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Molecular Mechanisms for Synchronous, Asynchronous, and Spontaneous Neurotransmitter Release

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Abstract

Most neuronal communication relies upon the synchronous release of neurotransmitters, which occurs through synaptic vesicle exocytosis triggered by action potential invasion of a presynaptic bouton. However, neurotransmitters are also released asynchronously with a longer, variable delay following an action potential or spontaneously in the absence of action potentials. A compelling body of research has identified roles and mechanisms for synchronous release, but asynchronous release and spontaneous release are less well understood. In this review, we analyze how the mechanisms of the three release modes overlap and what molecular pathways underlie asynchronous and spontaneous release. We conclude that the modes of release have key fusion processes in common but may differ in the source of and necessity for Ca^{2+} to trigger release and in the identity of the Ca^{2+} sensor for release.

Keywords

synaptic vesicles; exocytosis; calcium; SNARE complex; presynaptic active zone

INTRODUCTION

There are three primary modes of neurotransmitter release: Synchronous release occurs within several milliseconds after an action potential invades a presynaptic bouton, asynchronous release persists for tens of milliseconds to tens of seconds after an action potential or series of action potentials, and spontaneous neurotransmitter release occurs in the absence of presynaptic depolarization (Figure 1). Here we discuss the mechanisms underlying these forms of release, the extent to which these different modes of release are similar, and the degree to which they are specialized.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

NOTE ADDED IN PROOF

A recent study found that synaptotagmin 7 acts as a sensor for asynchronous release in the absence of the fast Ca^{2+} sensor synaptotagmin 1 in cultured hippocampal neurons. Ca^{2+} binding to synaptotagmin 7 is necessary for this form of asynchronous release.

SYNCHRONOUS RELEASE

Key Features of Synchronous Release

A hallmark of neuronal communication is its speed, which requires fast synaptic transmission. When an action potential invades a presynaptic bouton, voltage-gated Ca^{2+} channels open briefly, which results in a sharp, local rise of the intraterminal Ca^{2+}. Ca^{2+} then binds to a Ca^{2+} sensor to induce fusion of synaptic vesicles with the presynaptic plasma membrane. At most synapses, vesicles fuse after a delay of less than a millisecond, leading to fast release of neurotransmitters (1–4).

Synchronous release is remarkably temporally precise, with most vesicles fusing within hundreds of microseconds (1, 2, 5) (Figure 2). To achieve such a high degree of synchrony, Ca^{2+} channels must open only very briefly. This is the case because Ca^{2+} channels deactivate and close very quickly following action potential repolarization (4, 6). Synchronous release is driven by the high local Ca^{2+} concentration near open Ca^{2+} channels (Ca_{local}), and the time course of this signal is closely approximated by the time course of the Ca^{2+} current. Prolongation of action potential repolarization in turn prolongs Ca^{2+} entry and reduces the synchrony of release (7, 8). Another important factor for release synchrony is that the molecular machinery must have fast kinetics that allow fusion to turn on rapidly in response to Ca^{2+} increases and to terminate abruptly when Ca_{local} drops.

Synchronous release has been extensively characterized, and there is broad agreement about many aspects of the molecular mechanisms. Like all eukaryotic exocytotic pathways, synchronous release requires SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins and Sec-1/Munc18 (S/M) proteins for fusion of synaptic vesicles with the presynaptic plasma membrane (Figure 3). SNARE proteins, localized on the plasma membrane and the synaptic vesicle, form a tight complex with their α-helical SNARE motifs, bridging the membranes to fuse. The predominant presynaptic SNARE proteins are syntaxin-1 and SNAP-25 on the plasma membrane and synaptobrevin 2 (also termed VAMP2) on the synaptic vesicle. When they zipper their α-helical SNARE motifs into a SNARE complex between two membranes, they release energy, and this energy may be used to force the membranes close together, enabling them to fuse (9–14). Munc18-1 is the main S/M protein in vertebrate synapses, it is required for neurotransmitter release, and it engages in multiple interactions with the plasma membrane SNARE syntaxin-1 and with SNARE complexes. Through these interactions, it controls SNARE complex assembly during exocytosis (15–20). Excellent recent reviews provide detailed insight into these essential mechanisms for neurotransmitter release (21, 22). Here we focus on specializations that synchronize release and on aspects of synchronous release that provide insight into asynchronous and spontaneous release.

Four mechanisms are critical for synchronous release (Figure 3). First, a nerve terminal needs to generate and maintain a pool of readily releasable vesicles that can be quickly exocytosed upon Ca^{2+} entry. Second, presynaptic voltage-gated Ca^{2+} channels have to open briefly, but with minimal delay upon arrival of the action potential to trigger synchronous release. Third, the release machinery must contain a mechanism to quickly respond to sharp, action potential–gated Ca^{2+} signals, and the same machinery must also quickly respond to

the sharp decay of the Ca^{2+} signal. Fourth, the presynaptic Ca^{2+} channel has to be spatially coupled to the Ca^{2+}-sensing mechanism so that Ca^{2+} increases and decreases quickly at the sensor when Ca^{2+} channels open and close, respectively. In combination, these four cellular mechanisms produce a release apparatus that enables synchronous release when an action potential invades the nerve terminal.

Generation of a Readily Releasable Pool of Vesicles

In a presynaptic nerve terminal, only a small percentage of the vesicles, typically less than 5%, are releasable upon arrival of the action potential, and they belong to the readily releasable pool (RRP) of vesicles (23, 24). The RRP is determined by using stimuli that deplete it, such as short stimulus trains (25, 26), brief application of hypertonic sucrose (27), or a strong presynaptic depolarization (28, 29). These methods release vesicles from the same pool, as pool depletion with one method depletes a pool stimulated by another method (27, 30). Some studies suggest that the RRP is composed of vesicles that are docked and primed for release at the presynaptic active zone (31), whereas other studies find that the vesicles making up the RRP are distributed throughout the vesicle cluster within the presynaptic bouton rather than being localized to the release site (32).

Two classes of molecules have proven to be critical for generating an RRP: active zone proteins for synaptic vesicle docking and priming, and SNARE complexes and molecules that guide their assembly. Proteins at the presynaptic active zone are critical to generating an RRP. Active zones consist of at least six families of multidomain proteins, including Munc13, Rab3-interacting molecule (RIM), RIM-binding protein (RIM-BP), liprin-α, ELKS (named for its high content in the amino acids E, L, K, and S), and piccolo/bassoon (Figure 3). Genetic removal of a prominent active zone protein, Munc13, results in a near complete loss of the RRP (33). Munc13s operate during priming via their MUN domain. The MUN domain interacts with SNARE complexes (34, 35), and it activates priming most likely by opening syntaxin-1 for SNARE complex assembly (19, 36, 37). Munc13s, however, are unable to promote priming on their own, because they are inhibited by homodimerization of their N-terminal C_2 domain (38, 39, 40). RIM proteins, scaffolding molecules at the active zone, recruit Munc13 to the active zone and activate it by breaking up the Munc13 homodimer, which may expose the MUN domain to assembling SNARE complexes (38–42). This model explains the necessity of RIM for efficient vesicle priming (43–47). RIMs also function in generating the RRP by docking synaptic vesicles via Rab3 (45, 47, 48). Additional mechanisms must be involved in the docking and priming of synaptic vesicles, because genetic removal of RIMs does not eliminate the entire RRP. Synaptotagmin and CAPS (Ca^{2+}-dependent activator protein for secretion) may be involved (49, 50), but the molecular mechanisms in neurons are not well understood.

SNARE complex assembly itself is a second critical step for vesicles in the RRP (21, 22). Experimental evidence suggests that an RRP vesicle may be arrested in a fusion-ready state with a partially zippered SNARE complex (51–55). In this model, complexin, a small, soluble protein, inhibits SNARE complex zippering by preventing synaptobrevin 2 from incorporating its complete SNARE motif into the SNARE complex (55). This clamp is relieved by Ca^{2+} binding to synaptotagmin 1 (Syt1), possibly via a direct SNARE-Syt1

interaction. Analysis of SNARE complex biochemistry, however, suggests that it may be energetically challenging to halt SNARE assembly in a partially zippered state (56, 57), which recently led to an alternative model: SNARE zippering proceeds after Ca^{2+} triggering (22), and a partially zippered, arrested SNARE complex is thus not needed to generate an RRP vesicle. Whichever model turns out to be correct, SNARE complex assembly is undoubtedly critical for synchronous release, as genetic removal of SNAREs strongly impairs action potential–triggered synaptic vesicle exocytosis (58–61).

Ca^{2+} Sensors for Synchronous Release

The properties of synchronous release indicate that the Ca^{2+} sensor responds rapidly to Ca^{2+}; has a low affinity for Ca^{2+}; has a fast off-rate once the Ca^{2+} signal is terminated; and is steeply dependent on Ca^{2+} entry, typically to the third to fifth power (63, 64). Syt1, −2, and −9 satisfy all these criteria and have been identified as the fast Ca^{2+} sensors that mediate synchronous release at different synapses (65–69). For the purpose of this review, we refer to them as fast synaptotagmins. Syt1 was the first isoform identified as a fast Ca^{2+} sensor, and it has been the most extensively studied. It is composed of a transmembrane domain, a linker sequence, and two C-terminal C_2 domains (70). C_2 domains are universal Ca^{2+}-binding modules, and they also bind to membrane phospholipids and SNARE complexes. The observations that abolishing Syt1 in flies, mice, or worms impairs synchronous release (65, 71–73) and causes a shift to asynchronous release (65) established the importance of Syt1 in synchronous release. Furthermore, subtle mutations in Syt1 that change Ca^{2+}-dependent phospholipid binding in vitro also altered vesicular release probability, thereby establishing Syt1 as a Ca^{2+}-dependent switch to activate fusion (66, 74).

Several partially overlapping mechanisms have been proposed for how Syt1, −2, and −9 trigger fusion. In the first two proposed models, a clamp that depends on synaptotagmin-SNARE interactions halts SNARE complex assembly. Synaptotagmin may clamp SNARE complexes directly prior to fusion; in this model Ca^{2+} binding to synaptotagmin relieves this clamp to induce SNARE zippering (75, 76). Alternatively, complexin acts as the SNARE clamp, and synaptotagmin activates fusion by relieving the complexin clamp from SNARE complexes (51, 53, 77), a model that is more compatible with the Ca^{2+} dependence of the synaptotagmin C_2-domain interactions with the SNARE complex (77–79). Two distinct mechanisms were proposed during which membrane binding of synaptotagmin predominates. Fusion may be triggered by Ca^{2+}-induced phospholipid binding of synaptotagmin. This binding may lower the energy barrier for fusion by reducing the distance between the membranes to fuse (80–82) and may also support or induce fusion by curving membranes at release sites (83, 84). Further studies are needed to determine how each of these mechanisms contributes to synchronous release.

