested full collapse of synaptic vesicles and clathrin-mediated recapture. Ceccarelli and colleagues (1973) observed little change in the ultrastructure of stimulated synapses, which suggested that synaptic vesicles may form transient links with the plasma membrane and be locally recaptured, a model later to become known as 'kiss-and-run'. Starting with these initial observations, electron microscopy has been used to investigate several aspects of the synaptic vesicle cycle. Although electron microscopy can provide valuable information, with exquisite (sub-nanometer) spatial resolution, about the synaptic vesicle cycle, it is not a real-time technique, making it difficult to use for studying rapid events.

Biochemical methods

Numerous biochemical approaches for investigating synaptic transmission and the synaptic vesicle cycle have been developed over the past several decades. Using molecular biology and genetics, several synaptic vesicle proteins have been identified and their interactions with other proteins and lipids have been investigated. Subcellular fractionation permits the isolation of specific membrane components, as well as the identification of the proteins within these fractions. To understand the roles of specific presynaptic proteins and their domains, it has become indispensable to combine biochemical approaches and genetic methods (knock-outs or knock-ins, for example). Direct biochemical approaches are not easily adapted to intact cells and, therefore, other methods must be used in intact cells. Recently, fluorescence methods have begun to be used to study protein-protein interaction in intact living cells, and are rapidly approaching biochemical rigor.

Electrophysiology

Electrical recordings made from individual neurons or in some cases at individual synapses have provided us with a wealth of information regarding the synaptic vesicle cycle. A particular strength of electrophysiology is the impressive temporal resolution (microseconds) it affords. Exocytosis of individual vesicles from a single synapse can be evoked using brief stimuli, and individual release events may be detected by monitoring postsynaptic current influx or changes in postsynaptic membrane potential. Changes in membrane capacitance arising from the fusion of synaptic vesicles with the presynaptic active zone can be observed using electrophysiological techniques in some large synapses. Capacitance measurements have provided detailed information regarding the kinetics of some steps in the synaptic vesicle cycle. Another electrophysiological method called amperometry can be used to detect certain transmitters, such as dopamine, that are oxidizable. A combination of these electrophysiological techniques has provided us with information regarding the number of vesicles within a given synaptic pool (i.e., the readily releasable pool). Electrophysiology, however, has the inherent weakness of monitoring only vesicles that interact with the plasma membrane. In addition, in many cases it is not easy to identify which synapses are being studied.

Fluorescence microscopy

Although optical microscopy has lower spatial resolution than electron microscopy and lower temporal resolution than electrophysiology, it provides an optimal combination of resolution (spatial and temporal resolutions of micron and millisecond, respectively). In fact, it is possible to optically monitor the exocytosis and endocytosis of an individual synaptic vesicle. Using fluorescent styryl dyes such as FM1-43, it has been shown that not all synaptic vesicles recycle through an endosome and that individual vesicles may be reused. Additionally, the sizes of different synaptic vesicle pools as well as the kinetics of traffic between the different pools have been measured using fluorescence microscopy. Genetically encoded fluorescent probes have also become useful recently. In particular, synaptopHluorin, a pH sensitive fusion protein, has been used to investigate synaptic vesicle dynamics. Compared to other real-time methods, fluorescence microscopy has poorer signal-to-noise ratio and poorer temporal resolution at present.

trafficking and recycling. In fact, when axons are severed from their soma, the terminals are capable of remaining functional for quite some time. In addition to synaptic vesicles, the presynaptic terminal is enriched with components required for both exocytosis and endocytosis: these include specialized neurotransmitter transporters to repack empty vesicles; endosome organelles that might mediate some aspects of vesicle recycling; elements of smooth endoplasmic reticulum that may regulate intracellular Ca^{2+} ; mitochondria to meet the energy demands placed on the vesicle cycle; and a matrix of cytoskeletal elements and scaffolding proteins thought to facilitate synaptic vesicle sorting. A large number of cytoplasmic and plasma membrane proteins that appear to play regulatory roles are also found in synapses.

How are synaptic vesicles distributed within the presynaptic terminal? Typical presynaptic terminals in the mammalian forebrain contain about 200 synaptic vesicles. Under resting conditions, less than half of these vesicles participate in synaptic transmission. It is unclear why the remaining vesicles do not participate in the synaptic vesicle cycle. A subset of the recycling vesicles — about 10 typically — are in apparent contact with the active zone and are called 'docked vesicles'. Whereas docked vesicles can only be observed through ultrastructural examination, they are thought to be the morphological correlate of a physiologically defined 'readily releasable pool'. Members of the readily releasable pool are the first vesicles to undergo fusion upon invasion of an action potential into the presynaptic terminal (Figure 1A).

How do vesicles dock and what triggers their exocytosis?

Once an action potential propagates into a presynaptic terminal, information transfer from the presynaptic to the postsynaptic neuron occurs in about a millisecond. What is the sequence of events responsible for

What is in the presynaptic terminal?

