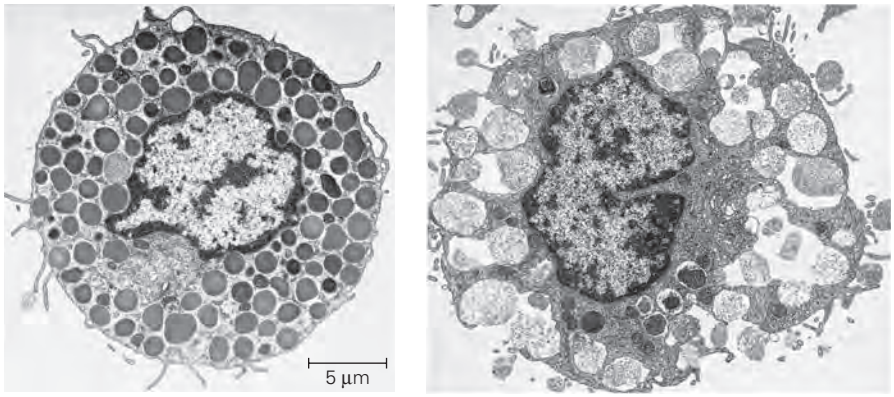
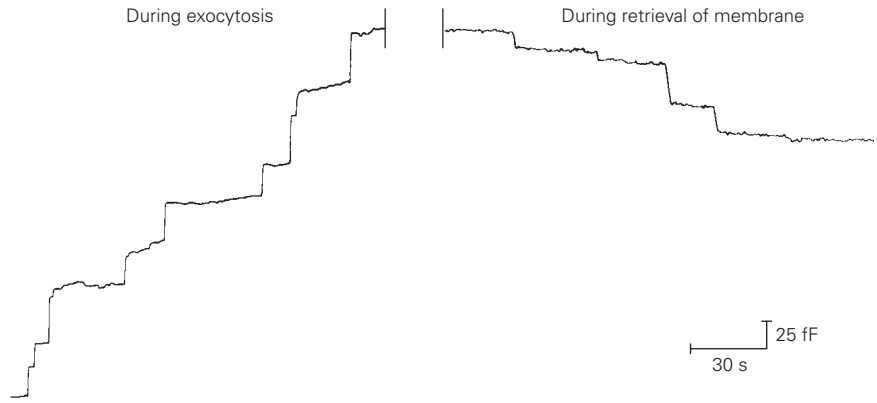
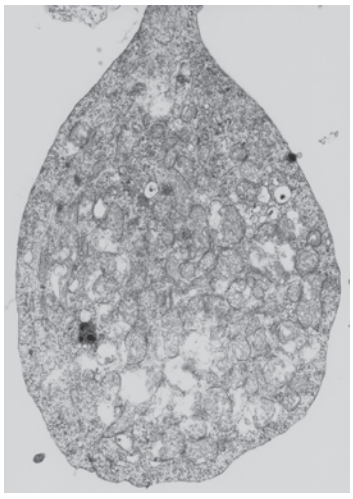
A Mast cell before and after exocytosis of secretory vesicles

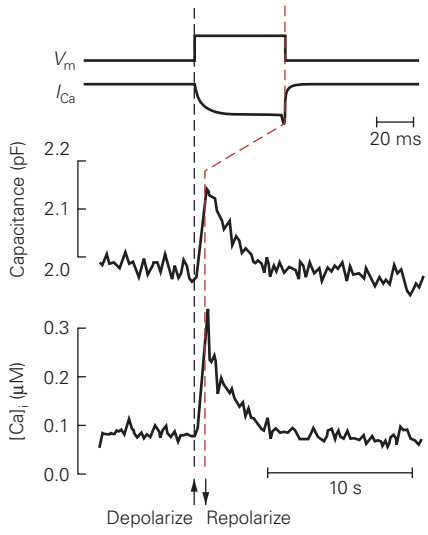


B Membrane capacitance during and after exocytosis of mast cell vesicles



C Retinal bipolar neuron terminal





current, corresponding to the exocytosis of the contents of a single dense-core vesicle. In some instances, these large transient increases are preceded by smaller, longer-lasting current signals that reflect leakage of transmitter through a fusion pore that flickers open and closed several times prior to complete fusion (Figure 15–9B).

It is possible that transmitter can also be released solely through transient fusion pores that fleetingly connect vesicle lumen and extracellular space without full collapse of the vesicle membrane into the plasma membrane. Capacitance measurements for exocytosis of large dense-core vesicles in neuroendocrine cells show that the fusion pore can open and close rapidly and reversibly. The reversible opening and closing of a fusion pore represents a very rapid method of membrane retrieval. The circumstances under which the small clear vesicles at fast synapses discharge transmitter through a fusion pore, as opposed to full membrane collapse, are uncertain.

The Synaptic Vesicle Cycle Involves Several Steps

When firing at high frequency, a typical presynaptic neuron is able to maintain a high rate of transmitter release. This can result in the exocytosis of a large number of vesicles over time, more than the number morphologically evident within the presynaptic terminal. To prevent the supply of vesicles from being rapidly depleted during fast synaptic transmission, used vesicles are rapidly retrieved and recycled. Because nerve terminals are usually some distance from the cell body, replenishing vesicles by synthesis in the cell body and transport to the terminals would be too slow to be practical at fast synapses.

Synaptic vesicles are released and reused in a simple cycle. Vesicles fill with neurotransmitter and cluster in the nerve terminal. They then dock at the active zone where they undergo a complex *priming* process that makes vesicles competent to respond to the Ca^{2+} signal that triggers the fusion process (Figure 15–10A). Numerous mechanisms exist for retrieving the synaptic vesicle membrane following exocytosis, each with a distinct time course (Figure 15–10B).

