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THE SYNAPTIC VESICLE CYCLE

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■ **Abstract** Neurotransmitter release is mediated by exocytosis of synaptic vesicles at the presynaptic active zone of nerve terminals. To support rapid and repeated rounds of release, synaptic vesicles undergo a trafficking cycle. The focal point of the vesicle cycle is Ca^{2+}-triggered exocytosis that is followed by different routes of endocytosis and recycling. Recycling then leads to the docking and priming of the vesicles for another round of exo- and endocytosis. Recent studies have led to a better definition than previously available of how Ca^{2+} triggers exocytosis and how vesicles recycle. In particular, insight into how Munc18-1 collaborates with SNARE proteins in fusion, how the vesicular Ca^{2+} sensor synaptotagmin 1 triggers fast release, and how the vesicular Rab3 protein regulates release by binding to the active zone proteins RIM1α and RIM2α has advanced our understanding of neurotransmitter release. The present review attempts to relate these molecular data with physiological results in an emerging view of nerve terminals as macromolecular machines.

NEUROTRANSMITTER RELEASE AND THE SYNAPTIC VESICLE CYCLE

Synaptic transmission is initiated when an action potential triggers neurotransmitter release from a presynaptic nerve terminal (Katz 1969). An action potential induces the opening of Ca^{2+} channels, and the resulting Ca^{2+} transient stimulates synaptic vesicle exocytosis (Figure 1). After exocytosis, synaptic vesicles undergo endocytosis, recycle, and refill with neurotransmitters for a new round of exocytosis. Nerve terminals are secretory machines dedicated to repeated rounds of release. Most neurons form >500 presynaptic nerve terminals that are often widely separated from the neuronal cell bodies. Action potentials, initiated in the neuronal cell body, travel to all of the cell body's nerve terminals to be transformed into synaptic secretory signals. Nerve terminals do not convert reliably every action potential into a secretory signal but are "reliably unreliable" (Goda & Südhof 1997). In most terminals, only 10%–20% of action potentials trigger release. The relationship between action potentials and release in a nerve

terminal is regulated by intracellular messengers and extracellular modulators and is dramatically altered by repeated use of a synapse. Thus in addition to secretory machines, nerve terminals are computational units where the relation of input (action potential) to output (neurotransmitter release) continuously changes in response to extra- and intracellular signals.

All presynaptic functions, directly or indirectly, involve synaptic vesicles. Synaptic vesicles undergo a trafficking cycle in the nerve terminal (Figure 1) that can be divided into sequential steps: First, neurotransmitters are actively transported into synaptic vesicles (step 1), and synaptic vesicles cluster in front of the active zone (step 2). Then synaptic vesicles dock at the active zone (step 3), where the vesicles are primed (step 4) to convert them into a state of competence for Ca^{2+}-triggered fusion-pore opening (step 5). After fusion-pore opening, synaptic vesicles endocytose and recycle probably by three alternative pathways: (*a*) Vesicles are reacidified and refilled with neurotransmitters without undocking, thus remaining in the readily releasable pool (step 6, called "kiss-and-stay"); (*b*) vesicles undock and recycle locally (step 7, called "kiss-and-run") to reacidify and refill with neurotransmitters (back to steps 1 and 2); or (*c*) vesicles endocytose via clathrin-coated pits (step 8) and reacidify and refill with neurotransmitters either directly or after passing through an endosomal intermediate (step 9). In the two-dimensional representation depicted here (Figure 1), each step in the vesicle cycle is illustrated by a shift in the position of the vesicle. In reality, however, most successive steps occur without much vesicle movement except for docking (step 3) and recycling (steps 7–9). Investigators sometimes propose that different types of release reactions exist that differ in fusion-pore dynamics (e.g., "kiss-and-run" is used as a description of exocytosis instead of recycling). However, synaptic vesicles are so small (radius 17–22 nm) that even an unstable fusion pore is likely to empty the vesicle rapidly, as reflected in the fast rise times of spontaneous release events (<100 μs). The dynamics of fusion pores are thus unlikely to influence release but are probably of vital importance for endocytosis: Fast recycling may preferentially utilize transient fusion pores, whereas slow recycling likely involves a full collapse of the vesicle into the plasma membrane.

A major goal in neurobiology in recent years has been to gain insight into the molecular machinery that mediates neurotransmitter release. More than 1000 proteins function in the presynaptic nerve terminal, and hundreds are thought to participate in exocytosis. In this protein zoo, which proteins are actually important, and which are only bystanders? How do proteins collaborate in shaping the vesicle cycle, and how can we understand the functions of so many proteins? To approach this fundamental problem, I posit that all of the presynaptic functions ultimately converge on the vesicle cycle and that all steps in the vesicle cycle collaborate, directly or indirectly, to make possible rapid, regulated, and repeated rounds of release (Südhof 1995). Because release is mediated by the interaction of synaptic vesicles with the active zone during exocytosis, this interaction is the common final pathway of all nerve terminal functions. In the discussion below, I therefore pursue a "vesicocentric" perspective that focuses on synaptic vesicles as the central

organelle of neurotransmitter release. This perspective is guided by the promise that synaptic vesicles as small, relatively simple organelles are in principle amenable to a complete molecular analysis.

Instead of providing a comprehensive overview of the entire literature (which would be impossible within the constraints of this review), I concentrate on a limited number of proteins for which key functions have been proposed. For many topics, other recent reviews provide a good summary [e.g., see Jahn et al. (2003) for a review of membrane fusion, Slepnev & De Camilli (2000) or Galli & Haucke (2001) for reviews on endocytosis, and Zucker & Regehr (2002) or von Gersdorff & Borst (2002) for reviews on short-term synaptic plasticity]. Furthermore, I primarily focus on work done in mammals because space constraints do not allow a discussion of the extensive and outstanding work in flies and worms (reviewed in Richmond & Broadie 2002). Before discussing the molecular machines that are anchored on synaptic vesicles and drive the vesicle cycle, one must review the salient properties of the two principal limbs of the cycle—exocytosis versus endocytosis and recycling—and the methodology used to elucidate how a given molecule could participate in the cycle.

Ca^{2+} TRIGGERING OF NEUROTRANSMITTER RELEASE

In preparation for neurotransmitter release, synaptic vesicles dock at the active zone and are primed to become Ca^{2+} responsive (steps 3 and 4; Figure 1). When an action potential invades a nerve terminal, voltage-gated Ca^{2+} channels open, and the resulting pulse of intracellular Ca^{2+} triggers fusion-pore opening of release-ready vesicles (step 5). In most synapses, release is stimulated by Ca^{2+} influx through P/Q- ($Ca_V2.1$) or N-type Ca^{2+} channels ($Ca_V2.2$), whereas the related R- ($Ca_V2.3$) or the more distant l-type Ca^{2+} channels (Ca_V1 series) are involved only rarely (e.g., see Dietrich et al. 2003). Even at rest, synapses have a finite but low probability of release, causing spontaneous events of exocytosis that are reflected in electrophysiological recordings as miniature postsynaptic currents (Katz 1969). Ca^{2+} influx triggers at least two components of release that are probably mechanistically distinct: A fast, synchronous phasic component is induced rapidly, in as little as 50 μs after a Ca^{2+} transient develops (Sabatini & Regehr 1996), and a slower asynchronous component continues for >1 s as an increase in the rate of spontaneous release after the action potential (Barrett & Stevens 1972, Geppert et al. 1994a, Goda & Stevens 1994, Atluri & Regehr 1998). Both components of release are strictly Ca^{2+} dependent but change differentially upon repetitive stimulation (Hagler & Goda 2001).

The best physiological description of how an action potential induces phasic, synchronous neurotransmitter release was obtained for the synapse formed by the calyx of Held, the only synapse for which models are available that accurately account for all properties of release (reviewed in Meinrenken et al. 2003). The calyx of Held forms a large nerve terminal ($\sim$15 μm diameter) that envelops the soma of the postsynaptic neuron like a cup, hence its name. The calyx terminal makes

~500–600 synaptic contacts with the postsynaptic cell (in young rats at ~P10, where most studies were performed). The synaptic contacts are distributed over the entire inner surface of the calyx and account for 2% of the plasma membrane area (Sätzler et al. 2002). Each synaptic contact acts like an independent synapse. A single action potential triggers release at ~200 synaptic contacts, which suggests that approximately one third of the synapses fire per Ca^{2+} signal (Bollmann et al. 2000). As a result, the calyx is effectively coupled to the postsynaptic cell by hundreds of parallel synapses that guarantee a reliable postsynaptic response to a presynaptic action potential. This is important physiologically because the calyx synapse is part of the auditory pathway where reliable fast transmission is essential for sound localization.

Serial electron micrographs revealed that, similar to other synapses, active zones in the calyx of Held synapse measure ~0.05–0.10 μm^2 (Sätzler et al. 2002, Taschenberger et al. 2002). Each active zone is associated with a cluster of vesicles (125 ± 82 per active zone). On average, only 2–3 vesicles are docked, and 5 vesicles are within 20 nm of an active zone (Sätzler et al. 2002, Taschenberger et al. 2002). Compared to cerebellar, cortical, or hippocampal synapses where vesicle clusters usually include >200 vesicles, and where more than 8–10 docked vesicles per active zone are generally reported (Harris et al. 1992, Schikorski & Stevens 2001, Xu-Friedman et al. 2001), the calyx thus contains fewer docked and free vesicles.

The size of the calyx allows direct electrophysiological recordings from a terminal, making it possible to monitor pre- and postsynaptic events simultaneously (Figure 2). Major advances in our understanding of neurotransmitter release were made in the calyx of Held by relating the precise concentration and dynamics of Ca^{2+} to synaptic vesicle exocytosis (reviewed in Schneggenburger et al. 2002, Meinrenken et al. 2003). When an action potential invades the calyx terminal, the Ca^{2+} current begins at the peak of the action potential and ends before the calyx terminal is fully repolarized (Figure 2; Helmchen et al. 1997). During the repolarization phase of the action potential, the Ca^{2+} current is much smaller than the K^+ current, which suggests that the Ca^{2+} current does not contribute markedly to the waveform of the action potential. The size of the Ca^{2+} current indicates that if all Ca^{2+} channels were located in active zones (probably an overestimate), each action potential would open ~20 Ca^{2+} channels per active zone (Sätzler et al. 2002), a value similar to estimates for cortical synapses (Koester & Sakmann 2000). Ca^{2+} influx results in a Ca^{2+} transient lasting ~400–500 μsec whose time course closely follows that of the Ca^{2+} current because Ca^{2+} is buffered effectively and rapidly (Meinrenken et al. 2002). Ca^{2+} thus invades the nerve terminal as a brief Ca^{2+} pulse that triggers fast release with a short delay (50–500 μsec depending on the synapse and temperature). The immediate dissipation of the Ca^{2+} transient rapidly terminates release, while the bolus of neurotransmitters released into the synaptic cleft activates postsynaptic receptors to elicit a postsynaptic reponse (Figure 2).

To determine how much Ca^{2+} is necessary to trigger neurotransmitter release, photolysis of caged Ca^{2+} was used (Bollmann et al. 2000; Schneggenburger &

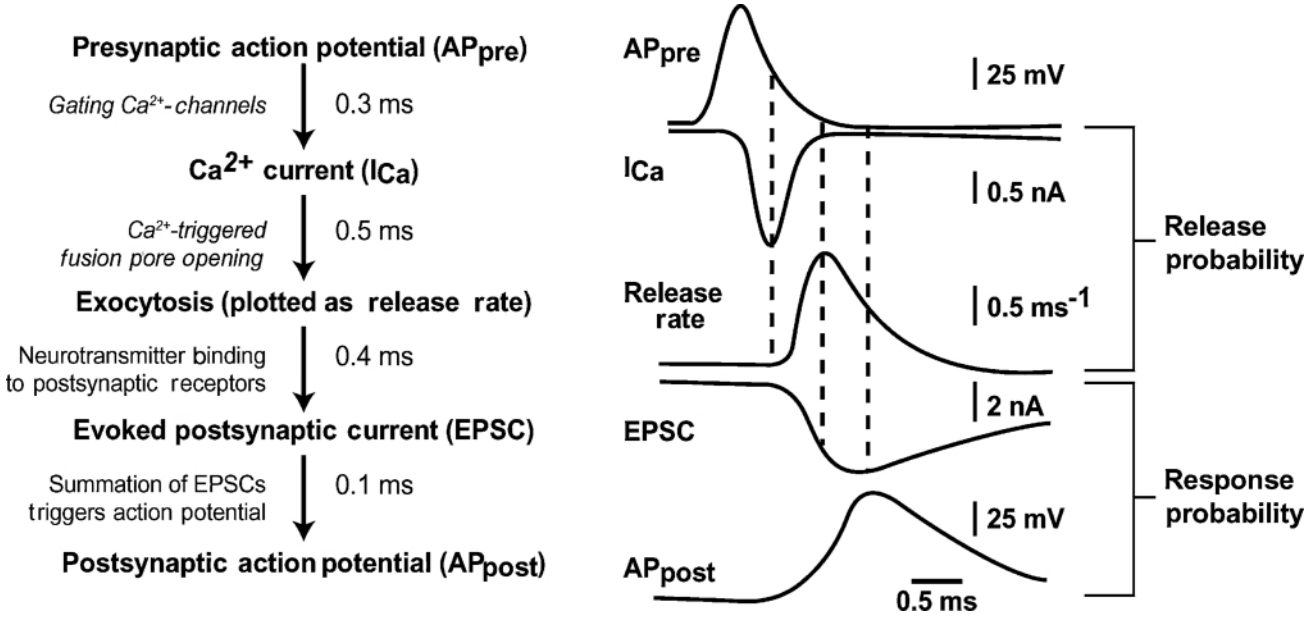


Figure 2 Reaction sequence and timing of synaptic transmission. The principal reactions with the associated time constants are shown on the left, and traces from the corresponding reactions in the calyx of Held synapses are illustrated on the right (modified from Meinrenken et al. 2003). The time calibration bar at the bottom applies to all traces.