Ca^{2+} Channels and Their Spatial Coupling to Ca^{2+} Sensors

Fast triggering of vesicle exocytosis requires a close spatial relationship between the Ca^{2+} sensor and the source of Ca^{2+}, the voltage-gated Ca^{2+} channels (64, 85, 86). When an action potential invades the terminal, Ca^{2+} levels at the Ca^{2+} sensor rise quickly from 0.1 μM to 30–100 μM (64, 87, 88). To quickly achieve such a high Ca^{2+} concentration at the Ca^{2+} sensor, a release site is organized to create a spatial domain of high intracellular Ca^{2+}.

Within this domain, Ca^{2+} channels are closely associated with the vesicular Ca^{2+} sensor (89). At some synapses, this is achieved in a Ca^{2+} domain that spans less than 100 nm, termed a nanodomain, by tight molecular coupling of one or two Ca^{2+} channels to a vesicle (90). At other synapses, release is driven by cooperative action of Ca^{2+} flowing into the terminal through many Ca^{2+} channels in a larger area termed a microdomain (91). The spatial organization of Ca^{2+} channels and sensors is also regulated during development, as exemplified by a developmental transition from microdomain to nanodomain coupling in the calyx of Held (92).

How does a synapse organize Ca^{2+} micro- and nanodomains? Tomography of the frog neuromuscular junction (NMJ) suggested that a protein scaffold tethers Ca^{2+} channels and vesicles in close proximity (93). The identity of the molecules within this scaffold is under intense investigation. Several potential molecular mechanisms organizing spatial Ca^{2+} domains have recently been identified (47, 94–96). RIM proteins take a central role in tethering Ca^{2+} channels to presynaptic release sites (Figure 3). One mechanism operates via central and C-terminal RIM domains, which form a tripartite complex with RIM-binding proteins and Ca^{2+} channels (47, 97). In addition, RIM N-terminal sequences bind to the synaptic vesicle protein Rab3 and to the priming molecule Munc13 (39, 41, 98). In hippocampal synapses and in the calyx of Held, these interactions tether vesicles close to Ca^{2+} channels, thereby promoting rapid, synchronous release (40, 45, 47). However, genetic ablation of presynaptic RIMs only partially abolishes Ca^{2+}-channel tethering at active zones (45, 47), suggesting that additional parallel mechanisms operate. Further candidate molecules that may promote Ca^{2+}-channel clustering are the *Drosophila* protein bruchpilot (brp) and its vertebrate homolog ELKS (95, 99), SNARE proteins (100, 101), the active zone protein bassoon (102), and presynaptic neurexins (103), but their contributions and mechanisms are less well understood.

In summary, synchronous release depends on a transmitter release apparatus that appears largely conserved among different neurons. The critical factors for synchrony are the availability of an RRP, the tight spatial organization of a release site containing a fast Ca^{2+} sensor close to presynaptic Ca^{2+} channels, and a Ca^{2+} signal at the Ca^{2+} sensor that increases and decreases quickly.

ASYNCHRONOUS RELEASE

Although most studies on synaptic transmission have focused on the synchronous component of release, there is often also an asynchronous component that in some cases can be quite large. At most synapses, synchronous release accounts for almost all (>90%) release at low-frequency stimulation (104–107). However, asynchronous release is prominent at specialized synapses, such as synapses from cholecystokinin (CCK) interneurons (108), glutamatergic synapses onto magno-cellular neurosecretory cells in the hypothalamus (109), dorsal horn synapses (110), and synapses from deep cerebellar nuclei (DCN) to the inferior olive (IO) (111). The DCN→IO synapse is the most extreme example, with essentially all release being asynchronous (>90%).

The pattern of presynaptic activation can profoundly influence the properties of release. At some synapses, asynchronous release is apparent even after a single stimulus (Figure 1*a*). For most synapses, however, low-frequency stimulation evokes primarily synchronous release, but sustained moderate- to high-frequency stimulation additionally evokes asynchronous release (Figure 4*a*). The magnitude of asynchronous release evoked by repetitive stimulation often reveals a steep frequency dependence, which may be tuned to the range of firing frequencies experienced by a synapse in vivo. For DCN→IO synapses, presynaptic cells fire at tens of hertz, and asynchronous release dominates at 20 Hz and above (111). Sustained activation can also lead to asynchronous release that persists long after all presynaptic firing has ceased, and asynchronous release often persists for much longer following a burst of presynaptic activity than following a single stimulus (Figure 4*b*). Repetitive presynaptic activation often leads to asynchronous release that consists of different temporal components (112, 113), as at the frog NMJ, where four kinetic components of release decay with time constants of 50 ms, 500 ms, 7 s, and 80 s (107). Although complicated Ca^{2+} dynamics may allow a single mechanism to mediate asynchronous release on different timescales, each of these temporal components of release may correspond to a different mechanism of asynchronous release.

Physiological Relevance of Asynchronous Release

Numerous roles for asynchronous release have been proposed. In the cochlear nucleus and in the IO, asynchronous γ-aminobutyric acid (GABA) release from inhibitory inputs during high-frequency activation provides a smooth and graded inhibition that is insensitive to the precise timing of individual action potentials (111, 114). In the hippocampus, asynchronous release is prominent at synapses made by CCK interneurons, whereas synchronous release dominates release from parvalbumin-positive interneurons (115–118). This suggests that CCK interneurons are specialized to provide prolonged inhibition (117). In the cortex, high-frequency presynaptic activation of fast-spiking interneurons produces asynchronous release that lasts several seconds and that may prevent widespread synchronous firing and suppress epileptiform activity (119).

Multiple roles for asynchronous release have also been proposed at excitatory synapses. Asynchronous release can be so prominent that synaptic activation of excitatory inputs can elevate firing in postsynaptic cells for hundreds of milliseconds following brief bursts of presynaptic activity (109). This late postsynaptic spiking triggered by asynchronous release may be highly effective at activating postsynaptic Ca^{2+} channels and *N*-methyl-D-aspartate receptors (NMDARs), thereby producing strong dendritic Ca^{2+} signals capable of evoking peptide release from dendrites. The powerful climbing fiber→Purkinje cell (PC) synapse triggers a complex spike that evokes multiple action potentials that propagate down PC axons; it is thought that desynchronization of vesicle fusion is more effective at triggering multiple action potentials in PC axons (120).

Another possible role for asynchronous release is coincidence detection. The acuity of coincidence detection depends on the time course of excitatory postsynaptic potentials (EPSPs) (121), which in turn depends on the degree of synchrony of release. The observation that at the calyx of Held single stimuli evoke prominent asynchronous release in

young animals but primarily synchronous release in more mature animals (122) raises the possibility that asynchrony allows broadly tuned coincidence detection in young animals, whereas narrowly tuned coincidence detection dominates for mature synapses.

Another possibility is that asynchronous release contributes to overall synaptic transmission by simply elevating neurotransmitter release. This elevation may cause neurotransmitter to pool and spread to activate high-affinity receptors, but in a manner relatively ineffective at triggering spiking. Thus, a presynaptic spike train may provide two types of signals: synchronous release more effectively evoking spiking and asynchronous release preferentially activating high-affinity metabotropic or ionotropic receptors. The finding that synchronous release and asynchronous release can be differentially modulated (116) raises the possibility that these different phases of release and their influence on postsynaptic cells can be independently regulated.

Finally, in mouse models for degenerative disorders of the nervous system, such as spinal muscular atrophy (123) and Alzheimer's disease (124), a shift from synchronous to asynchronous release during stimulation was observed. Asynchronous release from interneurons is also elevated in epileptiform tissue of rats and humans (125). These studies suggest asynchronous release may become more prominent in pathological conditions.

Studying Asynchronous Release

Numerous methods are employed to study asynchronous release, but for each method several factors complicate the interpretation. For example, asynchronous release is often studied by determining average synaptic currents and then attributing long-lasting components of this current to asynchronous release (Figure 4*c*). However, average synaptic currents may not provide a good quantitative estimate of the time course of release because of neurotransmitter accumulation, spillover, receptor saturation and desensitization, and activation of high-affinity receptors (126–130). For these reasons, it is preferable to quantify asynchronous release by detecting quantal events and using multiple trials to construct a histogram in order to identify a component of release corresponding to asynchronous release (Figure 4*d*). It is also important to avoid experimental conditions in which intrinsic neuronal firing properties or reverberating circuit activity produces the appearance of asynchronous release by allowing a single stimulus to trigger a burst of firing or a train of activity to evoke long-lasting spiking in presynaptic cells.

An additional complication is that synchronous release and asynchronous release may draw from the same pool of vesicles. The properties of synchronous and asynchronous release during action potential trains—in the presence of Sr^{2+} or EGTA to manipulate the contribution of asynchronous release (131–133) or upon an increase in asynchronous release by preventing Ca^{2+} uptake by mitochondria (134)—led researchers to conclude that synchronous release and asynchronous release share the same pool of vesicles. In all these studies, the basic finding was that the amplitudes of synchronous and asynchronous release are negatively correlated. This effect is also observed when synchronous release is abolished by removal of its Ca^{2+} sensor, Syt1, which results in an increase in the magnitude of asynchronous release (65, 135). Likewise, enhanced asynchronous release was observed in complexin knockout mice (136), suggesting that complexin may suppress asynchronous

release, possibly by enhancing synchronous release (Table 1). Thus, fast synaptotagmins and complexin suppress asynchronous release, but this suppression may, at least in part, reflect competition for the same pool of vesicles.

Inhibiting dynamin and increasing presynaptic activity produced similar reductionsinboth synchronous release and asynchronous release (quantified by using average synaptic current rather than event detection), which indicates that both rely on dynamin-dependent recycling, consistent with these modes of release sharing a vesicle pool (137). If synchronous release and asynchronous release compete for vesicles from the same pool, then manipulations that enhance synchronous release deplete the vesicle pool available for asynchronous release and indirectly reduce its magnitude. Moreover, both forms of release are Ca^{2+} dependent, which may explain the apparent contradiction that at the calyx of Held, Ca^{2+}-channel antagonists increase the magnitude of asynchronous release (138). Ca^{2+}-channel blockade may increase asynchronous release because fewer vesicles are released synchronously.