The first, most rapid mechanism involves the reversible opening and closing of the fusion pore, without the full fusion of the vesicle membrane with the plasma membrane. In the *kiss-and-stay* pathway, the vesicle remains at the active zone after the fusion pore closes, ready for a second release event. In the *kiss-and-run* pathway, the vesicle leaves the active zone after the fusion pore closes, but is competent for rapid rerelease. These pathways are thought to be used preferentially during stimulation at low frequencies.

Jorgensen and colleagues have described a second pathway of *ultrafast* clathrin-independent endocytosis that is 200 times faster than the classical clathrin-mediated pathway. Beginning just 50 ms after exocytosis, ultrafast endocytosis occurs just outside of the active zone.

Stimulation at higher frequencies recruits a third, slower recycling pathway that uses clathrin to retrieve the vesicle membrane after fusion with the plasma membrane. Clathrin forms a lattice-like structure that surrounds the membrane during endocytosis, giving rise to the appearance of a coat around the coated pits observed by Heuser and Reese. In this pathway, the retrieved vesicular membrane must be recycled through an endosomal compartment before the vesicles can be reused. Clathrin-mediated recycling requires up

Figure 15–8 (Opposite) Changes in capacitance reveal the time course of exocytosis and endocytosis.

A. Electron micrographs show a mast cell before (*left*) and after (*right*) exocytosis. Mast cells are secretory cells of the immune system that contain large dense-core vesicles filled with the transmitters histamine and serotonin. Exocytosis of the secretory vesicles is normally triggered by the binding of antigen complexed to an immunoglobulin (IgE). Under experimental conditions, massive exocytosis can be triggered by the inclusion of a nonhydrolyzable analog of guanosine triphosphate (GTP) in an intracellular recording electrode. (Reproduced, with permission, from Lawson et al. 1977. Permission conveyed through Copyright Clearance Center, Inc.)

B. Stepwise increases in capacitance reflect the successive fusion of individual secretory vesicles with the mast cell membrane. The step increases are unequal because of variability in the membrane area of the vesicles. After exocytosis, the membrane added through fusion is retrieved through endocytosis. Endocytosis of individual vesicles gives rise to the stepwise decreases in membrane capacitance. In this way, the cell maintains a constant size. (Units are femtofarads, where 1 fF = 0.1 μm^2 of membrane area.) (Adapted, with permission, from Fernandez, Neher, and Gomperts 1984.)

C. The giant presynaptic terminals of bipolar neurons in the retina are more than 5 μm in diameter, permitting direct patch-clamp recordings of membrane capacitance and Ca^{2+} current. A brief depolarizing voltage-clamp step in membrane potential (V_m) elicits a large sustained Ca^{2+} current (I_{Ca}) and a rise in the cytoplasmic Ca^{2+} concentration, $[Ca]_i$. This results in the fusion of several thousand small synaptic vesicles with the cell membrane, leading to an increase in total membrane capacitance. The increments in capacitance caused by fusion of individual vesicles are too small to resolve. As the internal Ca^{2+} concentration falls back to its resting level upon repolarization, the extra membrane area is retrieved and capacitance returns to its baseline value. The increases in capacitance and Ca^{2+} concentration outlast the brief depolarization and Ca^{2+} current (note different time scales) because of the relative slowness of endocytosis and Ca^{2+} metabolism. (Micrograph reproduced, with permission, from Zenisek et al. 2004. Copyright © 2004 Society for Neuroscience.)