Neher 2000). Ca^{2+} uncaging creates a uniform cytosolic Ca^{2+} signal that can be directly measured, which thereby circumvents the problem that local Ca^{2+} concentrations usually cannot be determined and that vesicles may see different Ca^{2+} concentrations during an action potential because they are separated from the mouth of the Ca^{2+} channels by a variable distance. Upon photolysis of caged Ca^{2+}, Ca^{2+} triggers release at the calyx with a high degree of cooperativity, similar to other synapses. Release was undetectable at Ca^{2+} concentrations of <1 μM, became measurable at 1–2 μM Ca^{2+}, resembled the release observed during a normal action potential at >5 μM Ca^{2+}, and saturated at >20 μM Ca^{2+} (Bollmann et al. 2000). Thus fast release, at least at this synapse, is triggered by Ca^{2+} binding to a highly cooperative Ca^{2+} sensor with relatively high apparent Ca^{2+} affinity (K_d 5–25 μM).

The precise Ca^{2+} dependence of release was fitted by different models that led to qualitatively similar but numerically distinct conclusions. In both models, the Ca^{2+} sensor contains 5 Ca^{2+}-binding sites (Bollmann et al. 2000, Schneggenburger & Neher 2000). No intrinsic heterogeneity in the Ca^{2+} responsiveness of readily releasable vesicle was observed when release was stimulated by Ca^{2+} uncaging, as opposed to action potential–induced release (Sakaba & Neher 2001). The model by Bollmann et al. (2000) predicts that an action potential induces a roughly Gaussian Ca^{2+} transient with a median duration of 400 μs and that an average readily releasable vesicle sees 9 μM Ca^{2+}. In this model, an action potential results in a 25% release probability per readily releasable vesicle during an action potential with a pool size of 800 vesicles. Schneggenburger & Neher's model (2000) predicts a broader Ca^{2+} transient (median duration of 500 μs) with a peak amplitude of 28 μM Ca^{2+} that induces a release probability of 10% per readily releasable vesicle (pool size $=$ 2000 vesicles). More recent studies in which release induced by uncaged Ca^{2+} was monitored by capacitance recordings also suggested that half-maximal release (measured 50 ms after Ca^{2+} uncaging) requires 5 μM free Ca^{2+}, with saturation at 10 μM Ca^{2+} (Wölfel & Schneggenburger 2003). Although this release probably includes not just the readily releasable pool, it gives a good estimate of the aggregate apparent affinity of all Ca^{2+} sensors. Under these conditions, 3–5 vesicles were estimated to fuse at maximal Ca^{2+} in <1 ms, which corresponds to the number of vesicles docked at the active zone by electron microscopy (Sätzler et al. 2002). As a result of the high cooperativity of the Ca^{2+} sensor, the relationship of the Ca^{2+} concentration to release is supralinear. This supralinearity restricts release to the brief time period during which the Ca^{2+} transient is above threshold and terminates release rapidly even though residual Ca^{2+} remains (Meinrenken et al. 2003). These studies quantitatively described the Ca^{2+}-binding properties of a Ca^{2+} sensor only for fast release but provided little information about the Ca^{2+} sensor for asynchronous release or the adaptations of the release machinery during repetitive stimulation.

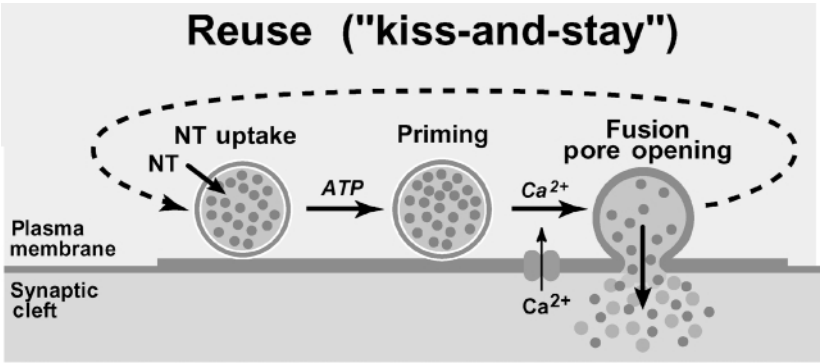
One of the key questions about how an action potential induces fast release is how Ca^{2+} channels are organized with respect to the vesicles. At the calyx, multiple Ca^{2+} channels contribute to the Ca^{2+} transient that triggers exocytosis of a readily releasable vesicle (Borst & Sakmann 1996). The relatively slow Ca^{2+}

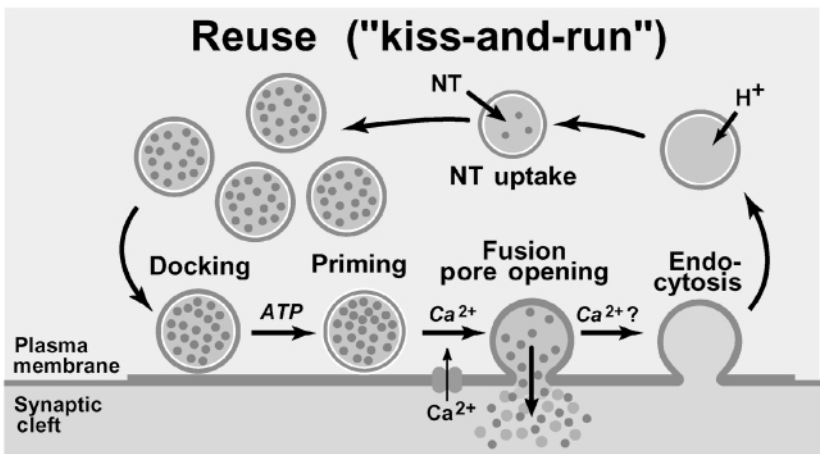
buffer EGTA, at 10 mM concentration, inhibits release 50% (Borst et al. 1995), which suggests that the majority of the vesicles are not linked to Ca^{2+} channels. Modeling of release observed in the presence of various Ca^{2+} buffers (Meinrenken et al. 2002) indicated that vesicles may be randomly distributed in an active zone but Ca^{2+} channels clustered. According to this model, the distance of a vesicle to the Ca^{2+} channel cluster ranges from 30 to 300 nm (average $\sim$100 nm), and vesicles in different locations are exposed to different Ca^{2+} transients and exhibit different release probabilities (from $<$0.01 to 1). Consistent with this hypothesis, vesicles exhibit a heterogeneous release probability during an action potential (Sakaba & Neher 2001). The clustering of Ca^{2+} channels accounts for the reliability of the Ca^{2+} signal: Even though Ca^{2+} channels open stochastically during an action potential, individual action potentials seem to create a similar Ca^{2+} signal. The model proposed by Meinrenken et al. (2002) predicts that the only step in the signaling cascade whose speed depends on the Ca^{2+} concentration is Ca^{2+} binding to the Ca^{2+} sensor, which explains why the synaptic delay (i.e., the time between the action potential and release) is relatively independent of the Ca^{2+} concentration, whereas the magnitude of release is supralinearly dependent on the Ca^{2+} concentration. However, an alternative model for the Ca^{2+} dynamics during an action potential that postulates additional intrinsic heterogeneity of vesicles also explains the characteristics of release (Trommershäuser et al. 2003).

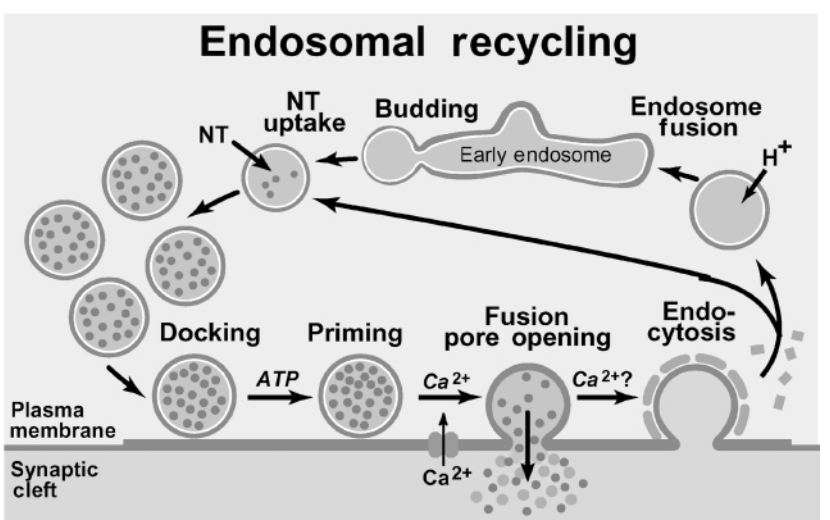
The speed with which Ca^{2+} triggers release ($<$400 μsec) suggests that Ca^{2+} binding to the Ca^{2+} sensor only induces fusion-pore opening and does not initiate a complex reaction cascade. Fusion pores form an aqueous connection across fusing bilayers and are likely to be at least partly lipidic (reviewed in Jahn et al. 2003). Because of the small size of synaptic vesicles, fusion pores cannot be readily measured but have been studied extensively in cells with large secretory vesicles (e.g., see Breckenridge & Almers 1987, Zimmerberg et al. 1987). Not surprisingly, the probability of fusion-pore opening depends on the tension and composition of the participating membranes. Thus any change, even indirect, that alters the tension of the plasma membrane influences neurotransmitter release. This is most prominently observed when neurotransmitter release is triggered by hyperosmotic solutions or by simply stretching the membrane (see Katz 1969). Changes in fusion-pore dynamics are also observed upon overexpression of a large number of proteins in nonneuronal cells [e.g., transfection of CSP (Graham & Burgoyne 2000), Munc18 (Fisher et al. 2001), Complexins (Archer et al. 2002), and synaptotagmin 1 (Wang et al. 2001a)], possibly because the plasma membrane tension is altered in the transfected cells.

SYNAPTIC VESICLE ENDOCYTOSIS AND RECYCLING

More than 30 years ago, biochemical experiments with synaptosomes (Barker et al. 1972) suggested that after exocytosis, vesicles undergo endocytosis and refill rapidly, and that a subpopulation of vesicles associated with the active zone recycles locally (the pathway referred to as kiss-and-stay; Figure 3). In parallel studies, Ceccarelli et al. (1973) demonstrated at the neuromuscular junction that







vesicles endocytose and recycle rapidly without a clathrin-coated intermediate (referred to as kiss-and-run because the vesicles did not remain attached to the active zone), whereas Heuser & Reese (1973) described that extensive stimulation of the neuromuscular junctions causes vesicles to endocytose via parasynaptic cisternae and coated pits (endosomal recycling; Figure 3). On the basis of these three observations and subsequent studies (e.g., see Koenig & Ikeda 1996, Pyle et al. 2000, Richards et al. 2000, Wucherpfennig et al. 2003), three vesicle recycling pathways are proposed here (Figure 3): two fast pathways in which the vesicles either remain at the active zone for refilling (kiss-and-stay) or are recycled locally without clathrin-mediated endocytosis (kiss-and-run), and a slower pathway that involves clathrin-mediated endocytosis. The fast pathway is used preferentially to recycle vesicles rapidly into the readily releasable pool at low stimulation frequencies, whereas the slow clathrin-dependent pathway kicks in at higher stimulation frequencies (Koenig & Ikeda 1996, Pyle et al. 2000, Richards et al. 2000).