The presynaptic terminal contains all the necessary molecular

machinery that permits it to function as an autonomous, subcellular compartment highly specialized for local vesicle

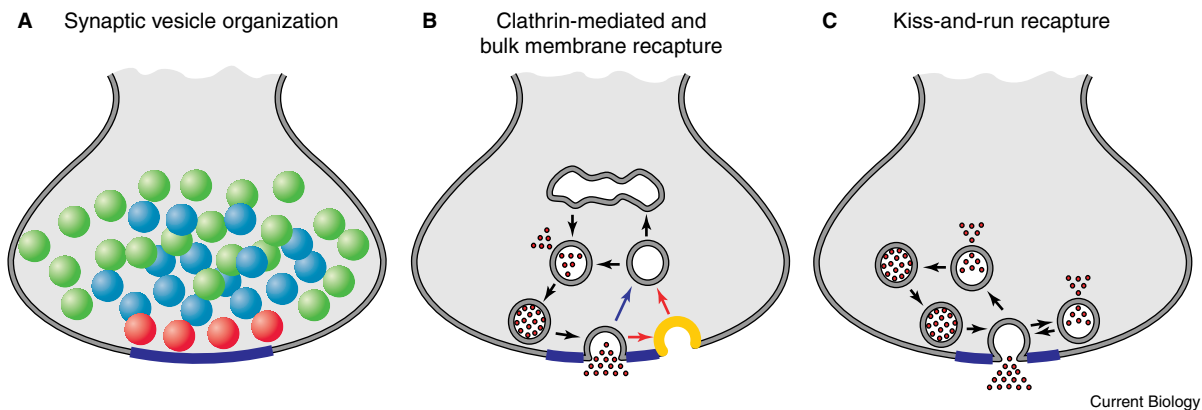


Figure 1. Synaptic vesicle organization and recapture.

(A) General organization of vesicles within the presynaptic terminal. The resting pool of synaptic vesicles is depicted in *green*, the recycling pool is depicted in *blue*, and the readily releasable pool is depicted in *red*. (B) Clathrin-mediated (*red arrows*) and clathrin-independent (*blue arrow*) modes of synaptic vesicle recapture. (C) Kiss-and-run mode of recapture.

ensuring synaptic vesicle fusion on such a timescale? The architecture of the active zone and the protein assemblies located there are specialized for rapid release of neurotransmitter. Evoked neurotransmitter release is triggered by Ca^{2+} influx through voltage-gated Ca^{2+} channels located in and near the presynaptic active zone.

Although we are beginning to understand the molecular machinery responsible for synaptic vesicle fusion, we still do not fully understand the protein interactions that are initially responsible for recruiting synaptic vesicles to docking sites. One candidate mechanism involves the interaction between the small GTPase Rab3a, located on vesicles, and a protein called *Rab3a interacting molecule* (RIM) in the active zone.

Once synaptic vesicles have docked at the active zone, they must be transformed into releasable vesicles. This step (or steps) is called 'priming', but the exact mechanisms involved are unclear. At least three proteins — RIM, Munc13 and Munc18 — have been implicated in this process.

Following vesicle docking and priming, synaptic vesicles may fuse with the presynaptic membrane to release their contents. As with other intracellular membrane fusion events, synaptic vesicle exocytosis is mediated by SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins. Two plasma

membrane SNARE proteins, Syntaxin and SNAP-25, interact with the vesicular SNARE protein Synaptobrevin (also called VAMP or vesicle-associated membrane protein) to promote bilayer fusion. Interestingly, in addition to the SNARE proteins, several other proteins — including Munc13 and Munc18 — appear to have an essential role in synaptic vesicle fusion. Another synaptic vesicle membrane protein, Synaptotagmin, is thought to be the calcium sensor for synaptic vesicle exocytosis. The exact kinetics of exocytosis during normal activity are highly regulated by many factors, including calcium and cyclic nucleotides.

What is the fate of a vesicle following exocytosis?

In the central nervous system, neurons may sustain action potential firing at rates of 10 Hz or more for several seconds. Given that there are only around 50 synaptic vesicles contributing to synaptic transmission at any given time, neurotransmitter containing vesicles might become depleted within a few seconds, if there were no efficient mechanisms for their replenishment. Synaptic vesicles are recycled following exocytosis and release of neurotransmitter.

Conventional wisdom suggests that exocytosed vesicles are replaced by prepackaged vesicles that are waiting in line. In parallel, exocytosed vesicles are

recovered at sites away from the active zone and return back to the recycling pool for later reuse. An alternative (or complementary) route of reuse invokes a mode of exocytosis called 'kiss-and-run', in which vesicles are quickly recovered at the site of exocytosis and prepared for reuse on location.

How are vesicles retrieved?

The current debate about the exact mode of exo-endocytosis can be cast as questions highlighting three key issues. First, during synaptic vesicle fusion, do the synaptic vesicle's bilayer and proteins mix with the plasma membrane by fully collapsing into the active zone, or do synaptic vesicles merely form a fusion pore with the presynaptic membrane? Second, if the vesicle does not fully collapse and is retrieved intact, does it stay in place or does it immediately leave the active zone to allow an existing vesicle to take its place? Third, if a synaptic vesicle undergoes full fusion, do the vesicular components remain at the release site or do they move to a peri-active zone region for subsequent retrieval?