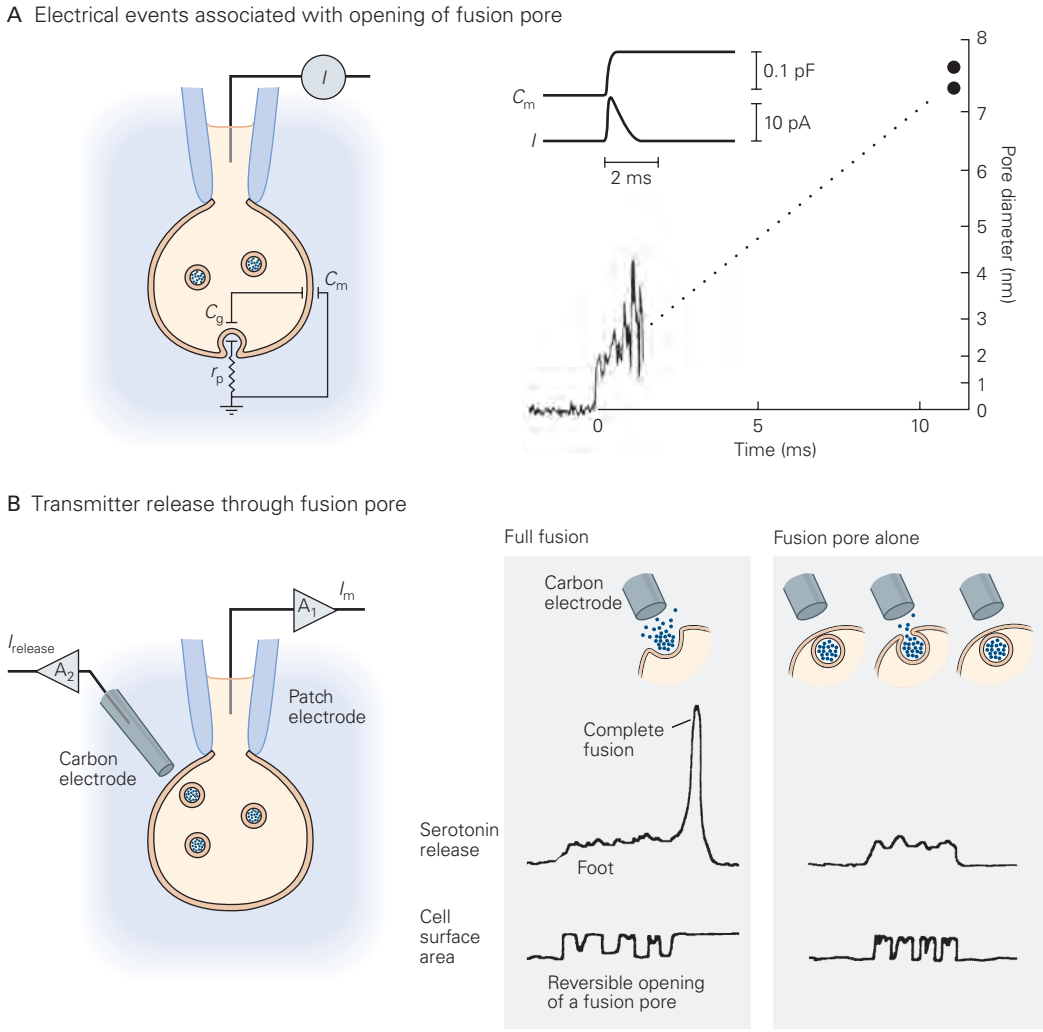


Figure 15–9 Reversible opening and closing of fusion pores.

A. A whole cell patch clamp is used to record membrane current associated with the opening of a fusion pore. As a vesicle fuses with the plasma membrane, the capacitance of the vesicle (C_g) is initially connected to the capacitance of the rest of the cell membrane (C_m) through the high resistance of the fusion pore (r_p). Because the membrane potential of the vesicle (lumenal side negative) is normally much more negative than the membrane potential of the cell, charge flows from the vesicle to the cell membrane during fusion. This transient current (I) is associated with the increase in membrane capacitance (C_m).

The magnitude of the conductance of the fusion pore (g_p) can be calculated from the time constant of the transient current according to $\tau = C_g r_p = C_g / g_p$. The pore diameter can be calculated from the pore conductance, assuming that the pore spans two lipid bilayers and is filled with a solution whose resistivity is equal to that of the cytoplasm. The plot on the right shows the pore has an initial conductance of approximately 200 pS, similar to the conductance of a gap-junction channel, corresponding to a pore diameter of approximately 2 nm. The pore diameter and conductance rapidly increase as the pore dilates to approximately 7 to 8 nm in 10 ms (**filled circles**). (Reproduced, with permission, from Monck and Fernandez 1992. Permission conveyed through Copyright Clearance Center, Inc; and adapted, with permission, from Spruce et al. 1990.)

B. Transmitter release is measured by amperometry. A cell is voltage-clamped with a whole cell patch electrode while an extracellular carbon fiber is pressed against the cell surface. A large voltage applied to the tip of the carbon electrode oxidizes certain amine transmitters (such as serotonin or norepinephrine). This oxidation of one molecule generates one or more free electrons, which results in an electrical current that is proportional to the amount of transmitter release. The current can be recorded through an amplifier (A_2) connected to the carbon electrode. Membrane current and capacitance are recorded through the patch electrode amplifier (A_1). Recordings of serotonin release (**top traces**) and capacitance measurements (**bottom traces**) from mast cell secretory vesicles are shown at the *right*. The records indicate that serotonin may be released through the reversible opening and closing of the fusion pore prior to full fusion (*traces on left*). During these brief openings, small amounts of transmitter escape through the pore, resulting in a low-level signal (a *foot*) that precedes a large spike of transmitter release upon full fusion. During the foot, the cell surface area (proportional to membrane capacitance) undergoes reversible step-like changes as the fusion pore opens and closes. Sometimes the reversible opening and closing of the fusion pore are not followed by full fusion (*traces on right*). (Adapted, with permission, from Neher 1993.)

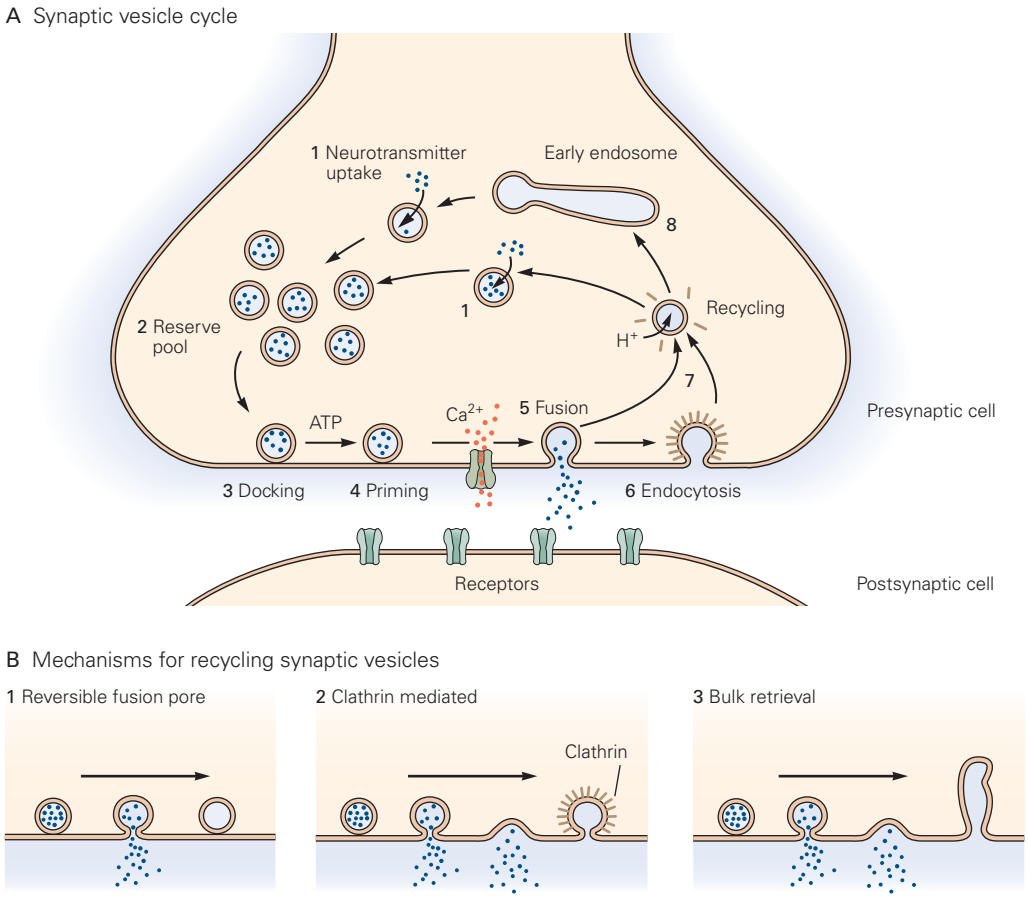


Figure 15–10 The synaptic vesicle cycle.