How fast synaptic vesicle endocytosis happens after exocytosis is a critical question. Recently, this question was addressed directly in the calyx of Held using capacitance measurements (Sun et al. 2002). After single-vesicle exocytosis during spontaneous release, endocytosis was extremely fast (56 ms time constant). When ~200 vesicles were stimulated by a single action potential, or thousands of vesicles by multiple action potentials at low frequency (<2 Hz), the time constant of endocytosis was only two-fold slower (~115 ms), which is not a significant increase considering that many more vesicles were endocytosed after action potential–induced release than were after spontaneous release events. However, when the stimulation frequency was increased, endocytosis slowed down dramatically. After 10 stimuli at 20 or 333 Hz, the time constants of endocytosis increased to 2.3 and 8.3 s, respectively (Sun et al. 2002). The decrease in endocytosis rate did not depend on the cytosolic Ca^{2+} concentration or number of stimuli but on the net increase in membrane area produced by the sum of exo- and endocytosis (Sun et al. 2002). Thus the number of unretrieved vesicles at a given time determined the speed of endocytosis, which suggests that local membrane tension may drive not only exocytosis (see discussion above) but also endocytosis. The speed of endocytosis measured by Sun et al. (2002) implies that vesicle recycling is very rapid, even for the slow component of endocytosis. Nevertheless, this speed does not mean that endocytosis cannot be carried out by a clathrin-independent mechanism because the molecular machinery for clathrin-mediated endocytosis in nerve terminals is highly developed, and could potentially achieve this speed (Brodin

Figure 3 Synaptic vesicle recycling pathways. Three pathways are proposed: a pathway in which vesicles endocytose by closure of the fusion pore and are refilled with neurotransmitters while remaining docked to the active zone (kiss-and-stay); a local recycling pathway that is clathrin independent but results in mixing vesicles with the reserve pool after endocytosis (kiss-and-run); and a pathway whereby vesicles undergo clathrin-mediated endocytosis and recycle either directly or via endosomes.

et al. 2000, Slepnev & De Camilli 2000). Gandhi & Stevens (2003) confirmed in principle the presence of activity-dependent modes of endocytosis using optical recordings, although the time constants measured were much slower, possibly because cultured neurons were investigated and because the signal-to-noise ratio in optical recordings makes resolution of fast responses more difficult.

Whereas endocytosis can be directly measured by capacitance recordings, recycling can be assessed only indirectly by the uptake of tracers such as fluorescent dyes. Clearly, most vesicles recycle directly without passing through an endosomal intermediate. In fact, endosomes are almost never observed by electron microscopy in normal nerve terminals, raising doubts about the endosomal recycling pathway. However, multiple lines of evidence unequivocally establish that the endosomal pathway is physiologically relevant for synaptic vesicle recycling under some conditions (Figure 3).

1. Purified synaptic vesicles contain as a stoichiometric component (i.e., a component present on all vesicles and not only a small subset) the SNARE protein Vti1aβ (Antonin et al. 2000). Vti1aβ is a neuronal splice variant of the ubiquitous SNARE protein Vti1aβ, which, like other SNARE proteins, functions in membrane fusion (reviewed in Jahn et al. 2003; see discussion below). Vti1a participates in fusion reactions involving endosomes and the trans-Golgi network but not the plasma membrane. Its presence on all synaptic vesicles implies that all vesicles at one point fuse with intracellular membranes and not only the plasma membrane.
2. Rab5 is also an obligatory synaptic vesicle component (Fischer von Mollard et al. 1994). Rab5 is a ubiquitous protein involved in endosomal fusion and recruits effector proteins such as phosphatidylinositol-3-kinases and rabenosyn to transport vesicles destined to fuse into endosomes (Nielsen et al. 2000). The high concentration of Rab5 on synaptic vesicles suggests a function in endosome fusion during the vesicle cycle. This suggestion is supported by the finding that Rab5 mutations interfere with efficient release during repetitive stimulation (Wucherpfennig et al. 2003).
3. The absence of endosomes from most nerve terminals at steady state is not surprising considering the transient nature of endosomal organelles. For example, in *Drosophila* neuromuscular junctions, endosomes were revealed as an obligatory component of the vesicle pathway in all synapses using endosome-specific green fluorescent protein (GFP)-labeled markers, even though regular electron microscopy did not detect them easily (Wucherpfennig et al. 2003).
4. Pharmacologic inhibition of endosome fusion in frog neuromuscular junctions using phosphatidylinositol 3-kinase blockers potently impaired neurotransmitter release and depleted synaptic vesicles (Rizzoli & Betz 2002).
5. The nerve terminal is endowed richly with proteins that specifically function in clathrin-mediated endocytosis (reviewed by Brodin et al. 2000, Slepnev

& De Camilli 2000). Particularly striking is the nerve terminal abundance of proteins that are dedicated to accelerating this clathrin-mediated endocytosis, consistent with the relatively fast time constants observed for "slow" endocytosis in capacitance measurements (Sun et al. 2002).

Why clathrin-dependent endosomal recycling is important, and why it is activated selectively upon high-frequency stimulation, are obvious questions. Although no conclusive answers are available, at least two hypotheses are consistent with the data. One hypothesis is that the capacity for fast local recycling is too low to maintain continuous steady-state release at high frequency. According to this hypothesis, the endosomal recycling pathway, although slower, makes up for its lack of speed because it has a much larger capacity. A second hypothesis is that endosomes serve as a sorting station to separate "defective" and "healthy" vesicles. According to this hypothesis, such sorting is particularly important when a synapse is stressed. These two hypotheses are not mutually exclusive and may only partially explain the phenomena.

POOLS AND PLASTICITY

Synaptic Vesicle Pools

When a nerve terminal is stimulated repeatedly at a high rate, release drops dramatically and eventually reaches a lower steady-state level. The use-dependent initial synaptic depression during high-frequency stimulation reflects the depletion of vesicles in the readily releasable pool. The steady-state level of release corresponds to the rate with which vesicles are replenished into the readily releasable pool by recycling or by recruitment from a reserve pool. The concept of equating release rates with vesicle pools has been useful, and different pools of synaptic vesicles were defined on the basis of the rates of release under various stimulation conditions. The size of the readily releasable pool that can be exocytosed by high-frequency stimulation generally agrees well with the amount of release obtained upon application of hypertonic sucrose as a mechanical stimulus (Rosenmund & Stevens 1996) or with the number of vesicles that can be measured as "docked" by electron microscopy (Schikorski & Stevens 2001, Sätzler et al. 2002). The total number of vesicles that participate in exo- and endocytosis during prolonged stimulation are referred to as the recycling pool. This pool is composed of the readily releasable pool and the reserve pool, which serves to replenish the readily releasable pool upon its depletion. In cultured hippocampal neurons, extensive stimulation in the presence of the fluorescent dye FM1-43 (which is taken up with endocytosed vesicles) allows labeling of the total recycling pool of vesicles. This pool has been estimated at only 21–25 vesicles per synapse, with ~4–8 vesicles in the readily releasable and ~17–20 vesicles in the reserve pool (Murthy & Stevens 1999). Thus in this synapse, the size of the recycling pool is surprisingly small compared to the total number of vesicles in the terminal (>200

vesicles), which suggests that a third, very large "resting pool" of vesicles exists (reviewed in Südhof 2000).

However, in other synapses the number and size of vesicle pools are different. For example, at the neuromuscular junction, 20% of all vesicles are in the readily releasable pool and 80% in the reserve pool, without any resting vesicles (Richards et al. 2003). In contrast, at the calyx, two different types of readily releasable pools—immediately and reluctantly releasable vesicles—were identified (Sakaba & Neher 2001), in addition to a large reserve pool (J. Sun, personal communication). Although the concept of vesicle pools thus far has been useful to describe the amount of release observed under different conditions, the differences between synapses in the size and nature of these pools suggest that their definition is operational and may not always reflect separate types of vesicles. This potential problem is shown clearly for the calyx of Held where estimates of the readily releasable pool, determined by summing up the synaptic responses during trains of action potentials and corrected for postsynaptic receptor desensitization, led to pool sizes of 900 vesicles (Bollmann et al. 2000). In contrast, continuous stimulation of the terminals, either by extended depolarizations or by photolysis of caged Ca^{2+}, reveals a readily releasable pool of 3000–5000 vesicles (Schneggenburger & Neher 2000, Sun & Wu 2001, Wölfel & Schneggenburger 2003).

The difference between the two estimates may be partly due to the fact that the second stimulation method also includes asynchronous release and that the capacitance measurements used for some of these studies may be more accurate. However, the difference is too large to be explained by technical factors and suggests an intrinsic problem of trying to define pools on the basis of the amount of release obtained under defined stimulation conditions. The concept of pools depends on the idea that defined numbers of vesicles are in different states of releasability and that, once released, the respective vesicle pools are refilled at a specific rate. If vesicles were present in distinct states that are in an equilibrium that can be quickly shifted in both directions, the pool concept would be difficult to apply. For example, equating the synchronous and asynchronous components of release with distinct vesicle pools would imply that they are physically different populations of vesicles. Equally possible, however, is that all vesicles are in principle components of both pools but are stimulated for release by distinct Ca^{2+} sensors: a low-affinity sensor for fast release, and a high-affinity sensor for slow release. Then only the vicinity of the vesicles to Ca^{2+} channels and the ambient Ca^{2+} concentration would determine its pool identity, and each vesicle would have a finite probability for both pools. Similar arguments apply for other pools of vesicles.

Presynaptic Plasticity

Release is dramatically modulated by signals impinging on presynaptic terminals and changes induced by repetitive stimulation. Two principal points of regulating release exist: (*a*) the peak Ca^{2+} concentration produced by an action potential, i.e., the conversion of an action potential to a Ca^{2+} current (Figure 2); and (*b*) the release probability per given Ca^{2+} concentration, i.e., the conversion of a Ca^{2+} signal to exocytosis. Both types of regulation are important. The peak Ca^{2+} concentration

depends in principle on the shape of the action potential, the open probability of the Ca^{2+} channels, and the Ca^{2+} concentration present at the time when the Ca^{2+} channels open. All of these three parameters can be regulated. For example, action-potential duration modulates neurotransmitter release (Qian & Saggau 1999), presynaptic receptors for endocannabinoids inhibit N-type Ca^{2+} channels (Wilson et al. 2001), and residual Ca^{2+} during repetitive action potentials causes short-term plasticity of release (reviewed in von Gersdorff & Borst 2002, Zucker & Regehr 2002). The release probability per peak Ca^{2+} concentration is also highly variable and depends on two principal parameters: the number of release-ready vesicles, and the Ca^{2+} responsiveness of these vesicles. The release probability per Ca^{2+} concentration varies characteristically between different types of synapses (e.g., Xu-Friedmann et al. 2001) and changes during cAMP-dependent presynaptic long-term potentiation (LTP) (Regehr & Tank 1991, Kamiya et al. 2002). Furthermore, the number of release-ready vesicles decreases during repetitive stimulation, causing short-term depression with a magnitude that depends on the pool size and kinetics (see discussion above). Pinpointing the mechanisms involved in regulating release beyond Ca^{2+} triggering has been difficult, and whether a difference in Ca^{2+} responsiveness at a synapse depends on the size of the readily releasable pool or on the Ca^{2+} responsiveness of synaptic vesicles is largely uncertain.

APPROACHES TO ANALYZING SYNAPTIC FUNCTION

Understanding synaptic transmission proceeds via a dialogue between physiological description and molecular analysis. The physiology describes the phenomena that the molecular biology tries to explain, and thus the physiology is always ahead. At this point, rapid physiological advances have produced a rich phenomenology of presynaptic events whose molecular basis is often obscure. That the criteria for what constitutes an explanation, as opposed to an epiphenomenal effect, differ dramatically from lab to lab is a major problem of the molecular analysis of presynaptic events.