The Dependence of Asynchronous Release on Intracellular Ca^{2+}

Ca^{2+} triggers asynchronous release, but there are important differences between these mechanisms and those of synchronous release. Introduction of the slow Ca^{2+} chelator EGTA into a presynaptic terminal eliminates asynchronous release and has minimal effects on synchronous release (104, 139, 140). This finding suggests that the Ca^{2+} sensor for asynchronous release is further from the source of Ca^{2+} compared with the fast Ca^{2+} sensor, likely responding to bulk cytosolic Ca^{2+} rather than to high local Ca^{2+}.

Studies of the Ca^{2+} dependence of vesicle fusion suggest that a specialized Ca^{2+} sensor mediates asynchronous release. At the crayfish NMJ, a linear relationship between the frequency of quantal events and presynaptic Ca^{2+} levels was revealed when the Ca^{2+} concentration was less than 600 nM; a much steeper dependence on Ca^{2+} was found for higher levels of Ca^{2+} (141). At cerebellar granule cell synapses, a component of asynchronous release lasting hundreds of milliseconds was linearly dependent on Ca^{2+}, and a component lasting tens of milliseconds was more steeply dependent on Ca^{2+} (104). These findings suggest that asynchronous release is mediated by a specialized Ca^{2+} sensor with a linear dependence on Ca^{2+}. Subsequently, Ca^{2+} photolysis was used to determine the Ca^{2+} dependence of asynchronous release at the calyx of Held (Figure 5) (67). At this synapse, Syt2 is the fast Ca^{2+} sensor, and asynchronous release was studied in Syt2 knockout mice. At Syt2 knockout calyces, the remaining release was predominantly asynchronous. The unknown Ca^{2+} sensor for this release had a surprisingly low affinity comparable to that of Syt2 (both affinities were ~40 μM), but the Ca^{2+} cooperativity n for the asynchronous component was 2, lower than for release mediated by Syt2 ($n = 5$). Similar experiments in autaptic hippocampal neurons found that the Ca^{2+} dependence of glutamate release is steep ($n \sim 3$), but in Syt1 knockout mice vesicle fusion is approximately linearly dependent on Ca^{2+} ($n \sim 0.9$) (142). Together, these studies suggest that a specialized Ca^{2+} sensor mediates asynchronous release and that this sensor is less steeply dependent on Ca^{2+} with a cooperativity of 1–2. This sensor mediates vesicle fusion when presynaptic Ca^{2+} levels are less than ~0.5 μM in the presence of the fast sensor. A model of release with multiple Ca^{2+}

sensors successfully accounts for asynchronous release at the crayfish NMJ (143). Intense efforts aim at determining the Ca^{2+} source(s) and sensor(s) for asynchronous release.

The observation that one isoform of synaptotagmin, Syt7, has slow kinetics led to the hypothesis that Syt7 mediates asynchronous release (144). This hypothesis was supported by studies using morpholino knockdown at the zebrafish NMJ. At this synapse, high-frequency stimulation evoked synchronous release early, but release was progressively desynchronized later in the train (145) (as in Figure 3*a*). The knockdown of Syt7 selectively reduced asynchronous release, whereas the knockdown of Syt2 selectively reduced synchronous release (145). It is important to determine whether Syt7's role in asynchronous release at this synapse is as a Ca^{2+} sensor, i.e., whether its function in asynchronous release depends on Ca^{2+} binding to the Syt7 C2 domains. Although these experimental findings suggest that Syt7 can play an important role in asynchronous release, Syt7 is not its sole mediator. At inhibitory cortical synapses, asynchronous release, quantified as an average tail current following a brief train, was unaltered in Syt7 knockout mice (144).

Doc2 proteins are also candidates for the Ca^{2+} sensor that mediates asynchronous release, but the role of Doc2 remains unclear. Doc2 proteins are similar to synaptotagmin in that they have two conserved C2 domains, but Doc2 is a soluble, cytosolic protein (146, 147), whereas fast synaptotagmins are attached to synaptic vesicles by a transmembrane domain. One group found that the in vitro properties of Doc2 are consistent with its potential functions as a slow Ca^{2+} sensor and that asynchronous release is increased or decreased when Doc2 levels are, respectively, upregulated or downregulated (148). However, Ca^{2+} binding to Doc2 was not shown to mediate asynchronous release, and how the kinetic changes in the time course of synaptic currents relate to asynchronous release is not clear. Furthermore, two other groups provide compelling evidence that asynchronous release is unaffected by reducing Doc2 levels (149, 150). Consequently, the hypothesis that Doc2 acts as a Ca^{2+} sensor to mediate asynchronous release is highly controversial.

Regulation of presynaptic Ca^{2+} signaling may provide an alternative means of regulating asynchronous release while employing the fusion machinery of synchronous release. For example, as a result of differences in presynaptic Ca^{2+} regulation due to specialized endogenous Ca^{2+} buffers and extrusion mechanisms, synapses with prominent asynchronous release may have a long-lasting presynaptic Ca^{2+} signal following presynaptic activation. It is also possible that the decay of presynaptic Ca^{2+} is greatly prolonged following sustained presynaptic activity, allowing short-lived and long-lasting asynchronous release. Measurements of presynaptic Ca^{2+} signaling corresponding to asynchronous release can provide important insight into these possibilities. Because alterations in presynaptic Ca^{2+} signaling lead to changes in asynchronous release, proteins implicated in it, such as RIM1α (43), likely influence asynchronous release through changes in the Ca^{2+} influx through presynaptic voltage-gated Ca^{2+} channels (47).

The apparent Ca^{2+} sensitivity of asynchronous release and its persistence in the absence of a fast Ca^{2+} sensor further support the notion that a functionally distinct Ca^{2+} sensor triggers asynchronous release (at least at some synapses). One of the initial approaches to characterize this Ca^{2+} sensor was to substitute extracellular Ca^{2+} with Sr^{2+}, which

suppresses synchronous release and greatly enhances asynchronous release (151). This led to the hypothesis that the sensor for asynchronous release is more sensitive to Sr^{2+} than is the sensor for synchronous release, Syt1 (105). Measurements of presynaptic Sr^{2+} and Ca^{2+} indicate that neurons do not buffer or extrude Sr^{2+} as well as they do Ca^{2+} and that presynaptic activation leads to larger and longer-lived elevation in Sr^{2+} compared with Ca^{2+}. When the differential signals are taken into account, it appears that Sr^{2+} is less effective than Ca^{2+} at triggering both synchronous release and asynchronous release (152, 153) and that the majority of Sr^{2+}-mediated release is triggered by the fast Ca^{2+} sensor Syt1 (154).

Ca^{2+} Sources for Asynchronous Release

Although most attention has focused on voltage-gated Ca^{2+} channels as the primary source of Ca^{2+} that evokes asynchronous release, presynaptic firing may activate presynaptic Ca^{2+}-permeable receptors, which in turn elevates Ca^{2+}. This was first proposed for P2X2 ATP receptors at hippocampal CA3→CA1 synapses (155). A P2X2 receptor antagonist suppressed asynchronous release by approximately 50% in half of the cells but did not affect it in the other cells. It was concluded that synaptic activation may liberate sufficient ATP to activate presynaptic, Ca^{2+}-permeable P2X2 receptors to trigger asynchronous release, although at this synapse most of the asynchronous release is not mediated by P2X2 receptors. Additionally, activity-dependent activation of Ca^{2+}-permeable TRPV1 receptors may promote asynchronous release from solitary tract afferents. Asynchronous release at this synapse was blocked by TRPV1 receptors and was much more prominent at physiological temperature than at room temperature, consistent with the temperature sensitivity of TRPV1 (156). In these studies, the identity of the Ca^{2+} sensor for asynchronous release is not known, and further studies are required to determine the contributions of P2X2, TRPV1, and other presynaptic Ca^{2+}-permeable receptors to asynchronous release and to identify the associated Ca^{2+} sensor.

It is well established that voltage-gated Ca^{2+} channels provide the brief Ca^{2+} signal that evokes synchronous release, but voltage-gated Ca^{2+} channels may also provide a longer-lasting phase of Ca^{2+} entry that may contribute to asynchronous release (157). In transfected nonneuronal cells, following a large and prolonged depolarization or a train of depolarizations, a fraction of either $Ca_V2.1$ (P-type) or $Ca_V2.2$ (N-type) Ca^{2+} channels open for hundreds of milliseconds. Intracellular EGTA eliminates this component, suggesting that it requires an increase in bulk cytosolic Ca^{2+}. Elevations of cytosolic Ca^{2+} may activate a Ca^{2+} sensor (other than calmodulin) to promote late opening of Ca^{2+} channels, which may in turn promote asynchronous release. In this mechanism, the Ca^{2+} sensor does not trigger asynchronous release but instead promotes late opening of voltage-gated Ca^{2+} channels to drive release. Given that both $Ca_V2.1$ and $Ca_V2.2$ Ca^{2+} channels can have late Ca^{2+} channel openings, which are highly effective at triggering release, these openings would have the potential to activate fast synaptotagmins. The late openings depended on the specific β subunit, with a delayed current prominent when β_2 subunits were present and absent in the presence of β_1. The other β subunits in brain, β_3 and β_4 (158), were not tested. This suggests that the presence of an appropriate specific β subunit may determine whether asynchronous release is prominent at a synapse. This mechanism alone would not account for the asynchronous release that remains when fast synaptotagmins are eliminated; an additional

Ca^{2+} sensor to trigger vesicle fusion would also be required. The properties of late Ca^{2+} channel openings are unlikely to contribute significantly to either asynchronous release evoked by single stimuli, given that Ca^{2+} channel openings are not prominent after single brief depolarization, or asynchronous release lasting for tens of seconds after prolonged presynaptic activation, because the duration of delayed Ca^{2+} channel openings is just hundreds of milliseconds. Thus, the role of late openings of voltage-gated Ca^{2+} channels in asynchronous release is yet to be established.

As expected from its Ca^{2+} dependence, factors that regulate presynaptic Ca^{2+} signaling influence asynchronous release. It appears that at the frog NMJ voltage-gated Ca^{2+} channels are located near ryanodine receptors on internal stores, and sustained high-frequency presynaptic activation of voltage-gated Ca^{2+} channels can trigger Ca^{2+}-induced Ca^{2+} release from internal stores that in turn promotes vesicle fusion (159, 160). However, other studies reported that preventing release from internal stores has minimal effects on presynaptic Ca^{2+} signals and asynchronous release, indicating that release from internal stores is not essential (161). Mitochondria can also influence asynchronous release, but they reduce asynchronous release by sequestering Ca^{2+} during high-frequency activation (134, 162).