A. Synaptic vesicles are filled with neurotransmitters by active transport (**step 1**) and join the vesicle cluster that may represent a reserve pool (**step 2**). Filled vesicles dock at the active zone (**step 3**) where they undergo an ATP-dependent priming reaction (**step 4**) that makes them competent for Ca^{2+}-triggered fusion (**step 5**). After discharging their contents, synaptic vesicles are recycled through one of several routes (see part B). In one common route, vesicle membrane is retrieved via clathrin-mediated endocytosis (**step 6**) and recycled directly (**step 7**) or via endosomes (**step 8**).

B. Retrieval of vesicles after transmitter discharge is thought to occur via three mechanisms, each with distinct kinetics. **1.** A reversible fusion pore is the most rapid mechanism for reusing vesicles. The vesicle membrane does not completely fuse with the plasma membrane, and transmitter is released through the fusion pore. Vesicle retrieval requires only the closure of the fusion pore and thus can occur rapidly, in tens to hundreds of milliseconds. This pathway may predominate at lower to normal release rates. The spent vesicle may either remain at the membrane (kiss-and-stay) or relocate from the membrane to the reserve pool of vesicles (kiss-and-run). **2.** In the classical pathway, excess membrane is retrieved through endocytosis by means of clathrin-coated pits. These pits are found throughout the axon terminal except at the active zones. This pathway may be important at normal to high rates of release. **3.** In the bulk retrieval pathway, excess membrane reenters the terminal by budding from uncoated pits. These uncoated cisternae are formed primarily at the active zones. This pathway may be used only after high rates of release and not during the usual functioning of the synapse. (Adapted, with permission, from Schweizer, Betz, and Augustine 1995; Südhof 2004.)

to a minute for completion and also appears to shift from the active zone to the membrane surrounding the active zone (see Figure 15–7). A fourth mechanism operates after prolonged high-frequency stimulation. Under these conditions, large membranous invaginations into the presynaptic terminal are visible, which are thought to reflect membrane recycling through a process called *bulk retrieval*.

Exocytosis of Synaptic Vesicles Relies on a Highly Conserved Protein Machinery

Many key proteins of synaptic vesicles as well as their interacting partners in the plasma membrane have been isolated and purified. Proteomic analysis of isolated synaptic vesicles has provided a census of the many types of proteins they contain (Figure 15–11).

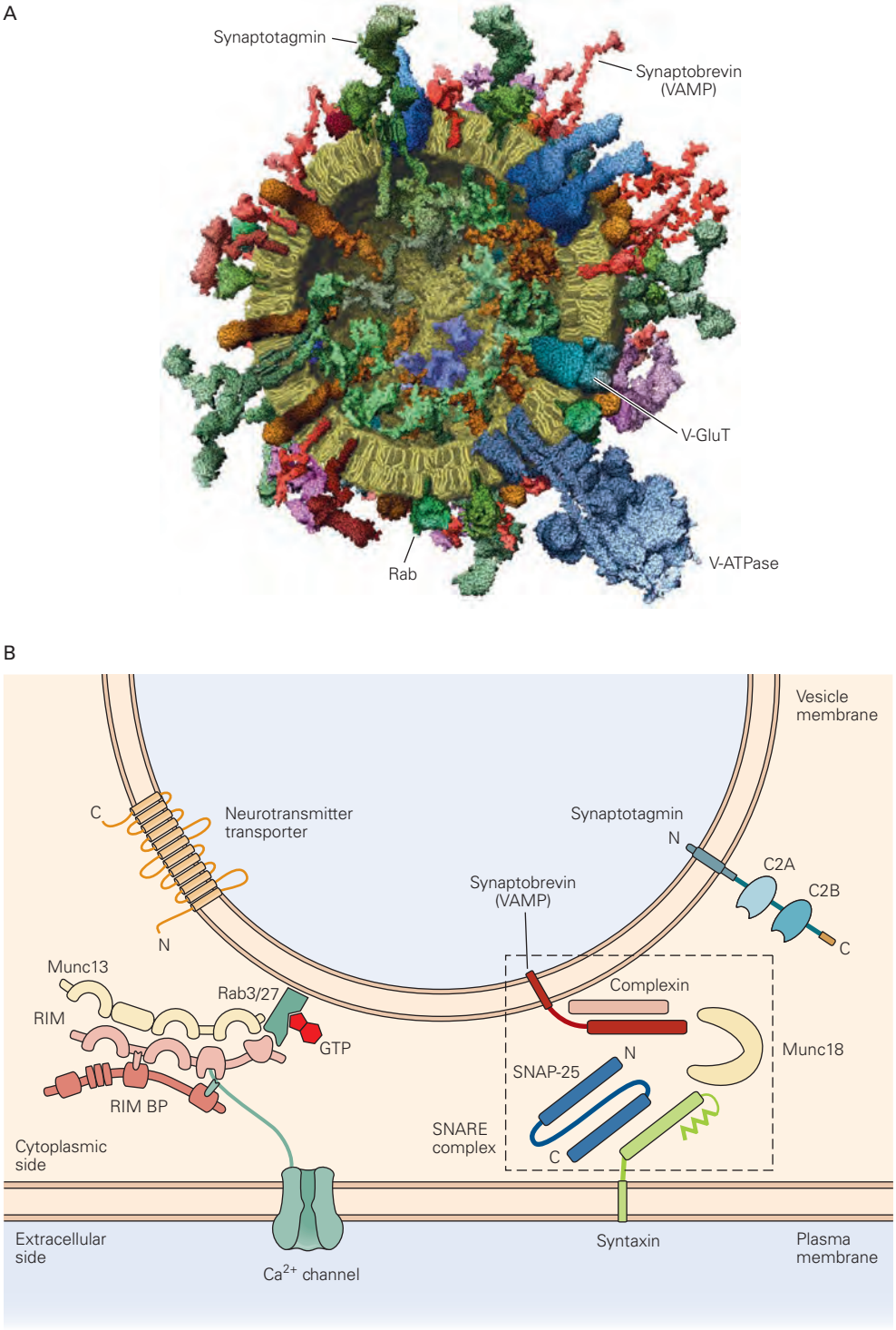


Figure 15–11 Molecular components of exocytosis.