A convergence of two approaches is required for a molecular explanation of presynaptic physiology. First, important molecules need to be characterized biochemically and structurally, i.e., their protein-protein interactions, cellular localizations, and atomic structures have to be determined. Second, the functions of such molecules need to be perturbed in vivo, and the consequences of such perturbations must be measured. In the vast literature on presynaptic molecules, fundamental limitations restrict the interpretation of many experiments. For example, many studies examine synaptic protein-protein interactions using a combination of GST (glutathione S-transferase)-fusion protein pulldowns, yeast two-hybrid assays, and immunoprecipitations. Such interactions have been most extensively reported for the SNARE protein syntaxin 1 with >40 purported binding partners. However, for a "sticky" protein such as syntaxin 1, this data may be insufficient. As recently shown for the apparent interaction of syntaxin 1 with K^+ channels, all of these methods can be positive and still give a wrong answer (Fletcher et al. 2003). A similar limitation applies to the location and activities of proteins deduced by analogy. For example,

on the basis of the paradigm of synaptotagmin 1, results of synaptotagmin 4 transfection studies were interpreted to reflect a Ca^{2+} sensor function of synaptotagmin 4 on secretory vesicles, even though the localization of synaptotagmin 4 or its Ca^{2+}-binding properties was not established (Wang et al. 2003). A molecular analysis first requires a description of the molecules that is often cumbersome and not publishable but without which the results of "functional" studies are not interpretable.

Three types of techniques are used to perturb a molecule to analyze its function: overexpression of a protein (full-length, truncated, or mutant), introduction of a chemical agent (drug) or peptide, and genetic manipulations with or without expression of wild-type or mutant molecules. Each technique has its own limitations:

1. Overexpression, the easiest approach, often gives the most dramatic results but is the most difficult to interpret, especially when performed on a wild-type background. For example, overexpression of wild-type Rab3A appears to block Ca^{2+}-induced exocytosis (Holz et al. 1994, Johannes et al. 1994) but in fact acts indirectly by activating constitutive exocytosis of secretory vesicles (Schlüter et al. 2002). This action depletes the vesicles and thereby abolishes Ca^{2+}-induced exocytosis indirectly.
2. Introduction of a chemical agent/peptide is potentially the best method because it allows direct and fast interference with a protein's function. However, few chemicals are known to interfere specifically with a function; even peptides are drugs that have no inherent specificity. For example, peptides from Rab3A activate exocytosis not by interfering with release, as proposed by Oberhauser et al. (1992), but by influencing Ca^{2+} signaling (Piiper et al. 1995). Overexpression and chemical/peptide interference experiments are only interpretable when the chemical or peptide involved has a validated mechanism of action.
3. Genetic approaches are the most definitive but often suffer from the failure to delineate the specificity of the changes caused by a genetic manipulation. Analysis of genetic changes is lengthy and laborious. For example, knockout (KO) of CaM kinase IIα creates an array of phenotypes that can be sorted out only when the pre- and postsynaptic contributions of CaM kinase IIα are separated (see Hinds et al. 2003). Thus a genetic manipulation is only interpretable when the effects are selective and when the particular genetic manipulation examined does not broadly alter downstream processes.

In view of these limitations, it is clear that the completeness of the analysis—and not the techniques used—determines the interpretability of the results obtained.

MOLECULAR ARCHITECTURE OF SYNAPTIC VESICLES AND INTERACTING PARTNERS

All activities in the presynaptic nerve terminal are directed toward neurotransmitter release, from single release events to repeated rounds of regulated release. As a result, all processes in a nerve terminal influence, directly or indirectly, the

interaction of synaptic vesicles with the presynaptic active zone. Understanding the composition of synaptic vesicles and of the active zone is a first step toward insight into the molecular mechanisms of release.

Synaptic Vesicles

Synaptic vesicles are uniformly small (~20-nm radius), abundant organelles whose only known function is to take up and release neurotransmitters. They are relatively simple because only a limited number of proteins fit into a sphere of 40-nm diameter. Purified vesicles have a protein:phospholipid ratio of 1:3 with an unremarkable lipid composition (40% phosphatidylcholine, 32% phosphatidylethanolamine, 12% phosphatidylserine, 5% phosphatidylinositol, 10% cholesterol, wt/wt; Benfenati et al. 1989). Many proteins that have been associated with synaptic vesicles are probably present only on a subset of vesicles or bind transiently to the vesicles, and the number of proteins that are constitutive parts of all synaptic vesicles may be comparatively small (i.e., less than 50).

Synaptic vesicles contain two classes of obligatory components: transport proteins involved in neurotransmitter uptake (step 1 of the vesicle cycle; Figure 1), and trafficking proteins that participate in synaptic vesicle exo- and endocytosis and recycling (steps 2–9). Transport proteins are composed of a vacuolar-type proton pump that generates the electrochemical gradient, which fuels neurotransmitter uptake and neurotransmitter transporters that mediate the actual uptake. The trafficking proteome of synaptic vesicles is complex. It includes intrinsic membrane proteins, proteins associated via posttranslational lipid modifications, and peripherally bound proteins (Figure 4). These proteins do not share a characteristic that would make them identifiable as synaptic vesicle proteins, and little is known about how these proteins are specifically deposited into synaptic vesicles. As summarized in Figure 4, many but not all of the known synaptic vesicle proteins interact with nonvesicular proteins and are linked to specific functions.

Active Zones and Presynaptic Plasma Membranes

The active zone is composed of an electron-dense, biochemically insoluble material located at the presynaptic plasma membrane precisely opposite the synaptic cleft. Electron microscopy revealed that active zones of central synapses are disk-like structures containing a hexagonal grid; synaptic vesicles are embedded in the depressions of the grid (Akert et al. 1971). In contrast, active zones of neuromuscular synapses are composed of an elongated ridge containing vesicles lined up like beads on a string (Harlow et al. 2001).

Investigators identified six large nonmembrane proteins that are each encoded by multiple genes, bind to each other, and probably form a humongous single complex at the active zone (reviewed in Dresbach et al. 2001, Lonart, 2002; see Figure 5): Munc13s (Munc13-1, -2, and -3; Brose et al. 1995) and RIMs (Rab3-interacting molecules) (RIM1α, 2$\alpha/\beta/\gamma$, 3γ, and 4γ; Wang et al. 1997a, 2000; Wang & Südhof 2003) are multidomain proteins that interact with each other and

many other synaptic components. Piccolo and Bassoon, homologous and very large proteins (Cases-Langhoff et al. 1996, tom Dieck et al. 1998), are part of the cytomatrix. ERCs (ELKS/Rab3-interacting molecule/CAST) (ERC1b and ERC2; Wang et al. 2002, Ohtsuka et al. 2002) are coiled-coil proteins whose C-terminus binds to RIMs, and RIM-BPs (RIM-BP1, 2, and 3; Wang et al. 2000) are SH3-domain proteins that also bind to RIMs. Finally, α-liprins (Liprin α1–α4) bind to RIMs (Schoch et al. 2003), ERCs (Ko et al. 2003), and receptor protein tyrosine phosphatases (Serra-Pages et al. 1998).

RIMs are central elements of active zones because they bind directly or indirectly to many other synaptic proteins. The largest RIM variants (RIM1α and RIM2α) include three types of domains: (*a*) an N-terminal zinc-finger domain that interacts with the synaptic vesicle protein Rab3 and the active zone protein Munc13-1 (Wang et al. 1997a, 2000; Betz et al. 2001; Wang et al. 2001b; Schoch et al. 2002); (*b*) a central PDZ domain that binds the C-terminus of ERCs (Ohtsuka et al. 2002, Wang et al. 2002); and (*c*) two C-terminal C_2 domains, of which the second, so-called C_2B domain binds to α-liprins (Schoch et al. 2002). The *RIM2* gene (but not the *RIM1* gene) produces two additional transcripts from internal promoters besides the RIM2α transcript: the RIM2β transcript, which lacks the zinc-finger domain but contains all other domains of RIM2α, and the RIM2γ transcript, which is composed only of the C_2B domain preceded by a short conserved sequence (Wang & Südhof 2003). The *RIM3* and *RIM4* genes produce only γ transcripts (RIM3γ and 4γ). RIMs directly bind to Munc13, ERCs, RIM-BPs, and α-liprins; in addition, all ERCs bind to α-liprins, Piccolo and Bassoon (see references cited above, and Y. Wang and T.C. Südhof, unpublished observations). The resulting macromolecular complex may be linked to synaptic vesicles via two interactions (Figure 5): the GTP-dependent binding of Rab3 to RIM1α/2α (Wang et al. 1997a, 2000), and the Ca^{2+}-dependent association of synaptotagmin 1 with RIM1α/2α/2β (Coppola et al. 2001, Schoch et al. 2002). Although conceptually attractive, the RIM/synaptotagmin interaction has not been validated, and whether this interaction is physiologically relevant is not clear. In addition, the complex may be linked to SNARE proteins via binding of Munc13 to syntaxin 1 and of RIMs to SNAP-25 (Betz et al. 1997, Coppola et al. 2001), but again these interactions have not been validated physiologically.

NEUROTRANSMITTER UPTAKE INTO SYNAPTIC VESICLES

Synaptic vesicles accumulate and store neurotransmitters at high concentrations by active transport, driven by a vacuolar proton pump whose activity establishes an electrochemical gradient across the vesicle membrane (Maycox et al. 1988). The vesicle proton pump is a large multiprotein complex ($\sim$1 million Dalton) that contains at least 13 subunits and measures $14 \times 14 \times 24$ nm (Arata et al. 2002), $\sim$10% of the vesicle volume. The proton pump is the single largest vesicle

component that extends from the vesicle by >15 nm, more than half of the vesicle radius; most vesicles contain only a single proton pump molecule (Stadler & Tsukita 1984). The proton pump is composed of a larger peripheral complex called V_1, which includes the ATPase activity, and an integral membrane complex called V_0, which mediates proton translocation. The two parts may be connected by the 116-kDa subunit of the pump, its largest component (Perin et al. 1991a). Although the generic composition of vacuolar proton pumps is well established, whether the subunits or properties of the synaptic vesicle proton pump differ from those of other vacuolar proton pumps (e.g., the endosomal or the Golgi proton pumps) is unclear.

The proton pump establishes an electrochemical gradient that drives all neurotransmitter uptake (reviewed by Fykse & Fonnum 1996). Vesicular uptake is mediated by only seven different transporters representing four distinct uptake systems. Glutamate is taken up into synaptic vesicles by three differentially expressed transporters (Fremeau et al. 2002, Gras et al. 2002, Schafer et al. 2002, Takamori et al. 2002, and references cited therein) and all monoamines (catecholamines, histamine, and serotonin) are taken up by two differentially expressed transporters (Erickson et al. 1992, Liu et al. 1992). A single transporter was identified for GABA and glycine (McIntyre et al. 1997, Sagne et al. 1997) and for acetylcholine (Alfonso et al. 1993, Roghani et al. 1994, Varoqui et al. 1994). The four families of transporters are distantly related to each other, but differ mechanistically. For some transporters (e.g., VGlut1–VGlut3), the main driving force for vesicular uptake is the membrane potential, whereas for other transporters (e.g., VGat), the membrane potential and the proton gradient both contribute to uptake (reviewed by Fykse & Fonnum 1996).

Expression of a particular transporter type is probably a major determinant of the type of neurotransmitter used by a neuron. When VGlut1 was transfected into GABAergic neurons, their synapses became glutamatergic, in addition to remaining GABAergic (Takamori et al. 2000). Thus simple expression of the glutamatergic transporter was enough to specify neurotransmitter type. VGlut3 is present in many neurons not previously considered glutamatergic (e.g., the cholinergic interneurons of the striatum; Fremeau et al. 2002, Schafer et al. 2002), which suggests that coexpression of glutamate transporters with other neurotransmitter transporters may confer onto a small set of neurons the ability to use multiple classical transmitters in violation of Dale's principle.

Whether and how the proton pump and/or transmitter transporters are regulated is a major question. The amount of transmitter released per synaptic vesicle exocytosis varies (reviewed in van der Kloot 1991). One explanation of this variation is that vesicles contain the same concentration of transmitters but have different sizes because the size variation of vesicles matches the variation in the amount of release mediated by a single vesicle (Bekkers et al. 1990, Bruns et al. 2000). Another explanation, though not mutually exclusive, is that neurotransmitter uptake is regulated via the activity of either the proton pump or the transporters. Overexpression of the vesicular monoamine transporter increases the amount of release mediated by exocytosis of a single vesicle (Pothos et al. 2000), whereas

hemizygous deletion of this transporter dramatically alters monoaminergic signaling in brain, which suggests that the levels of the transporter are physiologically important (Wang et al. 1997b). At least in the calyx, glutamate transport into vesicles is not saturated but can be enhanced simply by increasing the cytosolic glutamate concentration (Yamashita et al. 2003). Regulation of the proton pump is also important for understanding the vesicle cycle. For example, in the time between synaptic vesicle exo- and endocytosis—which can last seconds—the pump is presumably turned off to avoid pumping protons into the synaptic cleft, but this regulation has not actually been demonstrated. How fast neurotransmitter uptake operates is also unknown. Given the small size of the vesicles, only few protons and neurotransmitter molecules need to be pumped to fill a vesicle, which suggests that vesicle refilling after endocytosis could occur in milliseconds.