Other Presynaptic Proteins Implicated in Asynchronous Release

Additional proteins have been suggested to have roles other than serving as the Ca^{2+} sensor or source for asynchronous release. The role of vesicular SNAREs was studied by using cultured hip-pocampal cells from synaptobrevin 2 knockout mice in which viruses were used to express either VAMP4 or synaptobrevin 2 (163). Synaptobrevin 2 was highly effective at rescuing synchronous release, whereas VAMP4 rescued synchronous release less efficiently but produced a somewhat enhanced postsynaptic current late in a stimulus train, with increased susceptibility to EGTA. VAMP4/syntaxin-1/SNAP-25 SNARE complexes also did not interact with complexins 1 and 2 and Syt1, proteins critical to synchronous release. This study raised the intriguing possibility that SNARE proteins associated with a specific vesicle may determine whether that vesicle contributes to synchronous or asynchronous release (163). A simple distinction in which synaptobrevin 2– and VAMP4-containing vesicles mediate synchronous release and asynchronous release, respectively, seems unlikely because VAMP4 does not contribute prominent asynchronous release in synaptobrevin 2 knockout mice (58), VAMP4 partially rescues synchronous release, and VAMP4 knockdown reduces both synchronous release and asynchronous release in the presence of high levels of extracellular Ca^{2+} (163). The dependence of asynchronous release on canonical SNAREs (synaptobrevin 2, syntaxin-1, SNAP-25) and on the S/M protein Munc18 is further supported by knockout or knockdown of these proteins (Table 1) (15, 53, 58–60, 163).

Synapsin 2 has also been implicated in asynchronous release. Synapsins are a family of vesicle-associated proteins that are thought to regulate vesicle pools and to participate in short-term synaptic plasticity (164). In synapsin 2 knockout mice, synchronous release is enhanced and asynchronous release reduced at the inhibitory synapse onto hippocampal dentate granule cells, whereas asynchronous release of excitatory inputs onto these cells is unaffected (165). This suggests that synapsin 2 can regulate asynchronous release at some

but not all synapses. It is not known whether synapsin 2 is directly involved in asynchronous release or whether the lack of synapsin 2 reduces asynchronous release by enhancing synchronous release and decreasing the size of the RRP available for asynchronous release.

Remarkably, it appears that postsynaptic proteins can regulate asynchronous release. SAP97 is a member of the membrane-associated guanylate kinase family of proteins, is located postsy-naptically, and is involved in trafficking ionotropic receptors. Stimulation of synapses onto ciliary ganglion neurons evokes prominent asynchronous release in the presence of Sr^{2+}, and the knockdown of SAP97 decreased it by ~80% (166). SAP97 may control the expression of N-cadherin to allow the postsynaptic cell to regulate asynchronous release.

In summary, these studies show that diverse molecular mechanisms may mediate asynchronous release. Ultimately, this suggests that multiple mechanistically distinct forms of asynchronous release may operate at any given synapse and that different types of synapses may employ specific mechanisms for asynchronous release.

SPONTANEOUS RELEASE

The Functions of Spontaneous Release

Given that the difference between the release rates transiently evoked by an action potential (briefly as high as 1 vesicle per 500 μs, or $>10^3$ s^{-1}) or observed during spontaneous release ($<10^{-3}$ s^{-1}) is greater than 10^6, the synapse effectively limits action potential-independent exocytosis of synaptic vesicles. It is therefore reasonable to hypothesize that spontaneous release may simply reflect an imperfect suppression of spontaneous vesicle fusion and that, for many types of synapses, it does not have a function.

Nevertheless, a number of functional roles for spontaneous release, including regulating the excitability of neurons, have been proposed. The summed spontaneous quantal release from many inputs onto a neuron can contribute to basal extracellular levels of neurotransmitter and can regulate tonic activation of high-affinity receptors, thereby regulating the excitability of neurons (167, 168). Although individual quanta have a small effect on the excitability of large principal neurons, single quanta can trigger action potentials in small interneurons (169).

Spontaneous release has also been implicated in synaptic stabilization and long-term forms of synaptic plasticity. At the *Drosophila* NMJ, disruption of spontaneous release for minutes leads to the release, from the muscle, of a signal that acts retrogradely to induce a presynaptic form of homeostatic plasticity (170). Disruption of spontaneous glutamate release for hours also leads to homeostatic regulation of inhibitory synapses in the hippocampus through a mechanism that relies on activation of postsynaptic metabotropic glutamate receptors, release of endocannabinoids, and activation of cannabinoid receptors (171). Spontaneous vesicular glutamate release also acts as a trophic factor to prevent the loss of dendritic spines by activating AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (172). It also restricts the diffusion of GluR1 AMPA receptors at active synapses, thereby regulating the number and type of AMPA receptors

present at a synapse (173). In some cases, spontaneous activity may adjust synaptic strength by regulating protein synthesis. In cultured hippocampal pyramidal cells, spontaneous glutamate release activates NMDARs and tonically suppresses local protein synthesis in dendrites (174–176). Several hours of NMDAR blockade results in an increase in surface AMPA receptors, whereas it takes approximately 10 times as long to produce similar synaptic changes if evoked activity is eliminated by pharmacological blockage of spiking (175, 176). These findings indicate that preventing the activation of NMDARs by both spontaneous release and evoked release is much more potent than preventing only evoked release, and they suggest that spontaneous release may regulate protein synthesis in dendrites and in homeostatic plasticity. These findings are surprising given that NMDAR activation through spontaneous release is expected to be small due to the Mg^{2+} block of the receptors, and because the frequency of spontaneous release per site is low, typically less than one event every 1,000 s at most synapses (see below section, "What Is the Role of Ca^{2+} in Spontaneous Release?").

Studying Spontaneous Release

For the purpose of this review, a measure of spontaneous release is provided by spontaneous synaptic currents recorded in the presence of tetrodotoxin (TTX) to block voltage-gated Na^{+} channels and prevent action potentials. Such synaptic currents are also often referred to as miniature postsynaptic currents (mPSCs; Figure 1*b*). Synaptic currents measured in the absence of TTX, consisting of a combination of spontaneous vesicle fusion and fusion driven by the spontaneous firing of presynaptic cells, are not included here. There is considerable heterogeneity in the frequency of release and amplitude of quantal responses for different sites. The average quantal size is thought to provide a measure of the average number of receptors in a postsynaptic density, and the average event frequency is often thought to be the product of the number of release sites and the properties of the release.

The spontaneous event frequency is regulated in many ways and can be difficult to interpret. This is illustrated by the finding that removal of the vesicular GABA transporter in postsynaptic medium spiny neurons of the basal ganglia leads to an increase in spontaneous event frequency in these cells (177). This increase is a consequence of regulating the overall activity in a loop from the basal ganglia to the thalamus to the cortex and back to the basal ganglia, rather than a consequence of the postsynaptic cell retrogradely communicating to the presynaptic cell to control spontaneous release. This finding illustrates how circuit activity can control spontaneous release, an observation that complicates the interpretation of spontaneous event frequency in constitutive and conditional knockouts and transgenic animals.

It is also difficult to relate spontaneous release to evoked release, which has additional regulatory elements such as the presynaptic waveform and the action potential–driven opening of Ca^{2+} channels and may even involve a separate vesicle pool. Complexin, a presynaptic protein that binds to SNARE complexes, exemplifies this difficulty (Table 2). In addition to the well-established activating function of complexin on synchronous release (53, 178), genetic removal of complexin resulted in a dramatic increase in spontaneous event frequency, an effect that was first identified at the *Drosophila* NMJ (179). This finding was

later confirmed by knockdown experiments in cultured hippocampal neurons (53, 54) and by genetic deletion of complexin in *Caenorhabditis elegans* (180). These observations, together with biochemical and in vitro fusion studies in which complexin interfered with complete zippering of SNARE complexes during fusion (51, 52), led to an elegant model whereby complexin clamps spontaneous release by preventing full insertion of the synaptobrevin 2 SNARE motif into the assembling SNARE complex. This molecular clamp of SNARE complexes is lacking when complexin is removed, resulting in increased spontaneous fusion. However, in complexin knockout mice, activation of release is impaired, but spontaneous release is not affected or somewhat decreased (178, 181). It is currently difficult to reconcile these data with the increased spontaneous event frequency observed in complexin mutants in *C. elegans* and *Drosophila* and in knockdown experiments in cultured vertebrate neurons. Structure-function experiments with complexin further reveal that activation of synchronous release and clamping of spontaneous release are rescued independently by distinct complexin sequences (54, 180, 182). This suggests that the reduction in synchronous release is not simply a consequence of elevated spontaneous release depleting the RRP. More work is necessary to understand the functions of complexin in spontaneous release.

What Is the Role of Ca^{2+} in Spontaneous Release?

Numerous mechanisms may potentially mediate spontaneous release. Components of spontaneous release may be Ca^{2+} independent or may depend on bulk cytosolic Ca^{2+} or high local Ca^{2+}. The molecular machinery that mediates spontaneous release may be the same as or different from evoked release.

Many explorations of the Ca^{2+} dependence of spontaneous release have assessed the effect of altering extracellular Ca^{2+} levels. This is a powerful approach in studies of evoked release because it allows stimulus-evoked Ca^{2+} influx to be altered and reveals the steep Ca^{2+} dependence of synchronous release ($n \sim 4$; 63, 64). Although spontaneous release rates are much less steeply dependent on extracellular Ca^{2+} ($n \sim 0.3–1.5$) than evoked release rates are, this finding is difficult to interpret. Extracellular Ca^{2+} levels may influence spontaneous release by changing bulk Ca^{2+} levels either by activating Ca^{2+}-sensing G proteins (183), by decreasing Ca^{2+} entry through Ca^{2+}-permeable channels, or by changing the reversal potential of the Na^{+}/Ca^{2+} exchanger. Extracellular Ca^{2+} levels may change Ca_{local} near Ca^{2+} channels that open stochastically at rest and may alter the number of voltage-gated Ca^{2+} channels that open by changing the surface charge and thereby the voltage dependence of channel opening (184, 185). It is particularly difficult to interpret the dependence of spontaneous release on extracellular Ca^{2+} if presynaptic Ca^{2+} is not measured, which is typically the case.