A. Depiction of protein constituents of a glutamatergic synaptic vesicle (and their approximate copy numbers). Proteins are shown embedded in a synaptic vesicle, drawn to scale. Components include the vesicular ATPase (**V-ATPase**; 1–2 per vesicle), vesicular glutamate transporter (**V-GluT**; ~10 per vesicle), synaptobrevin/VAMP (~70 per vesicle), synaptotagmin (~15 per vesicle) and the small GTPases Rab3 and/or Rab27. Estimates are obtained as an average over many vesicles. (Reproduced from Takamori et al. 2006. Copyright © 2006 Elsevier.)

B. The molecular machinery mediating Ca^{2+}-triggered vesicle fusion with the presynaptic cell membrane. This depiction of a portion of a docked synaptic vesicle and the presynaptic active zone illustrates the interactions of several key functional proteins of the neurotransmitter release machinery. *Right:* The dotted box shows the core fusion machine, which is comprised of the SNARE proteins synaptobrevin/VAMP, syntaxin-1, and SNAP-25, along with Munc18-1. The Ca^{2+} sensor synaptotagmin-1 functions in coordination with complexin (shown bound to the SNARE complex). *Left:* The active zone protein complex also contains RIM, Munc13, and RIM-BP and a Ca^{2+} channel in the presynaptic plasma membrane. RIM plays a central role in this complex, coordinating multiple functions of the active zone by binding to specific target proteins: (1) vesicular Rab proteins (Rab3 and Rab27) to mediate vesicle docking; (2) Munc13 to activate vesicle priming; and (3) the Ca^{2+} channel, both directly and indirectly via RIM-BP, to tether Ca^{2+} channels within 100 nm of docked vesicles. The active zone protein complex puts into close proximity key elements that enable vesicles to dock, prime, and fuse rapidly in response to action potential–triggered Ca^{2+} entry near the docked vesicle. (Reproduced from Südhof 2013.)

Two of the most abundant proteins, *synaptobrevin* and *synaptotagmin-1*, are involved in vesicle fusion and are discussed later. Another key class of vesicle proteins are the neurotransmitter transporters (Chapter 16). These transmembrane proteins (exemplified by the *glutamate transporter* v-GluT) harness energy stored in the electrochemical gradient for protons to pump transmitter molecules against their concentration gradient from the cytoplasm into the vesicle. The protonmotive force is generated by a vesicular H^+ pump, the V-ATPase, that pumps protons into the lumen of the vesicle from the cytoplasm, leading to an acidic vesicular pH of around 5.0.

Other synaptic vesicle proteins direct vesicles to their release sites, participate in the discharge of transmitter by exocytosis, and mediate recycling of the vesicle membrane. The protein machinery involved in these three steps has been conserved throughout evolution, in species ranging from worms to humans, and forms the basis for the regulated release of neurotransmitter. We consider each of these steps in turn.

The Synapsins Are Important for Vesicle Restraint and Mobilization

The vesicles outside the active zone represent a reserve pool of transmitter. Paul Greengard discovered a family of proteins, *synapsins*, that are thought to be important regulators of the reserve pool of vesicles. Synapsins are peripheral membrane proteins that are bound to the cytoplasmic surface of synaptic vesicles. Synapsins contain a conserved central ATPase domain that accounts for most of their structure, but whose function remains unknown. In addition, synapsin-1 binds actin.

The synapsins are substrates for both protein kinase A and Ca^{2+}/calmodulin-dependent protein kinase II. When the nerve terminal is depolarized and Ca^{2+} enters, the synapsins become phosphorylated by the kinase and are thus released from the vesicles. Strikingly, stimulation of synapsin phosphorylation, genetic deletion of synapsins or intracellular injection of a synapsin antibody leads to a decrease in the number of synaptic vesicles in the nerve terminal and a resulting decrease in the ability of a terminal to maintain a high rate of transmitter release during repetitive stimulation.

SNARE Proteins Catalyze Fusion of Vesicles With the Plasma Membrane

Because a membrane bilayer is a stable structure, fusion of the synaptic vesicle and plasma membrane must overcome a large unfavorable activation energy. This is accomplished by a family of fusion proteins now referred to as *SNAREs* (soluble *N*-ethylmaleimide–sensitive factor attachment receptors) (Figure 15–12).

SNAREs are universally involved in membrane fusion, from yeast to humans. They mediate both constitutive membrane trafficking during the movement of proteins from the endoplasmic reticulum to the Golgi apparatus to the plasma membrane, as well as synaptic vesicle trafficking important for regulated exocytosis. SNAREs have a conserved protein sequence, the SNARE motif, that is 60 residues long. They come in two forms. Vesicle SNAREs, or v-SNAREs (also referred to as R-SNAREs because they contain an important central arginine residue), reside in the vesicle membranes. Target-membrane SNAREs, or t-SNAREs (also referred to as Q-SNAREs because they contain an important glutamine residue), are present in target membranes, such as the plasma membrane.