MEMBRANE FUSION DURING EXOCYTOSIS: SNARES AND COMPANY

Intracellular membrane fusion generally involves SNARE proteins that are present on both fusing membranes before fusion and that associate into tight core complexes during fusion (reviewed in Chen & Scheller 2001, Jahn et al. 2003). SNARE proteins are characterized by a homologous 70-residue sequence called the SNARE motif. The core complex is formed when four SNARE motifs (present in three or four separate SNARE proteins because some SNAREs contain two SNARE motifs) assemble into a parallel four-helical bundle, with the transmembrane regions of the SNAREs emerging on the C-terminus. Core-complex formation may force the membranes on which the SNAREs reside into close proximity, thereby initiating membrane fusion. Four different classes of SNARE motifs exist (R, Qa, Qb, and Qc SNARE motifs), and stable SNARE complexes only form when the four-helical bundle contains one SNARE motif from each class (reviewed in Jahn et al. 2003). The interaction of SNAREs is otherwise promiscuous in vitro, although in vivo they form highly specific complexes. The specificity is likely achieved, at least in part, via the sequences of SNARE proteins outside of their SNARE motifs.

Synaptic exocytosis is mediated by three SNARE proteins: synaptobrevin (also called vesicle-associated membrane protein) on synaptic vesicles, and syntaxin 1 and SNAP-25 on the presynaptic plasma membrane (Söllner et al. 1993). The synaptic core complex is formed by the R-SNARE motif from synaptobrevin, the Qa-SNARE motif from syntaxin 1, and the Qb- and Qc-SNARE motifs from SNAP-25 (which contains two SNARE motifs). The model shown in Figure 6 proposes that by pulling the synaptic vesicle and plasma membranes close together, the SNARE complex creates an unstable intermediate but does not open the fusion pore. The unstable intermediate can progress to a full-blown fusion pore or regress to the docked state of synaptic vesicles that do not contain engaged SNAREs. Complexins, small neuronal proteins, bind to assembled synaptic

core complexes (McMahon et al. 1995). Complexins insert into a groove in the C-terminal half of the SNARE complex that is absent from partially assembled SNARE complexes (Chen et al. 2002a). KO mice revealed that complexin is not essential for SNARE function or synaptic vesicle fusion (Reim et al. 2001) but promotes the action of synaptotagmin 1 (see discussion below).

SNARE complex formation at the synapse and in other intracellular fusion reactions is probably controlled by a class of essential fusion proteins called SM proteins for Sec1/Munc18-like proteins (reviewed in Jahn et al. 2003). SM proteins often interact with syntaxin-like SNAREs. Munc18-1, the SM protein that controls synaptic fusion, binds to a conformation of syntaxin that is closed (Dulubova et al. 1999) and blocks its SNARE motif from participating in SNARE complexes. Thus Munc18-1 must dissociate from syntaxin for SNARE complexes to form. However, regulation of SNARE complex formation by a steric mechanism is not the general function of SM proteins because several SM proteins bind to their cognate syntaxin SNARE proteins in the open conformation (Yamaguchi et al. 2002, Dulubova et al. 2002). It is striking that although SM proteins are generally required for membrane fusion and usually interact with syntaxin-like SNAREs, the interactions themselves vary between fusion reactions.

Several synaptic proteins may regulate the assembly of SNARE complexes. Two soluble proteins called tomosyn (Fujita et al. 1998) and amisyn (Scales et al. 2002) have a C-terminal R-SNARE motif that can substitute for synaptobrevin in the synaptic SNARE complex. Tomosyn and amysin exhibit no other similarity—tomosyn is a relatively large protein of >1100 residues that belongs to the family of lethal-giant-larvae proteins, whereas amisyn is a small protein of 222 residues—but both proteins, when introduced exogenously into a secretory system, inhibit exocytosis by interfering with the action of synaptobrevins (Scales et al. 2002, Hatsuzawa et al. 2003).

Another class of proteins that may regulate SNARE function at the synapse are synaptophysins, abundant synaptic vesicle proteins that bind directly to synaptobrevin (Johnston & Südhof 1990, Calakos & Scheller 1994, Edelmann et al. 1995, Washbourne et al. 1995). Synaptobrevin cannot simultaneously bind to synaptophysins and participate in the SNARE complex, which suggests that binding of synaptobrevin to synaptophysin restricts the availability of synaptobrevin for fusion (Edelmann et al. 1995, Becher et al. 1999). Consistent with this hypothesis, the synaptobrevin/synaptophysin complex is upregulated during development (Becher et al. 1999). Chronic blockade of glutamate receptors caused an increase in neurotransmitter release but a decrease in the synaptobrevin/synaptophysin complex (Bacci et al. 2001). In addition to heteromultimers, synaptophysin and synaptobrevin each assemble into homooligomers via their transmembrane regions (Thomas et al. 1988, Laage & Langosch 1997). Only dimers of synaptobrevin were observed, whereas synaptophysin associates into hexamers and larger oligomers (Johnston & Südhof 1990). The synaptic vesicle proteins synaptobrevin and synaptophysin thus engage in at least three complexes on the surface of synaptic vesicles: separate homooligomers and common heterooligomers.

There is general agreement that SNARE proteins function in fusion, but the precise nature of their activity is uncertain. Because most SNARE proteins contain other essential, conserved sequences besides the SNARE motif, it is important to differentiate between SNARE functions mediated by their SNARE motifs (e.g., core complexes) and SNARE functions mediated by their other sequences (e.g., interactions with Sec1/Munc18-like proteins). Two major hypotheses for SNARE function were advanced: (*a*) SNAREs are minimal fusion machines that single-handedly drive fusion via their SNARE motifs, and all other interactions are secondary; (*b*) SNAREs are components of fusion machines that include other proteins. At least at the synapse, two key observations support the second hypothesis. First, deletion of synaptobrevin caused a major impairment of synaptic vesicle exocytosis but left approximately 10% of exocytosis intact (Schoch et al. 2001). Ca^{2+}-triggered exocytosis was much more severely impaired than was spontaneous exocytosis or exocytosis triggered by hypertonic sucrose. No closely related R-SNARE was present in the affected synapses, which suggests that there is no simple redundancy of SNAREs. Similar results were reported for the SNAP-25 KO (Washbourne et al. 2002). Thus at least at the synapse, the proper SNARE complex was only essential for efficient, physiologically regulated fusion, but not for fusion as such. Second, although the synaptobrevin deletion did not abolish exocytosis, deletion of Munc18-1 eliminated release completely (Verhage et al. 2000). Thus at the mammalian synapse, Munc18-1 is more fundamental for fusion than is synaptobrevin. Munc18-1 probably acts by binding to the plasma membrane SNARE protein syntaxin 1 (Hata et al. 1993) and coupling it to as yet unidentified cytosolic factors to organize SNARE complex assembly (reviewed in Jahn et al. 2003).

Viewed together, the current data suggest that SNAREs still are the best candidates for initiating fusion by inducing transition states that ultimately lead to fusion-pore opening (Figure 6*B*). However, the critical step of bringing membranes close together may, at least in part, be substituted for by other cellular mechanisms. Besides forcing membranes close together, SNAREs likely perform additional functions that are as important, for example ensuring the temporal and spatial specificity of fusion reactions by embedding the approximation of membranes into an ordered sequence of reactions. This embedding is presumably guided by sequences of SNAREs outside of the SNARE motif and may, among others, require SM proteins. The best example for this embedding is the role of Munc18-1, which acts by binding to syntaxin 1 in fusion as discussed above.

In addition to synaptobrevin, synaptic vesicles contain the Qb-SNARE protein Vti1aβ (Antonin et al. 2000; Figure 4). Although as Qb- and R-SNAREs, Vti1aβ and synaptobrevin could, in principle, participate in the same SNARE complex, Vti1aβ functions in endosome and Golgi fusion reactions without interacting with synaptobrevin (Jahn et al. 2003). The selective interactions of SNARE proteins in vivo reinforces the notion that SNARE complex formation must be tightly regulated by mechanisms that are independent of the SNARE motifs of the participating SNARE proteins.

TRIGGERING FUSION VIA Ca^{2+} BINDING TO SYNAPTOTAGMINS

Studies of synaptic vesicle exocytosis in the calyx terminal showed that fast exocytosis is triggered by Ca^{2+} binding to a Ca^{2+} sensor with at least five Ca^{2+}-binding sites of micromolar affinity (reviewed in Meinrenken et al. 2003). Evidence accumulated over the past 13 years demonstrates that two synaptic vesicle proteins called synaptotagmins 1 and 2 (Figure 4) are such Ca^{2+} sensors.

Synaptotagmins 1 and 2 as Ca^{2+} Sensors for Fast Exocytosis

Synaptotagmins are composed of a short N-terminal intravesicular sequence, a single transmembrane region, a short linker sequence, and two cytoplasmic C_2 domains (the C_2A and C_2B domains; Perin et al. 1990, Geppert et al. 1991). Synaptotagmins 1 and 2 are abundant synaptic vesicle proteins that are differentially expressed (Geppert et al. 1991, Ullrich et al. 1994). Most experiments were performed on synaptotagmin 1, but the limited studies on synaptotagmin 2, its high degree of sequence similarity with synaptotagmin 1, and the reciprocal distributions of synaptotagmins 1 and 2 suggest that synaptotagmins 1 and 2 are functionally similar. Besides their differential distributions, the only major difference between synaptotagmins 1 and 2 is an approximately twofold difference in apparent Ca^{2+} affinity (Sugita et al. 2002; for synaptotagmins 3–15, see discussion below, which describes their substantially different properties).

The C_2A domain of synaptotagmin 1 binds three Ca^{2+} ions (Ubach et al. 1998), and the C_2B domain two Ca^{2+} ions (Fernandez et al. 2001). The intrinsic Ca^{2+} affinities of the C_2 domains are very low (0.5–5 mM) because the coordination spheres for the Ca^{2+} ions are incomplete. The apparent Ca^{2+} affinity of the C_2 domains increases dramatically (up to 1000-fold) when the C_2 domains bind to phospholipid membranes whose negatively charged headgroups provide additional coordination sites for the bound Ca^{2+} ions (Fernandez-Chacon et al. 2001). The number of Ca^{2+} ions bound by synaptotagmin 1 and its apparent Ca^{2+} affinity thus correspond to the Ca^{2+} sensor for exocytosis at the calyx (Bollmann et al. 2000, Schneggenburger & Neher 2000). Two experiments demonstrated that synaptotagmin 1 is an essential Ca^{2+} sensor for fast exocytosis but not for the slow component of exocytosis or for membrane fusion in general. First, a KO of synaptotagmin 1 in mice produced a lethal phenotype that consisted of a selective loss of fast Ca^{2+}-triggered exocytosis both in hippocampal synapses (Geppert et al. 1994a) and in chromaffin cells (Voets et al. 2001). No other parameter of synaptic membrane traffic examined was impaired. Second, a point mutation in the synaptotagmin 1 C_2A domain that alters the overall apparent Ca^{2+} affinity of synaptotagmin 1 induced an identical shift in the apparent Ca^{2+} affinity of exocytosis (Fernandez-Chacon et al. 2001). Experiments using flash photolysis of caged Ca^{2+} in chromaffin cells showed that this mutation shifted the apparent Ca^{2+} affinity of fast exocytosis (but not of the slow phase) in precisely the same manner as the apparent Ca^{2+}-binding

affinity of the cytoplasmic double C_2 domain fragment (Sorensen et al. 2003). The Ca^{2+}-binding properties and mouse mutants of synaptotagmin 1 established its function as a Ca^{2+} sensor for fast exocytosis but raised two new questions: How does it work, and what Ca^{2+} sensors are responsible for the slow phase of exocytosis?