The effects of Ca^{2+} buffers and Ca^{2+} channel antagonists indicate that spontaneous release has a significant Ca^{2+}-dependent component at many synapses. The Ca^{2+} chelator BAPTA reduces spontaneous release by 95% at cultured hippocampal synapses (186). In addition, antagonists of voltage-gated Ca^{2+} channels reduce spontaneous release by ~50% at some synapses (187, 188). At some brain stem neurons, the genetic elimination or blockade of TRPV1 receptors (which are Ca^{2+} permeable) eliminates more than 90% of spontaneous release observed in the presence of TTX (189). That TRPV1 is a Ca^{2+}-permeable receptor

suggests that tonically active TRPV1 leads to Ca^{2+} influx into presynaptic boutons, which in turn drives spontaneous glutamate release. At inhibitory synapses in the cerebellum, blocking Ca^{2+} channels or briefly eliminating extracellular Ca^{2+} has no effect on spontaneous transmission (190). This is because most spontaneous release at this synapse is evoked by Ca^{2+} transients produced by release from internal stores (191). These experiments establish that at a variety of synapses spontaneous release is Ca^{2+} dependent but that the source of Ca^{2+} depends on the type of synapse: voltage-gated Ca^{2+} channels for some synapses, Ca^{2+}-permeable ion channels such as TRPV1 for others, and in some cases release from internal Ca^{2+} stores (Figure 6).

The properties of spontaneous release are diverse, and in some cases a large fraction of release appears to be independent of Ca^{2+}. The blockade of voltage-gated Ca^{2+} channels did not alter spontaneous inhibitory or excitatory transmission onto CA3 pyramidal cells (192) or excitatory synaptic transmission onto cultured cortical cells (183) and left 50–70% of spontaneous release intact for inhibitory inputs onto hippocampal granule cells (187) and cultured inhibitory synapses (188). Chelating Ca^{2+} with BAPTA often leaves a significant fraction of spontaneous release intact. It is not known whether the remaining spontaneous release is Ca^{2+} independent, whether it is induced by local Ca^{2+} signals that are incompletely eliminated by BAPTA, or whether bulk Ca^{2+} levels that are predicted to be unaffected by Ca^{2+} buffering drive this remaining spontaneous release. If there is indeed a Ca^{2+}-independent component of release, the mechanism underlying this component is not known.

It is difficult to determine whether Ca^{2+} -dependent spontaneous release is produced by local elevations of Ca^{2+} or by increasing bulk cytosolic Ca^{2+} levels. To consider whether resting Ca^{2+} levels (Ca_{rest} ~ 50 nM) may evoke spontaneous release via the fast Ca^{2+} sensor synaptotagmin, which has a Ca^{2+} ion cooperativity of $n \approx 4$, it is helpful to estimate whether such a Ca^{2+} sensor could drive spontaneous release at a typical rate at Ca_{rest}. The ratio of the evoked and spontaneous release rates is estimated by $Release_{evoked}/Release_{spontaneous} \sim (Ca_{local}/Ca_{rest})^n$, where Ca_{local} is the local Ca^{2+} signal that evokes synchronous release. The observed ratio is ~10^5 (~10^2 s^{-1} /~10^{-3} s^{-1}), and the predicted ratio for Ca_{local} = 25 μM (87) is 5×10^2 for $n = 1$, 2.5×10^5 for $n = 2$, and >10^{13} for $n = 5$. This suggests that resting Ca^{2+} likely triggers negligible release for a sensor where $n = 5$ but that, for Ca^{2+} sensors that require the binding of one or two Ca^{2+} ions, Ca_{rest} may produce significant spontaneous release (Figure 5).

As discussed above with regard to asynchronous release, many synapses may contain a specialized Ca^{2+} sensor that is suited to mediating spontaneous release driven by modest increases in cytosolic Ca^{2+}. At the calyx of Held synapse, for modest increases in presynaptic Ca^{2+}, release is dominated by a component with a cooperativity of $n = 0.6–2$, but for higher Ca^{2+} levels cooperativity is n=5, which is consistent with the Ca^{2+}-binding properties of Syt2 (67, 193). Although the lower Ca^{2+} cooperativity could also be mediated by Syt2 if an allosteric modification reduced the cooperativity at low Ca^{2+} concentrations (193), this possibility seems unlikely because this component is also present in Syt2 knockout animals (67). This suggests that most spontaneous release driven by bulk Ca^{2+} levels is mediated by an unidentified sensor that has a lower Ca^{2+} cooperativity than does

synaptotagmin. If release is driven by high local Ca^{2+} near an open, Ca^{2+}-permeable channel, even low-affinity sensors with a steep Ca^{2+} dependence, such as Syt1 or −2, may be involved.

These findings establish that in many cases spontaneous release is Ca^{2+} sensitive. There are several possible Ca^{2+} sensors for spontaneous release, including the same Ca^{2+} sensor that mediates synchronous release (a fast synaptotagmin) and a specialized Ca^{2+} sensor.

Doc2 proteins have emerged as attractive candidates for such a Ca^{2+} sensor. Knocking out or knocking down Doc2 proteins reduces spontaneous release by more than 50% without altering synchronous release (149, 150). The Ca^{2+} -binding properties and membrane-binding properties of Doc2 combined with the reduction of spontaneous release suggested that Doc2 might be a Ca^{2+} sensor that mediates spontaneous release but not evoked release (149). Surprisingly, it was subsequently found that the decrease in spontaneous release produced by lowering Doc2 levels was rescued by a mutated Doc2 that did not bind Ca^{2+} (150). This finding suggests that, although Doc2 regulates spontaneous release, it may not operate as a Ca^{2+} sensor for spontaneous release.

The simplest possibility is that the molecular mechanisms for spontaneous release are fundamentally identical to those for evoked release but that, instead of concerted opening of many Ca^{2+} channels by an action potential, spontaneous release is evoked by stochastic openings of single channels. Indeed, it has been proposed that for cultured hippocampal cells Syt1 mediates most spontaneous release (186). The role of Syt1 in spontaneous release seems reasonable if high Ca_{local} near open Ca^{2+} channels drives release (Figure 5). The rate of Ca_{local}-driven fusion depends on the distance of the Ca^{2+} channels from the docked vesicles, the number of Ca^{2+} channels near each docked vesicle, the potential of the presynaptic bouton, and the number of Ca^{2+} channel openings per channel. At some synapses, a small number of Ca^{2+} channels are required to trigger vesicle fusion so that Ca^{2+} channels would need to open at very low rates to drive such release at the observed rate of one event every ~1,000 s per release site. It is difficult to exclude the possibility that these channels open once every 1,000 s, given that the rate at which $Ca_V2.2$ and $Ca_V2.1$ Ca^{2+} channels open at potentials near the resting potential of cells is not well characterized.

A particularly interesting possibility is that the resting potential of the presynaptic bouton controls the opening of voltage-gated Ca^{2+} channels to regulate the Ca^{2+}-dependent component of spontaneous release. At the calyx of Held, a modest presynaptic depolarization elevates spontaneous release. Small depolarizations of the presynaptic bouton can also elevate spontaneous release by promoting the stochastic opening of $Ca_V2.1$ channels (194, 195). In addition, alterations of extracellular potassium that are predicted to produce modest changes in the resting potential regulate spontaneous release (187).

Synaptic Vesicle Pools for Spontaneous Release

A long-standing and intensively debated question is whether spontaneous release draws from the same pool of vesicles that fuse in response to action potentials or whether evoked release and spontaneous release draw from different, nonoverlapping pools (196). It originally seemed likely that the same vesicles that account for spontaneous release are

released by action potentials, because action potential–evoked responses represent the summation of multiple events with the same properties as spontaneous events (197). However, examination of the functional properties of vesicles revealed that intense prolonged stimulation (>1,000 stimuli at >10 Hz) releases <20% of the vesicles in a presynaptic bouton (termed the recycling pool), and the remaining >80% of vesicles are not liberated by stimulation (the nonrecycling pool; 23, 24). It has been hypothesized that part of the nonrecycling pool selectively supports spontaneous release (198, 199).

The hypothesis that spontaneous release and evoked release arise from different vesicle pools has been extensively studied by fluorescently staining and destaining vesicles that undergo fusion and endocytosis. In the presence of a lipophilic dye, such as FM 1-43, vesicle fusion is followed by endocytosis, and the newly formed vesicle is fluorescently labeled. When the dye is removed from the extracellular solution, a previously labeled vesicle loses its dye when it fuses, and destaining occurs. This approach makes it possible to load vesicle pools either by stimulation or by waiting a long time and allowing labeling to occur following spontaneous release. If the spontaneous and evoked vesicles draw from the same pool, then stimulation should destain vesicles labeled spontaneously, and spontaneous activity should destain vesicles labeled with stimulation. However, this will not be the case if spontaneous release and evoked release involve different pools of vesicles.

Numerous studies using this and related approaches indicate that evoked release and spontaneous release share vesicles. This was first observed in studies of cultured hippocampal neurons in which a 20-Hz, 30-s stimulus destained vesicles equally effectively whether they had been labeled by a few action potentials or by spontaneous release in the presence of the Na^+-channel blocker TTX (200). Similar to hippocampal neurons, at the NMJs of *Drosophila*, frogs, and mice, spontaneously stained vesicles are destained by activity and vice versa, suggesting that the mechanism of sharing a pool of vesicles for spontaneous release and evoked release is common across species and at various types of synapses (201). It has also been found that an action potential train simultaneously unloads FM 5-95–labeled vesicles loaded spontaneously and FM 1-43–labeled vesicles loaded with stimulation (202). The same conclusion was reached when pools were labeled with intravesicular markers (203).

Other studies using similar approaches led to the opposite conclusion, namely that vesicle pools are segregated for different types of release (198, 199). Vesicles loaded spontaneously were destained much slower by K^+-induced depolarization or by action potentials than were vesicles loaded by stimulation (198). Another study found that vesicles formed during spontaneous vesicle fusion were labeled intraluminally with a synaptobrevin 2 biotin tag and were still present in presynaptic boutons even after prolonged stimulation (199), suggesting that these spontaneously labeled vesicles reside in a pool that is inaccessible to stimulation. These two studies suggest that vesicle pools for miniature release and evoked release are distinct. It is unclear why some studies suggest that a single pool is shared whereas other studies suggest that there are different pools for spontaneous release and evoked release.