Each synaptic vesicle contains a v-SNARE called *synaptobrevin* (also called vesicle-associated membrane protein or VAMP). By contrast, the presynaptic active zone contains two types of t-SNARE proteins, *syntaxin* and *SNAP-25*. (Synaptobrevin and syntaxin have one SNARE motif; SNAP-25 has two.) The first clue that synaptobrevin, syntaxin, and SNAP-25 are all involved in fusion of the synaptic vesicle with the plasma membrane came from the finding that all three proteins are substrates for botulinum and tetanus toxins, bacterial proteases that are potent inhibitors of transmitter release. James Rothman then provided the crucial insight that these three proteins interact in a tight biochemical complex. In experiments using purified v-SNAREs and t-SNAREs in solution, four SNARE motifs bind tightly to each other to form an α-helical coiled-coil complex (Figure 15–12B).

How does formation of the SNARE complex drive synaptic vesicle fusion? During exocytosis, the SNARE motif of synaptobrevin on the synaptic vesicle forms a tight complex with the SNARE motifs of SNAP-25 and syntaxin on the plasma membrane (Figure 15–12B). The crystal structure of the SNARE complex suggests that this complex draws the membranes together. The ternary complex of synaptobrevin, syntaxin, and SNAP-25 is extraordinarily stable. The energy released in its assembly is thought to draw the negatively charged phospholipids of the vesicle and plasma membranes in close apposition, forcing them into a prefusion intermediate state (Figure 15–12). Such an unstable state may start the formation of the fusion pore and generate the rapid opening and closing (flickering) of the fusion pore observed in electrophysiological measurements.

However, the SNAREs do not fully account for fusion of the synaptic vesicle and plasma membranes. Reconstitution experiments with purified proteins in

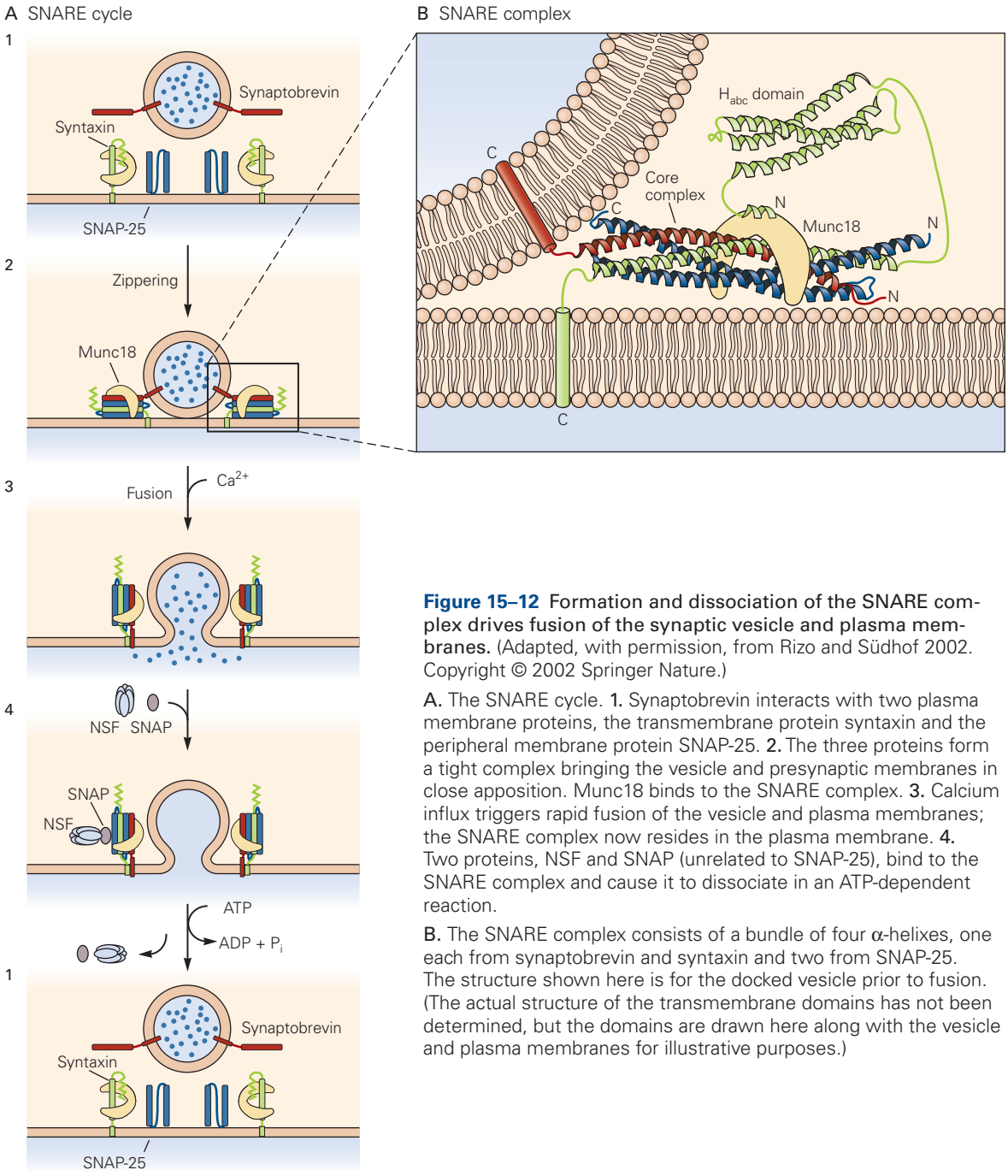


Figure 15–12 **Formation and dissociation of the SNARE complex drives fusion of the synaptic vesicle and plasma membranes.** (Adapted, with permission, from Rizo and Südhof 2002. Copyright © 2002 Springer Nature.)