Mechanism of Action of Synaptotagmin 1

In addition to forming Ca^{2+}-dependent complexes with phospholipids (which are essential for achieving a physiologically apparent Ca^{2+} affinity), synaptotagmins 1 and 2 also bind to SNARE complexes. Binding to SNARE complexes is partly Ca^{2+} independent (i.e., observed in the presence of an excess of EGTA) and partly Ca^{2+} dependent (Bennett et al. 1992, Chapman et al. 1995, Li et al. 1995a, Shin et al. 2003). In addition, synaptotagmins 1 and 2 form homomultimers both Ca^{2+}-independently (via its N-terminal transmembrane region; Perin et al. 1991b) and Ca^{2+}-dependently (via its C_2B domain; Chapman et al. 1996, Sugita et al. 1996). The Ca^{2+}-dependent interaction of synaptotagmin 1 with the SNARE complex is not essential for Ca^{2+} triggering of release because Sr^{2+} can substitute for Ca^{2+} in triggering release, but it is unable to stimulate SNARE binding (Shin et al. 2003). This finding established that triggering of fast exocytosis by Ca^{2+} binding to synaptotagmin 1 is not executed via Ca^{2+}-induced binding of synaptotagmin 1 to SNAREs but does not mean that SNARE binding is not important. In fact, Ca^{2+}-independent binding of synaptotagmin 1 could serve to position synaptotagmin 1 next to SNARE complexes after these have been assembled but before the fusion pore has opened (see model in Figure 6*B*). Even Ca^{2+}-dependent binding of synaptotagmin 1 to SNAREs could be functionally important, for example in recruiting vesicles into the readily releasable pool.

A possible model derived from these observations is based on the notion that SNARE complex formation forces synaptic vesicles into an unstable fusion intermediate that is stabilized by complexin binding to the SNARE complexes (Figure 6*B*). Synaptotagmin 1 binds to the SNARE complex in the absence of Ca^{2+} but switches to the phospholipid membrane as soon as Ca^{2+} enters via Ca^{2+} channels. Binding of synaptotagmin 1 C_2 domains to the phospholipids, with partial insertion of hydrophobic amino acids from synaptotagmin 1 and mechanical stress induced by the binding, may then destabilize the fusion intermediate and open the fusion pore (Figure 6*C*). This model has the advantage of explaining several key features of release. First is speed. Because all Ca^{2+} does in binding to synaptotagmin 1 is cause a switch in binding partners, which results in a mechanical stress, Ca^{2+} binding to synaptotagmin 1 is a fast reaction. Second is cooperativity. Synaptotagmin 1 binds five Ca^{2+} ions as predicted in the calyx model and thus has the requisite number of Ca^{2+}-binding sites. Third is the synaptobrevin KO phenotype (Schoch et al. 2001). Ca^{2+}-triggered release was much more severely depressed in synaptobrevin KO mice than in Ca^{2+}-independent release, which indicates that the proper SNARE complex may not be absolutely essential for fusion but is essential for Ca^{2+} triggering. Fourth is the complexin KO phenotype

(Reim et al. 2001). The complexin KO mouse exhibits a phenotype that has the properties of a milder version of the synaptotagmin 1 KO, consistent with the notion that complexin stabilizes the SNARE complex that is essential for the proper positioning of synaptotagmin 1 (see model in Figure 6*B*). In spite of this supporting evidence, however, the model is far from proven, and alternative interpretations are possible. For example, no mutant of synaptotagmin 1 that still binds Ca^{2+} but is unable to interact with SNARE complexes was reported, and the question of whether synaptotagmin 1 can bind to the SNARE complex simultaneously with complexin has not been investigated. Thus, although it seems assured that Ca^{2+} binding to synaptotagmin 1 (and synaptotagmin 2 in more caudal synapses) triggers fast release, the mechanism of triggering is probably intimately connected to SNARE proteins and likely requires clarification of SNARE function.

Ca^{2+} Sensors for Asynchronous or Delayed Exocytosis

A second major question considers how Ca^{2+} triggers slow, asynchronous release. Synaptotagmin 1 is part of a gene family containing 15 members in vertebrates (reviewed in Südhof 2002). Of these, only synaptotagmins 1–7 and 9–11 have sequences consistent with Ca^{2+} binding to at least one of the two C_2 domains. However, direct measurements revealed that not all C_2 domains of synaptotagmins with predicted Ca^{2+}-binding sites in fact bind Ca^{2+}. For example, the C_2B domains of synaptotagmin 3 and synaptotagmin 9, although including all predicted Ca^{2+}-binding residues, are inactive (Sugita et al. 2002; Shin et al. 2004). Despite the incomplete information, it is likely that other synaptotagmins function as Ca^{2+} sensors for the slow component of exocytosis, either alone or in collaboration with synaptotagmin 1. Support for this hypothesis comes from the observation that the "other" synaptotagmins 3, 6, and 7 exhibit higher apparent Ca^{2+} affinities (Sugita et al. 2001, 2002). Asynchronous release, although intrinsically slower than the fast component, is probably induced by lower Ca^{2+} concentrations. The Ca^{2+} transient induced by an action potential has only a very short ($\sim$400 μs) lifetime sufficient to trigger fast release, but the residual Ca^{2+} decays more slowly and still activates asynchronous release at lower Ca^{2+} concentrations. Consistent with this hypothesis, the C_2 domains from high-affinity synaptotagmins 3 and 7 are potent inhibitors of slow release in permeabilized neuroendocrine cells, whereas the C_2 domains of synaptotagmin 1 and 2 are inactive (Sugita et al. 2001, 2002).

REGULATING EXOCYTOSIS VIA Rab3

Rab proteins form a large set of GTP-binding proteins that regulate intracellular transport. Synaptic vesicles contain members of at least three families of rab proteins: Rab3 (Rab3A, 3B, 3C, and 3D; Schlüter et al. 2002), Rab5 (Fischer von Mollard et al. 1994), and Rab11 (Khvotchev et al. 2003). Of these, Rab3 proteins are the most abundant; Rab3A alone accounts for $\sim$25% of the total Rab GTP binding in brain (Geppert et al. 1994b). In addition to synaptic vesicles, Rab3

proteins are also found on other secretory vesicles in a variety of cell types and are the only mammalian Rab proteins that are specific for such vesicles (reviewed in Darchen & Goud 2000).

The Rab3 Cycle

Rab3 undergoes a cycle of synaptic vesicle association and dissociation in parallel with synaptic vesicle exo- and endocytosis (Fischer von Mollard et al. 1991). Rab3 is attached to synaptic vesicles in the GTP-bound state via covalently linked geranylgeranyl moieties (Johnston et al. 1991). During or after synaptic vesicle fusion, GTP on Rab3 is hydrolyzed to GDP, and the resulting GDP-bound Rab3 is dissociated from synaptic vesicles by GDI (named GDP dissociation inhibitor, although its general function is to dissociate rab proteins from membranes; Araki et al. 1990). The soluble GDI/GDP-Rab3 complex is then reattached to synaptic vesicles by a poorly understood process that involves GDP to GTP exchange. Rab3 dissociation from vesicles depends on Ca^{2+}-triggered exocytosis of synaptic vesicles (Fischer von Mollard et al. 1991), which suggests that the Rab3 cycle ensures directional interactions of Rab3 with effector proteins during exocytosis.

Analysis of KO mice revealed a discrete but important function of Rab3A in a late step of exocytosis. Two major phenotypes were observed:

1. In synapses of the hippocampal CA1 region, short-term plasticity was altered without a change in the readily releasable vesicle pool (Geppert et al. 1994b, 1997). Different from other Rab proteins that normally function in vesicle transport or docking (Jahn et al. 2003), Rab3A was found to be selectively essential for a late step in exocytosis that follows transport and docking. However, other Rab3 isoforms are still expressed in the Rab3A KO mice, and the phenotype may reflect only the essential part of Rab3A function, whereas other parts of Rab3A function may be redundant among isoforms.
2. In the hippocampal CA3 region, mossy-fiber LTP was abolished, but no change in short-term plasticity was detected (Castillo et al. 1997). Mossy fiber–type LTP is expressed as a presynaptic increase in release, triggered by stimulation of cAMP-dependent protein kinase A (PKA), which causes a direct modification of the secretory machinery (reviewed in Nicoll & Malenka 1995, Hansel et al. 2001). Mossy fiber–type LTP is expressed not only in hippocampal mossy fiber synapses in the CA3 region (Huang et al. 1994, Weisskopf et al. 1994), but also in corticothalamic and corticostriatal (Castro-Alamancos & Calcagnotto 1999, Spencer & Murphy 2002) and cerebellar parallel fiber synapses (Salin et al. 1996, Linden 1997). Rab3A is not a substrate for PKA, which suggests that although Rab3A is essential for mossy fiber–type LTP, LTP is not simply induced by Rab3A phosphorylation.

The presence of distinct phenotypes in synapses lacking Rab3A suggests that excitatory synapses fall into two principal classes: synapses capable of PKA-dependent presynaptic LTP where Rab3A is only required for LTP, and synapses

that lack this form of LTP and exhibit changes only in short-term plasticity upon deletion of Rab3A.

Rab3 Effectors

Two classes of Rab3 effectors that bind only to GTP-Rab3 but not to GDP-Rab3 have been identified, rabphilin (Shirataki et al. 1993, Li et al. 1994) and RIM1α/2α (Wang et al. 1997a, 2000; Wang & Südhof 2003). Both effectors have a similar N-terminal zinc-finger domain that interacts with all Rab3 isoforms, include central phosphorylation sites for PKA, and contain two C-terminal C_2 domains. Otherwise, however, rabphilin and RIM1α/2α are very different (Wang et al. 1997a). Rabphilin is a soluble protein that requires Rab3 for binding to synaptic vesicles (Geppert et al. 1994b, Li et al. 1994) and binds Ca^{2+} via its C_2 domains (Ubach et al. 1998). RIM1α/2α, in contrast, are larger, biochemically insoluble active-zone proteins whose C-terminal C_2 domains lack predicted Ca^{2+}-binding sites.

Rabphilin exhibits biologically interesting properties (Ca^{2+} binding, cycling on- and off-synaptic vesicles in a manner dependent on Rab3, stimulation-dependent phosphorylation by multiple kinases; see Shirataki et al. 1993, Li et al. 1994, Ubach et al. 1999). It was thus disappointing that deletion of rabphilin in mice failed to cause a major impairment (Schlüter et al. 1999). The rabphilin KO mice exhibited none of the phenotypic traits of Rab3A KO mice. The absence of a rabphilin KO phenotype may be due to a hidden redundancy, but no rabphilin isoform that binds to Rab3 and is expressed in neurons has been identified.

A very different picture emerged for RIM1α: KO of RIM1α caused a major phenotype in synaptic transmission that went beyond the features of the Rab3A KO phenotype (Castillo et al. 2002, Schoch et al. 2002). RIM1α KO mice displayed a large decrease in synaptic strength, exhibited major changes in short-term synaptic plasticity, and lacked mossy fiber–type LTP both in CA3 synapses in the hippocampus and in parallel fiber synapses in the cerebellum. In RIM1α KO mice but not Rab3A KO mice, the levels of the active-zone protein Munc13-1 (which binds to the N-terminal zinc-finger of RIMs; see discussion above in Molecular Architecture of Synaptic Vesicles and Interacting Partners) were substantially depressed (Schoch et al. 2002). The binding of Rab3A on synaptic vesicles to RIM1α in the active zone evokes a docking function (Figure 5), but RIM1α KO mice did not exhibit a change in the number of docked vesicles (Schoch et al. 2002), consistent with a lack of change in docking in the Rab3A KO mice (Geppert et al. 1997). Viewed together, these data suggest that RIM1α (and probably RIM2α) regulates neurotransmitter release via interactions of its N-terminal domain with Rab3 and Munc13-1, and possibly via interactions of its PDZ domains with ERCs and its C-terminal C_2 domain with α-liprins and synaptotagmin 1 (Betz et al. 2001, Ohtsuka et al. 2002, Schoch et al. 2002, Wang et al. 2002).

Possibly the most interesting question is how Rab3A and RIM1α participate in mossy fiber–type LTP. Recent studies showed that RIM1α is phosphorylated by PKA in vivo and that phosphorylation of RIM1α at a central PKA site is essential for mossy fiber–type LTP (Lonart et al. 2003). Thus the long-lasting increase in

release upon LTP induction appears to require the coincidence of two signals: GTP-dependent binding of Rab3A to the N-terminal zinc-finger domain of RIM1α, and phosphorylation of RIM1α by PKA at a site immediately C-terminal to the zinc-finger domain (Figure 5). Although the mechanism by which this convergence of signals induces LTP is unknown, this mechanism may include a restructuring of the active zone that leads to an increase either in the number of release-ready vesicles or in the efficiency of Ca^{2+} triggering of release. Interestingly, binding of Munc13-1 and Rab3 compete for binding to the N-terminal zinc-finger of RIM1α (Betz et al. 2001). A possible pathway is that Rab3 binding to RIMs inactivates Munc13 by displacing it from RIM and that this action potentiates release, possibly by allowing Munc13 to recruit additional RIM molecules to the active zone and thereby to increase the size of the active zone.