If some vesicles are liberated only by spontaneous activity and others only by evoked activity, then specific molecular markers must be present on the vesicles associated with a

particular mode of release. Two recent publications conclude that the noncanonical SNAREs Vti1a (204) and VAMP7 (205) mediate spontaneous release. Both studies found that ectopic expression of a noncanonical SNARE marked or generated a pool of vesicles that is more selectively used for spontaneous release. However, the protein levels of Vti1a and VAMP7 on synaptic vesicles are not nearly as high as the levels of synaptobrevin 2 (206–209). With an estimated average of 70 copies of synaptobrevin 2 per vesicle (208), low levels of Vti1a and VAMP7 are not likely to outcompete synaptobrevin 2–containing vesicles to generate a separate pool for spontaneous release. Indeed, genetic loss of synaptobrevin 2 greatly reduces spontaneous release (58, 204, 210, 211), whereas it has not been shown that the loss of VAMP7 impairs spontaneous release, and the knockdown of Vti1a has little effect on spontaneous release. Spontaneously cycled vesicles are labeled with biotinylated, ectopically expressed synaptobrevin 2 (199), showing that exogenous synaptobrevin 2 is localized to spontaneously cycling vesicles. The central role of the canonical SNARE pathway in spontaneous release is further supported by strong impairment of spontaneous release by knockout or knockdown of the plasma membrane SNAREs syntaxin-1 and SNAP-25 (59–61, 212) and by complete loss of such spontaneous release in the absence of Munc18-1 (15). Together, these findings indicate that most spontaneous events are mediated by synaptobrevin 2 and the other canonical SNARES. They also suggest that Vti1a and VAMP7 may not be molecular markers that specify an independent vesicle pool that accounts for most spontaneous release.

Further studies are needed to clarify discrepancies in the literature and determine whether some vesicles have a unique molecular marker that allows them to selectively contribute to spontaneous release. Although reconciling the conflicting results is difficult, the most parsimonious explanation is that most spontaneous release is mediated by a vesicular pool that is shared with evoked release and that employs synaptobrevin 2.

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SUMMARY POINTS

1. Synchronous release, asynchronous release, and spontaneous release are the primary modes of secretion of neurotransmitters in the central nervous system.
2. Synchronous release is well characterized and requires a readily releasable pool of synaptic vesicles; voltage-gated Ca^{2+} channels that open and close quickly upon arrival of the action potential; a Ca^{2+}-triggering mechanism with fast on- and off-kinetics; and a tight spatial coupling between the Ca^{2+} channel, the Ca^{2+} sensor, and the primed vesicle.
3. At the molecular level, the three modes of release appear to share key mechanisms, most important, the fusion mechanism that is mediated by the canonical SNARE proteins synaptobrevin 2/VAMP2, SNAP-25, and syntaxin-1.
4. Heterogeneous mechanisms have been implicated in asynchronous release and spontaneous release. Thus, these forms of release may be controlled by multiple alternative molecular mechanisms that differ in important ways from those involved in synchronous release.
5. Synchronous release and asynchronous release depend strictly on Ca^{2+}. Although a significant portion of spontaneous release is also triggered by Ca^{2+}, there may also be a Ca^{2+}-independent component.
6. The major Ca^{2+} sensors for synchronous release are synaptotagmins 1, 2, and 9, but Ca^{2+} sensors for asynchronous release have not been conclusively identified.

FUTURE ISSUES

1. Ca^{2+} sensors for asynchronous release need to be identified.
2. A better understanding of the Ca^{2+} dependence of spontaneous release is needed.
3. The sources of Ca^{2+} that triggers asynchronous and possibly spontaneous release should be further assessed.
4. Clarification of the functional significance of asynchronous and spontaneous release is needed.

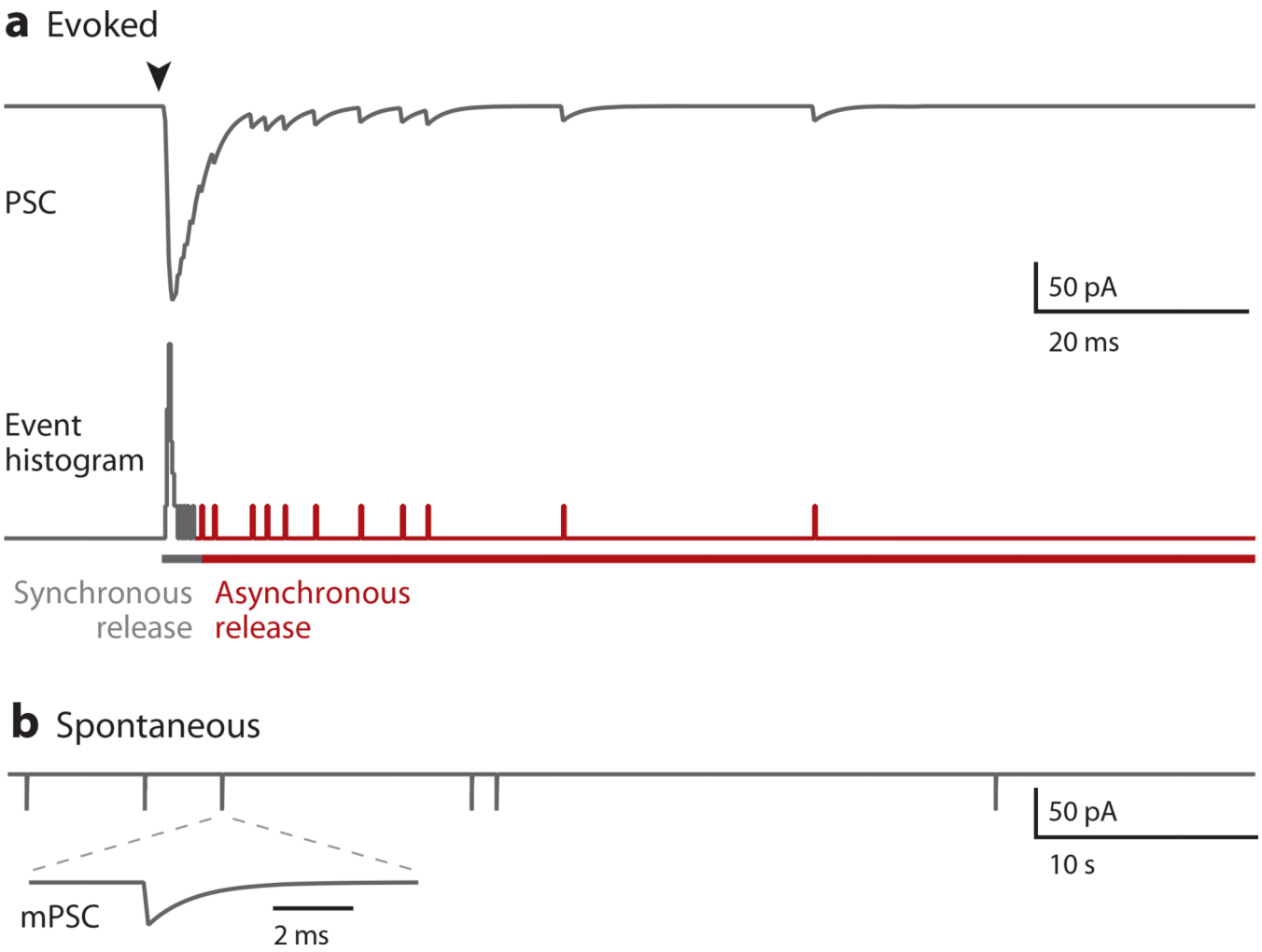

Figure 1.

Different types of synaptic transmission illustrated with simulated data. (*a*) Stimulation (*arrowhead*) evokes synchronous and asynchronous release. (*b*) Spontaneous neurotransmitter release is shown on a different timescale. The inset shows a miniature postsynaptic current on an expanded timescale. Abbreviations: mPSC, miniature PSC; PSC, postsynaptic current.

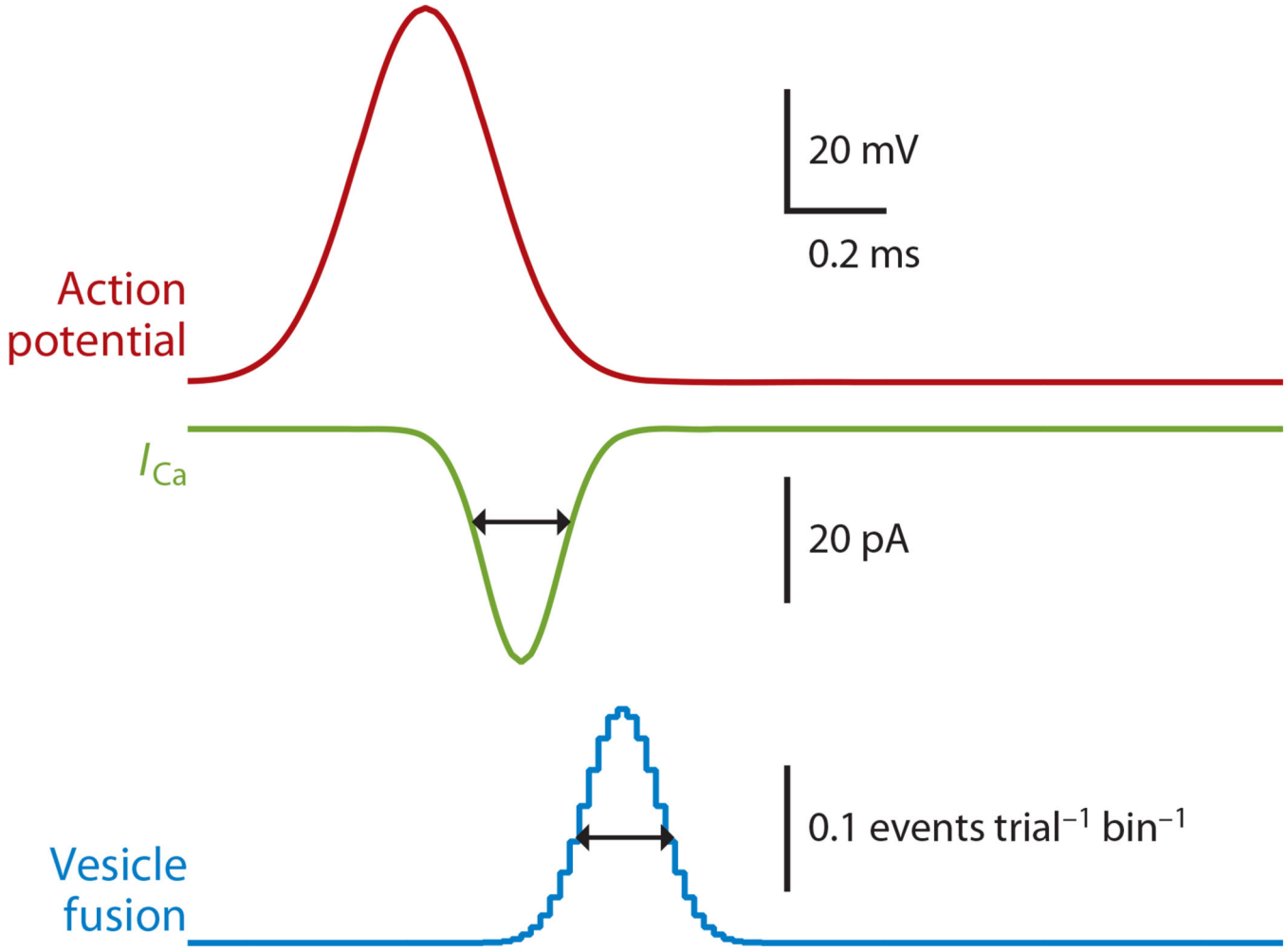

Figure 2.
Precision of synchronous release relies on brief Ca^{2+} channel signals and on the rapid kinetics of the Ca^{2+} sensor. The schematic illustrates the rapid depolarization and repolarization of an action potential, and the speed of the resulting Ca^{2+} current (I_{Ca}) and vesicle fusion.