A. The SNARE cycle. **1.** Synaptobrevin interacts with two plasma membrane proteins, the transmembrane protein syntaxin and the peripheral membrane protein SNAP-25. **2.** The three proteins form a tight complex bringing the vesicle and presynaptic membranes in close apposition. Munc18 binds to the SNARE complex. **3.** Calcium influx triggers rapid fusion of the vesicle and plasma membranes; the SNARE complex now resides in the plasma membrane. **4.** Two proteins, NSF and SNAP (unrelated to SNAP-25), bind to the SNARE complex and cause it to dissociate in an ATP-dependent reaction.

B. The SNARE complex consists of a bundle of four α-helixes, one each from synaptobrevin and syntaxin and two from SNAP-25. The structure shown here is for the docked vesicle prior to fusion. (The actual structure of the transmembrane domains has not been determined, but the domains are drawn here along with the vesicle and plasma membranes for illustrative purposes.)

lipid vesicles indicate that synaptobrevin, syntaxin, and SNAP-25 can catalyze fusion, but the in vitro reaction shows little regulation by Ca^{2+}, and the reaction is much slower and less efficient than vesicle fusion in a real synapse. One important additional protein required for exocytosis of synaptic vesicles is Munc18 (mammalian unc18 homolog). Homologs of Munc18, referred to as SM proteins (sec1/Munc18-like proteins), are essential for all SNARE-mediated intracellular fusion reactions. Munc18 binds to syntaxin before the SNARE complex assembles. Deletion of Munc18 prevents all synaptic fusion in neurons. The core fusion machinery is thus composed of SNARE and SM proteins that are modulated by various accessory factors specific for particular fusion reactions. Finally, the synaptic SNARE complex also interacts with a small soluble protein called *complexin*, which suppresses the spontaneous release of transmitter but enhances Ca^{2+}-dependent evoked release.

After fusion, the SNARE complex must be disassembled for efficient vesicle recycling to occur. Rothman discovered that a cytoplasmic ATPase called *NSF* (*N*-ethylmaleimide-sensitive fusion protein) binds to SNARE complexes via an adaptor protein called *SNAP* (soluble NSF-attachment protein, not related to the SNARE protein SNAP-25). NSF and SNAP use the energy of ATP hydrolysis to dissociate SNARE complexes, thereby regenerating free SNARE (Figure 15–12A). SNAREs and NSF also participate in the cycling of postsynaptic AMPA-type glutamate receptors in dendritic spines.

Calcium Binding to Synaptotagmin Triggers Transmitter Release

Because fusion of synaptic vesicles with the plasma membrane must occur within a fraction of a millisecond, it is thought that most proteins responsible for fusion are assembled prior to Ca^{2+} influx. According to this view, once Ca^{2+} enters the presynaptic terminal, it binds a Ca^{2+} sensor on the vesicle, triggering immediate fusion of the membranes.

Members of a family of closely related proteins, the synaptotagmins, have been identified as the major Ca^{2+} sensors that trigger fusion of synaptic vesicles. Synaptotagmins are membrane proteins with a single N-terminal transmembrane region that anchors them to the synaptic vesicle (Figure 15–13A,B). The cytoplasmic region of each synaptotagmin protein is largely composed of two domains, the C2 domains, which are a common protein motif homologous to the Ca^{2+} and phospholipid-binding C2 domain of protein kinase C. The finding that the C2 domains bind not only Ca^{2+} but also phospholipids is consistent with their importance in Ca^{2+}-dependent exocytosis. Synaptotagmin-1, -2, and -9 have been identified as Ca^{2+} sensors for fast and synchronous vesicle fusion. Each exhibits distinct Ca^{2+} binding affinities and kinetics, endowing different synapses with distinct release properties on the basis of the particular synaptotagmin isoform that is expressed. In contrast, synaptotagmin-7 mediates a slower form of Ca^{2+}-triggered exocytosis that is important for synaptic transmission during prolonged periods of activity periods of repeated firing of action potential. All of these synaptotagmins also function as Ca^{2+} sensors in other forms of exocytosis, such as exocytosis in endocrine cells and the insertion of AMPA-type glutamate receptors into the postsynaptic cell membrane from a pool of intracellular vesicles during NMDA-receptor-dependent long-term potentiation.

Studies with mutant mice in which synaptotagmin-1 is deleted or in which its Ca^{2+} affinity is altered through genetic engineering provide important evidence that synaptotagmin is the physiological Ca^{2+} sensor. When the affinity of synaptotagmin for Ca^{2+} is decreased two-fold, the Ca^{2+} required for transmitter release is changed by the same amount. When synaptotagmin-1 is deleted in mice, flies, or worms, an action potential is no longer able to trigger fast synchronous release. However, Ca^{2+} is still capable of stimulating a slower form of transmitter release referred to as asynchronous release (Figure 15–13A), mediated by synaptotagmin-7. Thus, nearly all Ca^{2+}-triggered neurotransmitter release depends on the synaptotagmins.

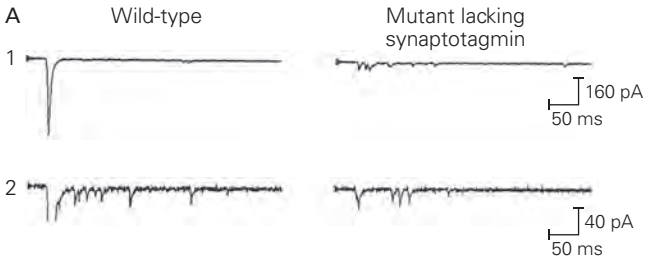
How does Ca^{2+} binding to synaptotagmin trigger vesicle fusion? The two C2 domains bind a total of five Ca^{2+} ions, the same minimal number of Ca^{2+} ions required to trigger release of a quantum of transmitter (Figure 15–13B). However, as multiple synaptotagmins may be engaged to trigger release, more than five bound Ca^{2+} ions may be distributed among the multiple synaptotagmin molecules on a single vesicle.