SV2s MAY REGULATE Ca^{2+} LEVELS

SV2, identified in a monoclonal antibody screen for synaptic vesicle proteins (Buckley & Kelly 1985), is a component of synaptic vesicles and neuroendocrine secretory granules in all vertebrates but is not conserved in invertebrates. Three SV2 genes in vertebrates encode highly homologous proteins referred to as SV2A, SV2B, and SV2C (see Janz & Südhof 1999, and references cited therein). SV2 proteins contain 12 potential transmembrane regions with cytoplasmic N- and C-termini and exhibit significant homology with carbohydrate transport proteins in eukaryotes and bacteria. Most loops connecting the transmembrane regions are short except for one large intravesicular loop that is highly glycosylated and may supply an ionic matrix to the synaptic vesicle interior (Buckley & Kelly 1985, Scranton et al. 1993). Comparisons between SV2 isoforms show that the transmembrane regions and the cytoplasmic loops are highly conserved, whereas the N-terminal cytoplasmic sequence and the intravesicular loops exhibit little homology (Janz & Südhof 1999).

The three SV2 proteins are differentially expressed in brain. SV2A is present in almost all neurons, SV2B exhibits a more restricted distribution (Bajjalieh et al. 1994), and SV2C is present only in a small subset of neurons in the basal forebrain and caudal brain regions (Janz & Südhof 1999). In addition to SV2 proteins, synaptic vesicles contain a distantly related protein called SVOP (Janz et al. 1998). SVOP has a similar transmembrane structure as SV2 but lacks the long glycosylated intravesicular loop. Furthermore, SVOP is highly conserved in invertebrates, whereas SV2 is not (Janz et al. 1998). These differences suggest that SV2 proteins may be evolutionarily late, vertebrate-specific descendants of SVOP.

Initially, investigators thought that SV2A may be a neurotransmitter transporter, but its presence in nerve terminals with distinct neurotransmitters dispelled this idea (Bajjalieh et al. 1994). Analysis of KO mice revealed that SV2B knockout mice are phenotypically unremarkable, whereas SV2A and SV2A/SV2B double knockout mice exhibit severe seizures and die postnatally (Janz et al. 1999a). A similar lethal phenotype was observed in independently generated SV2A KO

mice (Crowder et al. 1999). In electrophysiological recordings from cultured hippocampal neurons, SV2A- or SV2B-deficient cells exhibited no decrease in evoked release or in the size of the readily releasable pool (Janz et al. 1999a). Neurons lacking both SV2 isoforms, however, experienced sustained increases in Ca^{2+}-dependent synaptic transmission when two or more action potentials were triggered in succession. These increases could be reversed by a membrane-permeable EGTA-ester that is hydrolyzed to EGTA in cells where the EGTA then chelates free Ca^{2+} ions. This observation suggests that the deletion of SV2 caused presynaptic Ca^{2+} accumulation during consecutive action potentials (Janz et al. 1999a).

The phenotype of the SV2-deficient synapses indicates that SV2 may be a Ca^{2+} transporter in synaptic vesicles, which is corroborated by the presence of a pair of conserved negatively charged residues in the first transmembrane region (Janz et al. 1998, Janz & Südhof 1999). However, a separate analysis of SV2A-deficient mice came to a very different conclusion (Xu & Bajjalieh 2002). In chromaffin cells, the Ca^{2+}-induced exocytotic burst of chromaffin granule exocytosis, thought to correspond to the readily releasable pool of vesicles, was significantly decreased in mice lacking SV2A, and in brain, detergent-resistant SNARE complexes were reduced. One possible hypothesis to explain the difference in electrophysiological phenotype—a reduction in the readily releasable pool in chromaffin cells but not in synapses—is based on the role of intravesicular Ca^{2+} in stabilizing the chromaffin granule structure (Südhof 1983). A reduction in the Ca^{2+} content of chromaffin granules in the SV2 KO mice could lead to a destabilization of vesicles, which in turn would manifest as a loss of the readily releasable pool of vesicles without actually exhibiting a docking and priming phenotype. The reduction in SNARE complexes in the brain from SV2A KO mice (that still contain SV2B and SV2C) is more difficult to reconcile with the synaptic phenotype and is hard to explain mechanistically. The structure of SV2 is that of a transporter, with few sequences exposed to the cytoplasm. The reported interaction of the N-terminal region of SV2A with synaptotagmin 1 (Schivell et al. 1996) does not help to explain the purported SNARE-related phenotype because the deletion of synaptotagmin 1 does not change SNARE complexes or reduce the readily releasable pool of synaptic vesicles that is dependent on SNARE complexes (Geppert et al. 1994a). Thus it seems likely that SV2 is indeed a transporter, although the transport of Ca^{2+} remains to be demonstrated directly and other cations could potentially be substrates for the transport activity of SV2.

CONTROLLING POOLS VIA SYNAPSINS

Synapsins are abundant synaptic vesicle proteins that coat the vesicle surface as peripheral membrane proteins. Although synapsins were the first synaptic vesicle proteins identified when they were discovered as neuronal substrates for cAMP- and Ca^{2+}/calmodulin-activated kinases (reviewed in Greengard et al. 1994), their precise function remains unclear.

Three synapsin genes in mammals express alternatively spliced protein variants that have similar N-terminal and central domains but distinct C-terminal sequences (Südhof et al. 1989, Hosaka & Südhof 1998a, Kao et al. 1998, Porton et al. 1999). Most synapses express synapsin 1 and 2, whereas synapsin 3 variants are of lesser abundance. All synapsins contain (*a*) a short N-terminal domain (~20 residues) that features a conserved phosphorylation site for PKA and Ca^{2+}/calmodulin-dependent protein kinase I, (*b*) a linker sequence, and (*c*) a large central C domain (~300 residues) that accounts for more than half of the sequence of most synapsins. C-terminally, synapsins are composed of variable, alternatively spliced sequences that in each synapsin gene include one variant with a single conserved domain (the E domain).

The central C domain of synapsins forms a constitutive dimer and binds ATP with a high affinity (Hosaka & Südhof 1998a,b, 1999; Esser et al. 1998). The crystal structure of the C domain of synapsin 1 revealed a striking similarity to a family of ATPases (Esser et al. 1998). This family includes glutathione synthetase and D-alanine:D-alanine ligase, which suggests that synapsins may be ATP-utilizing enzymes. The ATP-binding domains of different synapsins exhibit distinct properties: Synapsin 1 binds ATP only in the presence of Ca^{2+}, synapsin 2 binds ATP irrespective of Ca^{2+}, and synapsin 3 binds ATP only in the absence of Ca^{2+} (Hosaka & Südhof 1998a,b).

Several mechanisms of binding synapsins to synaptic vesicles were proposed. Originally, investigators thought that synapsin 1 binds to vesicles via Ca^{2+}/calmodulin-dependent protein kinase II (Greengard et al. 1994). However, synapsin 2 does not bind to this kinase but is found on vesicles, and Ca^{2+}/calmodulin-dependent kinase II is not a regular component of synaptic vesicles. Transgenic experiments demonstrated that the N-terminal domains of synapsin 1 are sufficient for synaptic vesicle targeting (Geppert et al. 1994c). Synapsins avidly bind to lipid surfaces (Benfenati et al. 1989) via their short N-terminal phosphorylated domain (Hosaka et al. 1999). The N-terminal domain is phosphorylated by PKA and Ca^{2+}/calmodulin-dependent protein kinase I, and phosphorylation abolishes binding of synapsins to synaptic vesicles. As a result, synapsins cycle on and off vesicles in a stimulation-dependent manner (Hosaka et al. 1999). However, whether synapsins cycle on and off vesicles normally during exo- and endocytosis, or only upon extensive stimulation, is unclear. Some studies observed a depletion of synapsins from synaptic vesicles as the vesicles became docked (Pieribone et al. 1995), whereas others demonstrated that synapsins remain associated with the vesicles during exo- and endocytosis (Torri-Tarelli et al. 1990).

Analysis of KO mice showed that synapsins 1 and 2 are not essential for mouse survival or for synaptic vesicle exocytosis but are required to maintain normal numbers of synaptic vesicles and to regulate short-term synaptic plasticity (Rosahl et al. 1993, 1995; Li et al. 1995b). Deletion of synapsins decreased the number of vesicles without altering vesicle clustering or docking (Rosahl et al. 1995). The synapsin 2 KO phenotype was more severe than was the synapsin 1 KO phenotype, and the double KO exhibited the biggest changes, including a 50%

decrease in vesicle numbers (Rosahl et al. 1995). The loss of synaptic vesicles in the synapsins KO mice indicates a maintenance function of synapsins, possibly via an ATP-dependent activity. Deletion of synapsins also caused dramatic changes in short-term synaptic plasticity (Rosahl et al. 1993, 1995). Short-term plasticity involves changes in release when two or several action potentials are triggered within milliseconds. The short time frame of short-term synaptic plasticity suggests that this type of plasticity regulates the interaction of synaptic vesicles with the active zone. By inference, synapsins thus may regulate this interaction, but the mechanism of such regulation and its relation to the maintenance function of synapsins remains unclear. In addition, deletion of synapsins was found to delay axonal outgrowth and/or synaptogenesis of cultured neurons (e.g., see Ferreira et al. 1998). However, double KO mice lacking both synapsins exhibited no changes in the density of synapses and the appropriateness of synaptic connections (Rosahl et al. 1995). Synapsin 3 KO mice have also been analyzed, but only in isolation without crossing to synapsin 1 and 2 KO mice (Feng et al. 2002). These mice exhibited discrete changes, for example a 5% increase in synaptic vesicle density and a change in evoked GABAergic synaptic responses. The observation of distinct effects by deletion of various synapsins is corroborated by the differential regulation of ATP binding to synapsins by Ca^{2+}, which either stimulates ATP binding (synapsin 1), has no effect on ATP binding (synapsin 2), or inhibits ATP binding (synapsin 3).

What do synapsins actually do? Investigators have reported innumerable in vitro binding activities. Most prominently, synapsins bind to various elements of the cytoskeleton, especially actin, leading to the hypothesis that synapsins may anchor synaptic vesicles in the presynaptic vesicle cluster to the cytoskeleton (reviewed in Greengard 1994). However, with the realization that the mature vesicle cluster contains virtually no cytoskeleton (Dunaevsky & Connor 2000, Morales et al. 2000, Zhang & Benson 2001), this hypothesis is unlikely to explain synapsin function. At present there is a gap between the structural definition of synapsins as ATP-binding molecules and their functional role in maintaining vesicle integrity and regulating release. Key questions are whether ATP binding to synapsins is functionally important, whether synapsin phosphorylation plays a physiological role, and at what point exactly synapsins work in the synaptic vesicle cycle. Addressing these questions will provide crucial advances in understanding these enigmatic proteins.

PERSPECTIVE

In this review, I attempt to evaluate how well the molecular characterization of synaptic vesicle proteins and their interaction partners can account for the physiological properties of the vesicle cycle. Although significant progress has been made (e.g., in the definition of synaptotagmin 1 as the Ca^{2+} sensor for fast release), more progress will be required until investigators achieve a reasonable mechanistic understanding of the cycle. The gap between the physiological description and the molecular understanding of the vesicle cycle is immense. Two principal problems

make it difficult to close this gap. First, much of the physiological description is protocol dependent, and does not measure parameters that correspond to a unitary molecular event. One reason why our understanding of Ca^{2+} triggering of release is so advanced is that the physiological description of Ca^{2+} triggering is very precise. If one compares Ca^{2+} triggering, for example, with endocytosis and recycling where different protocols measure distinct time constants, it is clear that defining the molecular basis for endocytosis and recycling will be more difficult. Second, most proteins function in interaction networks that are difficult to define and validate, and many proteins participate in more than one function. Unraveling these protein networks and overlapping functions will be a major challenge, but it will be necessary to achieve a complete molecular understanding. In the molecular mosaic that composes the nerve terminal, many pieces do not yet have an appropriate fit—e.g., the vesicle proteins SVOP or synaptogyrin—and many more pieces are still missing. Nevertheless, the presynaptic nerve terminal promises to be one of the best systems to explore the relationship between the structure of a protein, its biochemical and cell-biological properties, and its physiological role.