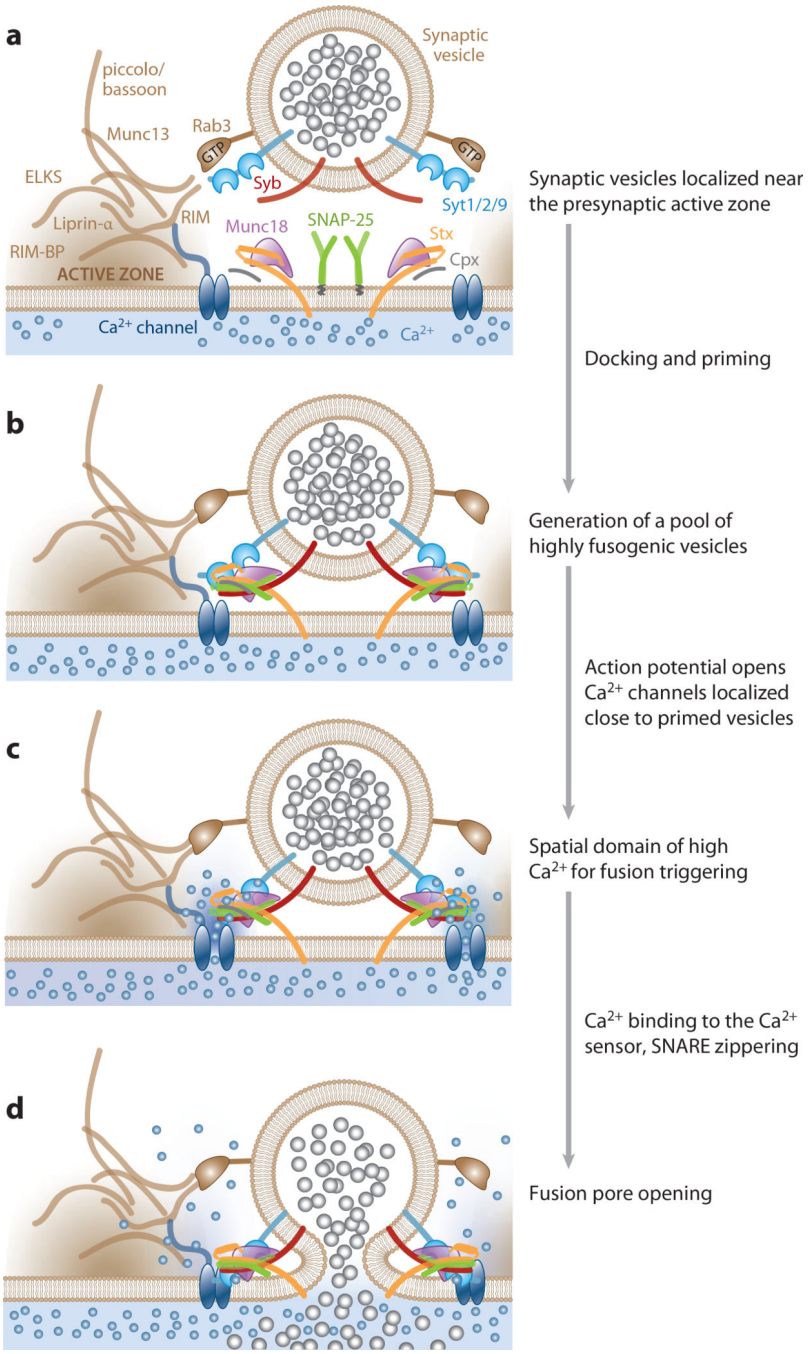

Figure 3.

Schematic of mechanisms for synchronous release of neurotransmitters at central nervous synapses. The four mechanisms are (*a*) tethering of synaptic vesicles close to presynaptic, voltage-gated Ca^{2+} channels at specialized sites for release; (*b*) rendering vesicles release ready during priming under the control of active zone molecules Munc13 and RIM, which may involve partial assembly of SNARE complexes; (*c*) action potential–induced, brief opening of voltage-gated Ca^{2+} channels to allow for a sharp rise and decay of Ca^{2+} near vesicles; and (*d*) fast binding of Ca^{2+} to the synchronous Ca^{2+} sensor synaptotagmin 1 to

trigger fusion of synaptic vesicles. The sensor also needs to have a fast off-rate for limiting high release rates to a few hundred microseconds. These steps synchronize release, and asynchronous release and spontaneous release may mechanistically differ in one of these four steps. Abbreviations: Cpx, complexin; ELKS, protein rich in the amino acids E, L, K, and S; RIM, Rab3-interacting molecule; RIM-BP, RIM-binding protein; Stx, syntaxin-1; Syb, synaptobrevin 2 (also termed VAMP2); Syt1/2/9, synaptotagmin 1, 2, or 9.

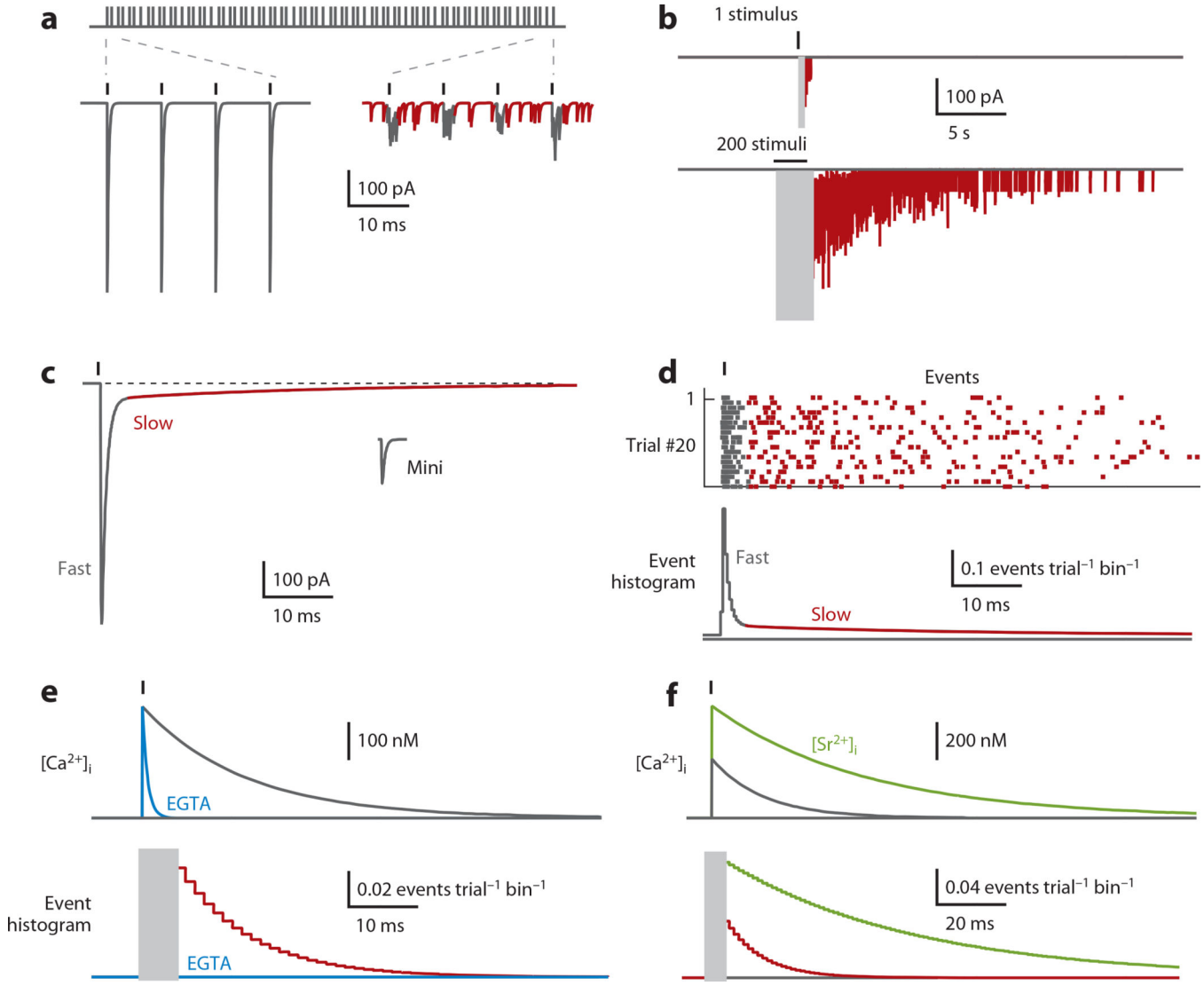

Figure 4.

Properties of asynchronous release. Schematics and simulated data are used to illustrate properties of asynchronous release (indicated by *red*). (*a*) At some synapses, sustained high-frequency activation initially evokes synchronous release (*left*), but after prolonged stimulation release is desynchronized (*right*). (*b*) At many synapses, a single stimulation evokes asynchronous release lasting tens of milliseconds (*upper*), whereas prolonged high-frequency stimulation produces asynchronous release lasting tens of seconds (*lower*). Large currents corresponding to synchronous release are blanked (*gray boxes*). (*c*) In some cases, average synaptic currents are used to estimate the time course of neurotransmitter release, and a slow component is used to estimate the amplitude and duration of asynchronous release. An average miniature synaptic current, during which all the transmitter is released at the same time, is shown for comparison. (*d*) Ideally, asynchronous release is characterized as individual quantal events, as illustrated by an example in which 20 trials (*upper*) are displayed in an event histogram of detected events (*lower*). (*e*) The slow Ca^{2+} buffer EGTA has been used to demonstrate that asynchronous release is Ca^{2+} dependent. EGTA has little

effect on peak Ca^{2+} levels (*upper*) or synchronous release (*lower*; blanked out by *gray box*), but it chelates Ca^{2+} to abolish asynchronous release (*lower blue*), which is present in the absence of EGTA (*lower red*). (*f*) Replacing extracellular Ca^{2+} with Sr^{2+} increases the amplitude and duration of asynchronous release (*lower green*). This is primarily a consequence of Sr^{2+} being less well buffered and extruded by presynaptic boutons, which results in larger, longer-lasting Sr^{2+} signals (*upper green*) that drive release via the machinery for synchronous release.