Three basic modes of synaptic vesicle retrieval are thought to exist at synapses: clathrin-mediated endocytosis of vesicular components from plasma membrane, a similar mechanism independent of clathrin (sometimes called 'bulk membrane

retrieval') and fast local recapture without loss of vesicle membrane identity (Figure 1B,C). Unlike the first two modes, which are experimentally supported by multiple methods, fast local recapture of synaptic vesicles has been inferred based mainly on physiological observations. The widely accepted and best understood mechanism of synaptic vesicle recapture is clathrin-mediated endocytosis. Clathrin-coated pits have been frequently observed near the active zone of stimulated synapses, but usually not in resting synapses. Through a series of protein-protein interactions, a clathrin coat recognizes vesicular components within the presynaptic membrane and forms an invagination that subsequently buds off as a clathrin-coated vesicle through the action of Dynamin. Lipid modifying and lipid deforming proteins such as Amphiphysin, Epsin and Endophilin also appear to play important roles in endocytosis. Following the uncoating of the recaptured vesicle, the endocytic vesicle is most likely to mature directly to become a fusion-competent vesicle — an additional sorting step through endosomes may exist at some synapses (Figure 1B).

Clathrin-mediated endocytosis clearly contributes to vesicle recycling, but it is believed to be inefficient due to its lack of speed, with a retrieval time of more than 10 seconds. The actual speed of clathrin-mediated recapture of an individual vesicle at the presynaptic terminal, however, remains to be determined and there may be surprises here. Slow membrane retrieval that does not depend on the formation of clathrin coats may mediate another mode of endocytosis. While the precise mechanisms of this clathrin-independent membrane retrieval remain vague, large membrane invaginations observed at sites away from the active zone may represent an intermediate step in this process. An even faster mode of exo- and endocytosis (thought to be too fast to be

mediated by clathrin-dependent endocytosis) has received considerable attention in recent years and is colloquially called 'kiss-and-run'. However, it is important to remember that fast endocytosis does not automatically imply kiss-and-run.

The kiss-and-run mode of synaptic vesicle retrieval is relatively fast, with a retrieval time of less than one second, and has gathered support recently from studies on central synapses. Compared to other modes of endocytosis, a crucial distinction of kiss-and-run is the way the synaptic vesicle interacts with the presynaptic active zone membrane to release its contents. During kiss-and-run, synaptic vesicles are thought to form a fusion pore, rather than to fully collapse, with the active zone to release their neurotransmitter content. The synaptic vesicle can either remain at its docked site for subsequent reuse or may undock while remaining in the recycling pool of synaptic vesicles (Figure 1C). Experimental evidence supporting kiss-and-run of small synaptic vesicles has relied on the kinetics of various fluorescent probes — there is a pressing need for further experiments addressing the underlying molecular mechanisms.

How are synaptic vesicles reused?

Synaptic vesicles must re-associate with several proteins that are presumably lost during exocytosis, and have to be refilled with neurotransmitter. The majority of neurotransmitter filling is thought to be carried out by synaptic vesicle neurotransmitter transporters. These transporters use electrochemical gradients generated by proton pumps to load synaptic vesicles with small non-peptide transmitters such as glutamate and GABA (γ -aminobutyric acid). We still lack a clear picture of synaptic vesicle sorting following exocytosis. Some synaptic vesicles have been shown to retain their identity and remain in the recycling pool for later reuse. The rate of reacidification of the

vesicle lumen (an acidic pH is necessary for neurotransmitter loading) and the rate of subsequent transmitter filling are unknown, as are the exact locations of vesicles at the time at which these processes occur. In the kiss-and-run mode of exo-endocytosis, it has been supposed that transporters refill synaptic vesicles while they remain docked at the active zone. It is not known how synaptic vesicles recaptured by clathrin-mediated endocytosis are placed back into the pool of synaptic vesicles. Endocytosed vesicles appear to exchange with the readily releasable (docked?) vesicles over just a few minutes at some synapses, indicating constant motion of at least some vesicles. It is puzzling that resting and recycling vesicles appear to be fairly well-mixed morphologically, especially in the face of their mobility during synaptic activity.

Concluding remarks

Recent studies in the presynaptic terminal have uncovered many interesting phenomena that require mechanistic explanation. Advances in techniques to study the vesicle cycle in intact living synapses, combined with precise molecular genetic manipulation are sure to accelerate progress in this field.

Further reading

- Ceccarelli, B., Hurlbut, W.P., and Mauro, A. (1973). Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. *J. Cell Biol.* 57, 499–524.
- Heuser, J.E., and Reese, T.S. (1973). Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. Cell Biol.* 57, 315–344.
- Murthy, V.N., and De Camilli, P. (2003). Cell biology of the presynaptic terminal. *Annu. Rev. Neurosci.* 26, 701–728.
- Rettig, J., and Neher, E. (2003). Emerging roles of presynaptic proteins in Ca^{++} -triggered exocytosis. *Science* 298, 781–785.
- Royle, S.J., and Lagnado, L. (2003). Endocytosis at the synaptic terminal. *J. Physiol.* 553, 345–355.