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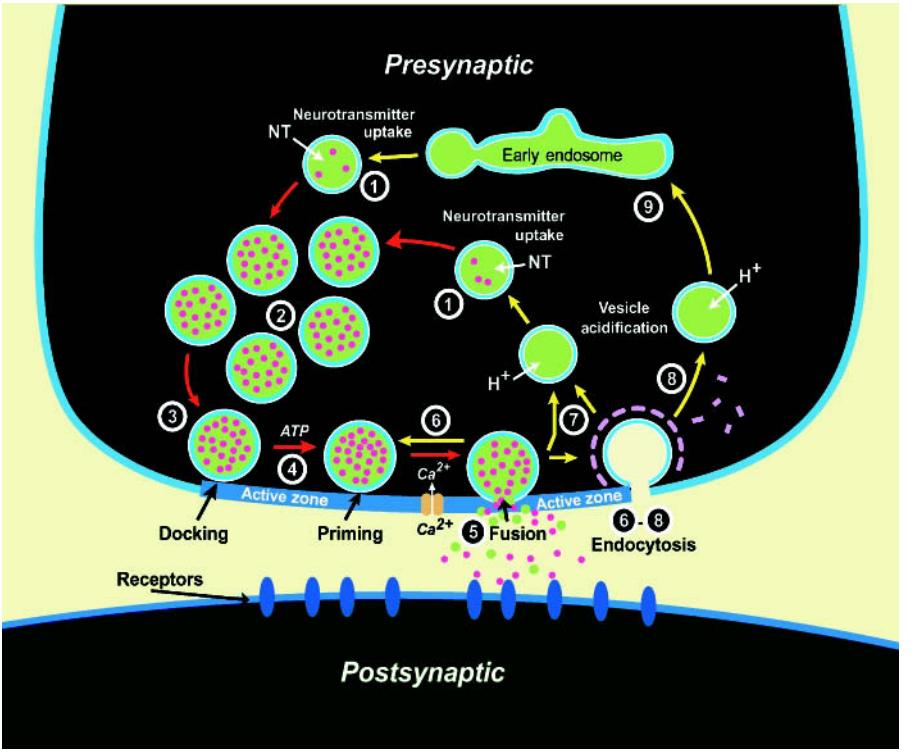

Figure 1 The synaptic vesicle cycle. Synaptic vesicles are filled with neurotransmitters by active transport (step 1) and form the vesicle cluster that may represent the reserve pool (step 2). Filled vesicles dock at the active zone (step 3), where they undergo a priming reaction (step 4) that makes them competent for Ca^{2+} triggered fusion-pore opening (step 5). After fusion-pore opening, synaptic vesicles undergo endocytosis and recycle via several routes: local reuse (step 6), fast recycling without an endosomal intermediate (step 7), or clathrin-mediated endocytosis (step 8) with recycling via endosomes (step 9). Steps in exocytosis are indicated by red arrows and steps in endocytosis and recycling by yellow arrows.

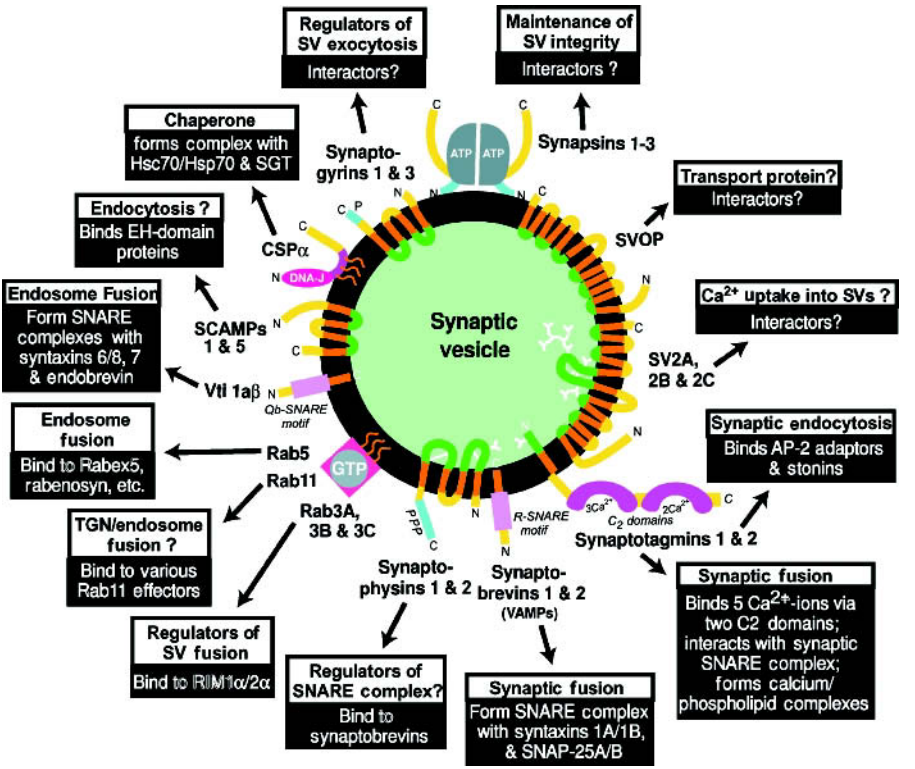

Figure 4 Structures, proposed interactions, and putative functions of synaptic vesicle trafficking proteins. Proteins are shown schematically (*green*, intravesicular sequences; *orange*, transmembrane regions; *blue*, phosphorylation domains; *pink*, SNARE motifs; *red* and *gray*, folded domains; *yellow*, other sequences). The white connecting lines in the intravesicular space identify disulfide bonds, and the branched white lines indicate sugar residues. In the boxes corresponding to the individual proteins, proposed functions are shown on a white background and purported interactions on a black background.

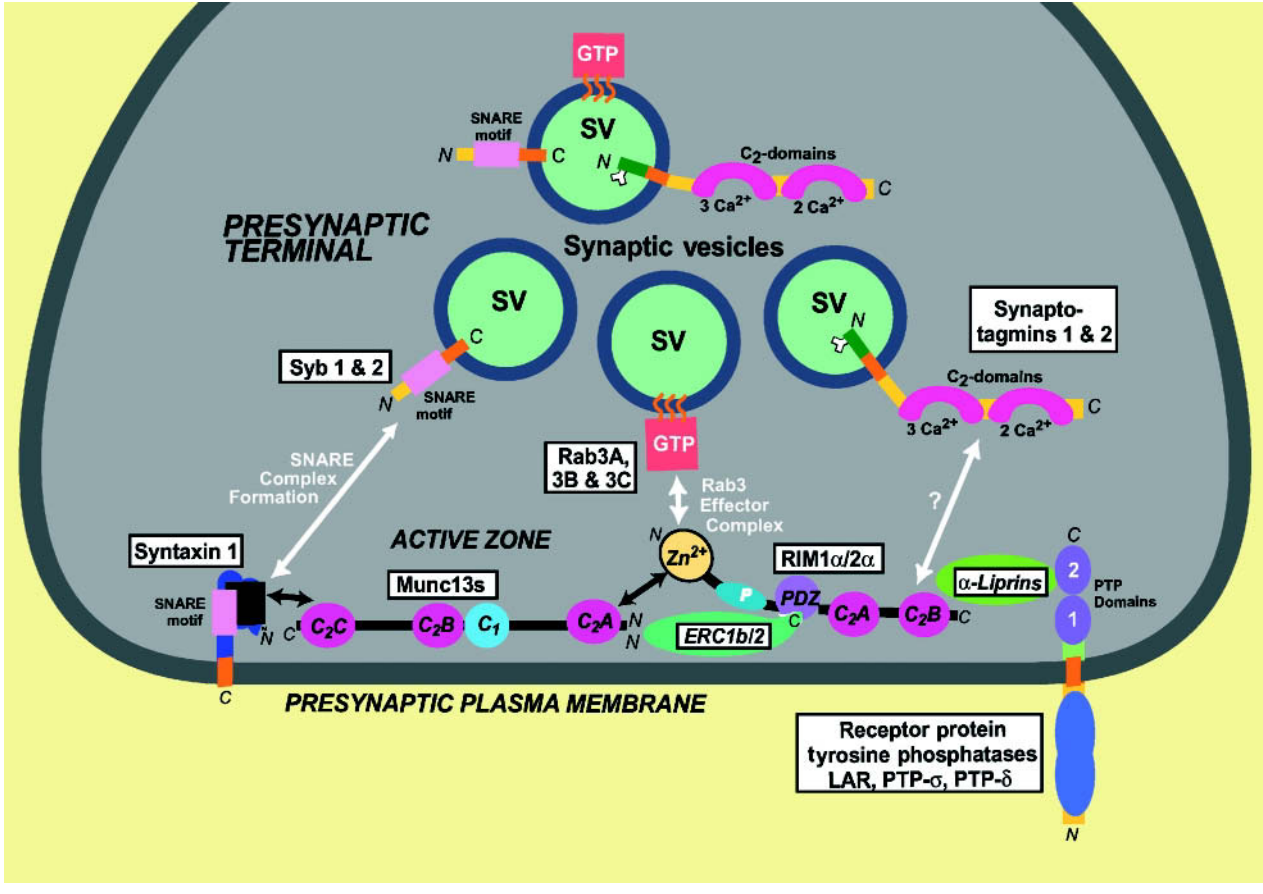

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Figure 5 Protein-protein interactions at the active zone: putative mechanisms of synaptic vesicle attachment. The active zone includes, among others, an interaction of the SNARE protein syntaxin 1 with the active zone component Munc13-1 (*left*), a direct binding of the N-terminal domain of Munc13-1 to the N-terminal zinc-finger of RIM1α/2α (*center*), and additional interactions of RIM1α/2α with the coiled-coil proteins ERC1a and ERC2 (*center*) and with α-liprins that in turn bind to receptor-tyrosine phosphatases (*right*). Two direct connections of synaptic vesicles with the active zone are established: binding of the vesicle SNARE synaptobrevin to syntaxin and SNAP-25 (*not shown*), and binding of the vesicle Rab proteins Rab3A, 3B, 3C, and 3D to the same N-terminal domain of RIM1α and RIM2α that also binds to Munc13-1. In addition, an interaction of the C_2 domains of synaptotagmins with the C_2B domain of RIMs has been observed but is not yet validated (*question mark*).

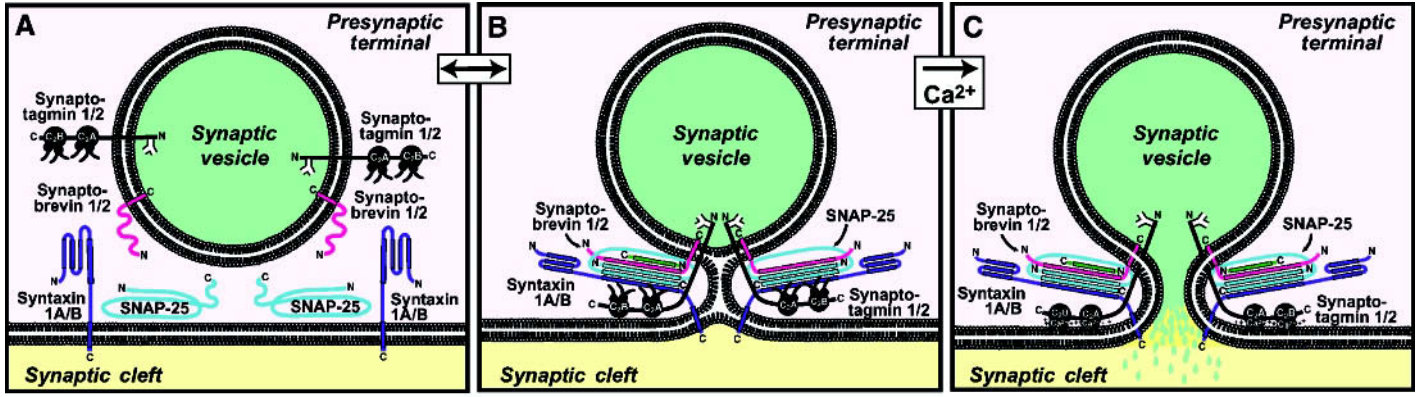

Figure 6 Model for the functions of SNARE proteins, complexins, and synaptotagmins 1 and 2 in synaptic vesicle exocytosis. In docked vesicles (*panel A*), SNAREs and synaptotagmins are not engaged in direct interactions. During priming (*panel B*), SNARE complexes form, complexins (*green*) are bound to fully assembled complexes, and synaptotagmins constitutively associate with the assembled SNARE complexes. The synaptic vesicle membrane and plasma membranes are forced into close proximity by SNARE complex assembly, which results in an unstable intermediate that is shown as a speculative fusion stalk. Ca^{2+} influx (*panel C*) further destabilizes the fusion intermediate by triggering the C_2 domains of synaptotagmin to partially insert into the phospholipids. This action is proposed to cause a mechanical perturbation that opens the fusion pore. Note that the nature and stability of the putative fusion intermediate is unclear and that SNARE complex assembly in panel B is suggested to be reversible, whereas Ca^{2+} triggering is not.

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