The binding of the Ca^{2+} ions to synaptotagmin is thought to act as a switch, promoting the interaction of the C2 domains with phospholipids. The C2 domains of synaptotagmin also interact with SNARE proteins and complexin. Crystal structures of synaptotagmin reveal a conserved primary interface with the associated SNARE complex. In addition, a second molecule of synaptotagmin forms a tripartite interaction with the same SNARE complex and complexin. Brunger and colleagues found that both the primary SNARE complex/synaptotagmin interface and the tripartite SNARE complex/synaptotagmin/complexin interface are essential for fast Ca^{2+}-triggered fusion. These findings have led to the hypothesis that: (1) at rest, synaptotagmin of primed vesicles exists in a complex with partially pre-zippered SNARE proteins and complexin; (2) upon action potential–triggered Ca^{2+} influx, Ca^{2+} binds to synaptotagmin. This triggers an interaction between synaptotagmin and the plasma membrane that causes the complex to rotate en bloc, which induces complexin to partly dissociate from the SNARE complex; (3) this rotation causes a dimpling of the plasma membrane, rearrangement of its cytoplasm-facing lipids, and ultimately the fusion of plasma and vesicle membranes (Figure 15–13C). In this way, the energy of the favorable interaction of synaptotagmin, Ca^{2+}, and the membrane can be harnessed to both relieve the complexin-mediated lock on fusion and promote the energetically unfavorable merging of a vesicle membrane with the plasma membrane.

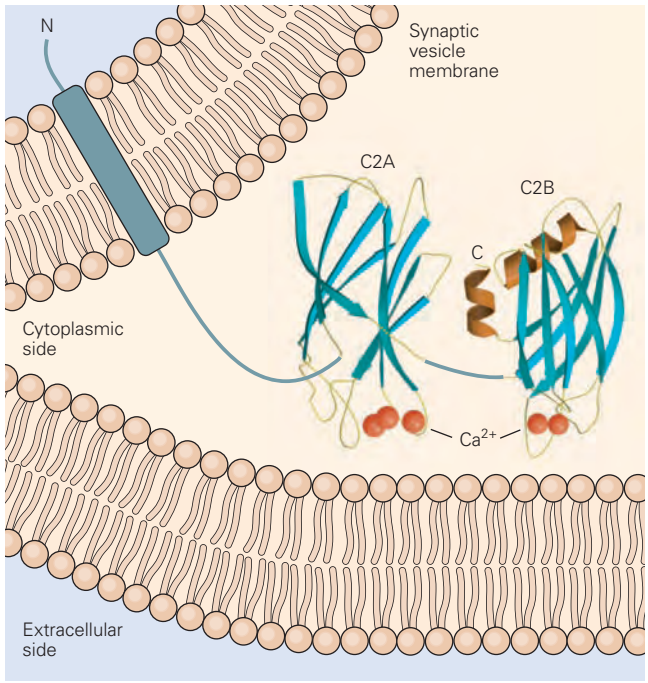
The Fusion Machinery Is Embedded in a Conserved Protein Scaffold at the Active Zone

As we have seen, a defining feature of fast synaptic transmission is that neurotransmitters are released by

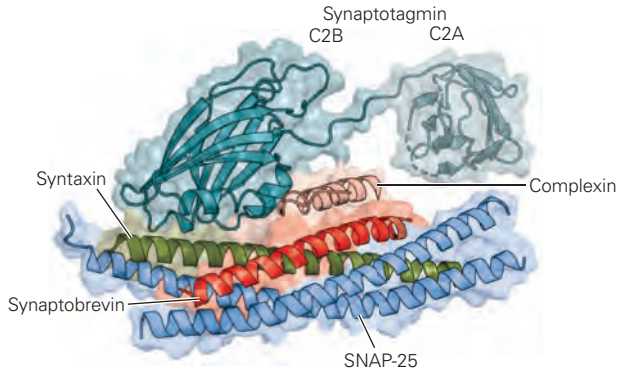
A



B1 Calcium-bound synaptotagmin

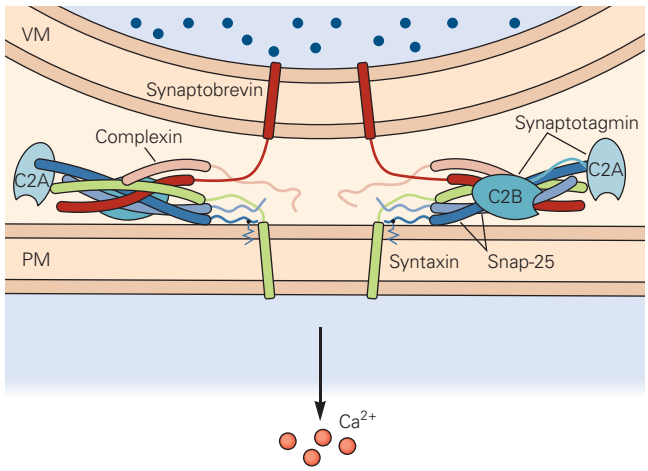


B2 Synaptotagmin/SNARE complex

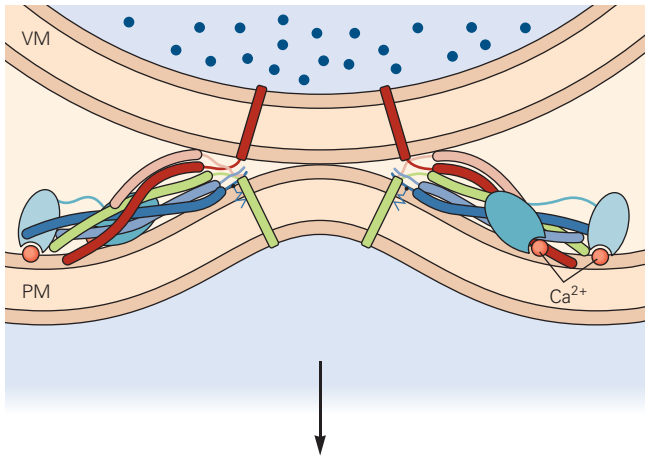


C

Locked



Unlocking and triggering



Fusion pore formation