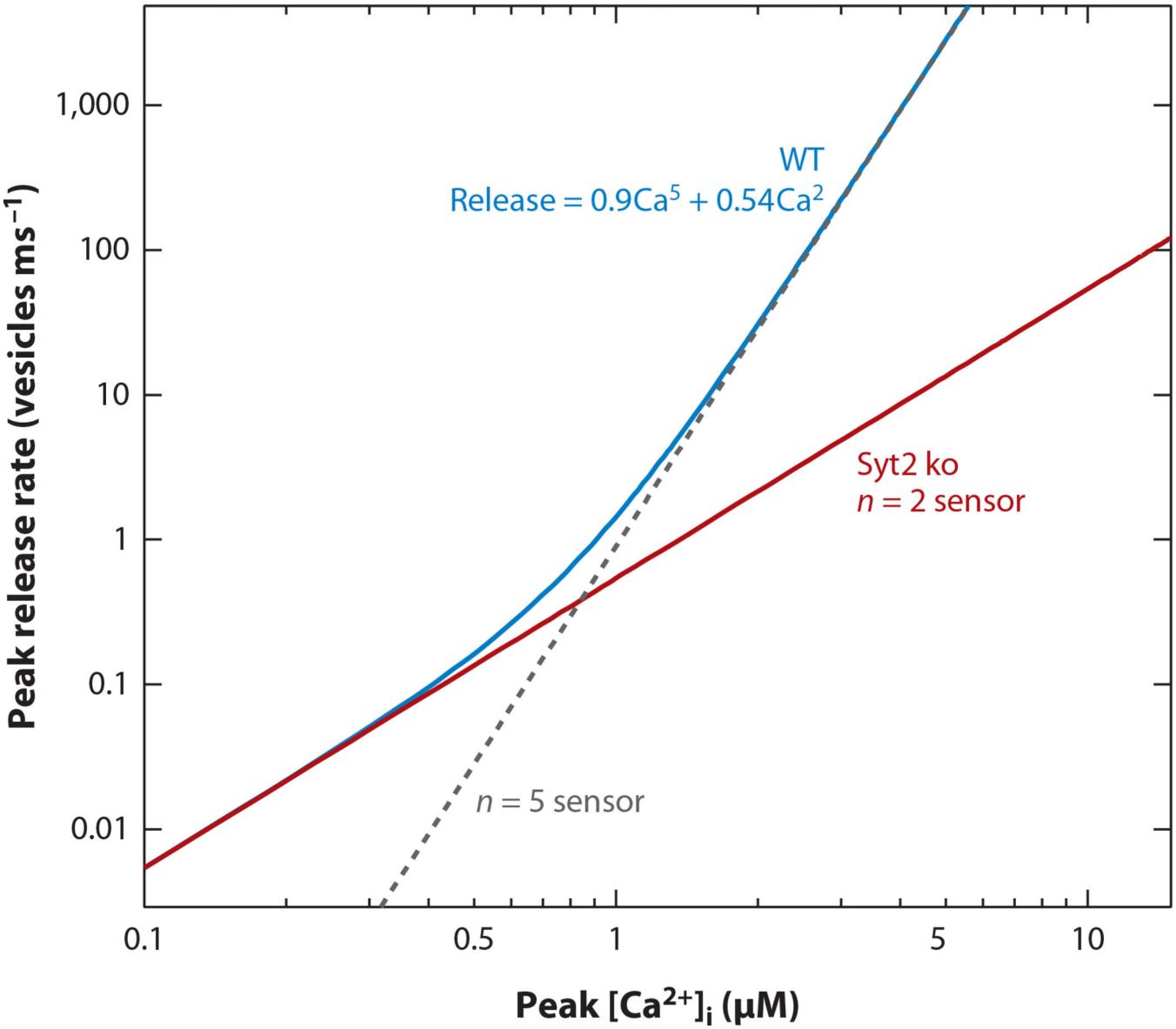

Figure 5.

The Ca^{2+} dependence of neurotransmitter release. The dependence of release on intracellular Ca^{2+} was established at the calyx of Held in wild-type (WT) animals and in knockout (ko) animals in which the fast Ca^{2+} sensor synaptotagmin 2 (Syt2) has been eliminated. In wild-type animals, a steep power law dependence is seen for Ca^{2+} levels above 0.5 μM (n= 5), and for lower Ca^{2+} levels there is a much shallower Ca^{2+} dependence (n= 2). In Syt2 ko animals, release for all ranges of Ca^{2+} is approximated by n= 2. A dashed line shows the Ca^{2+} dependence as a function of release for a sensor with n= 5. These findings indicate that, at the calyx of Held, a Ca^{2+} sensor other than Syt2 dominates release when Ca^{2+} levels are less than ~0.5 μM in WT animals. Simulated data inspired by Reference 67.

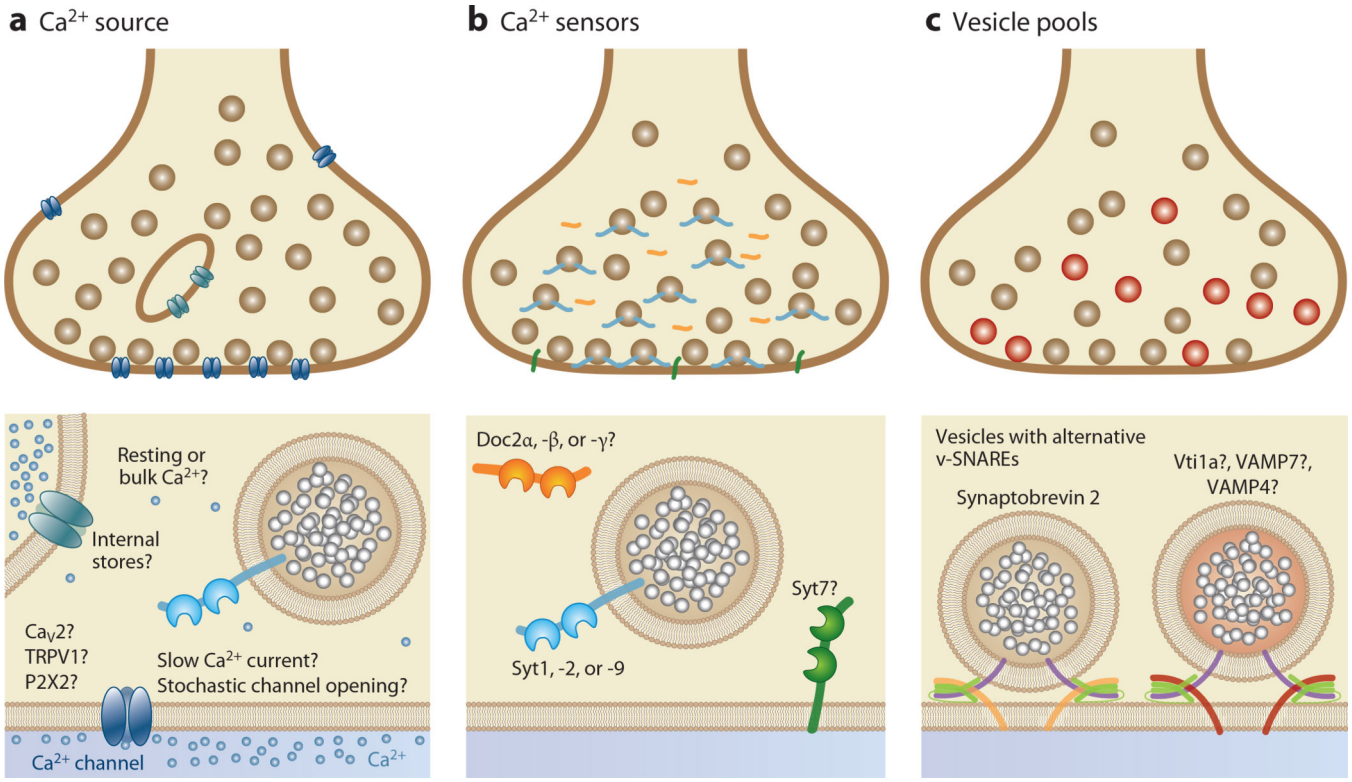

Figure 6.

Schematic of proposed points of divergence of the modes of neurotransmitter release. Several mechanisms may be involved in promoting asynchronous and/or spontaneous release as opposed to synchronous release. Top graphs depict a presynaptic nerve terminal filled with synaptic vesicles. Bottom graphs show an expanded schematic of the presynaptic plasma membrane and a synaptic vesicle to highlight potential mechanisms. (*a*) Asynchronous or spontaneous release may be driven by distinct sources of Ca^{2+}; potential sources include Ca^{2+} currents through Ca^{2+}-permeable TRPV1 channels or P2×2 purinergic receptors, slow or stochastic currents through Ca_V2 voltage-gated Ca^{2+} channels, bulk cytosolic or resting Ca^{2+}, and Ca^{2+} from internal stores. (*b*) Divergent Ca^{2+} sensors may control properties of release. Fast synaptotagmins (Syt1, −2, and −9) trigger synchronous release. Additional Ca^{2+} sensors may be involved in mediating asynchronous or spontaneous release. Synaptotagmin 7 (Syt7) was originally proposed to be a potential Ca^{2+} sensor that may be localized on the presynaptic plasma membrane. Recent experiments delivered arguments for and against Syt7 operating as a Ca^{2+} sensor for asynchronous release (Table 1). Doc2 proteins are cytosolic C_2-domain proteins that were recently promoted as Ca^{2+} sensors for spontaneous or asynchronous release, but the data are controversial (Tables 1 and 2). (*c*) Distinct vesicle pools may also be involved in the mechanisms of spontaneous and asynchronous release. In particular, alternative vesicular SNARE proteins (Vti1a, VAMP7, and VAMP4) have been proposed to be associated with vesicles that specifically promote asynchronous or spontaneous release (Table 2).

Table 1

Proteins involved in asynchronous neurotransmitter release

Protein	Proposed function	References[a]
Synaptotagmin 1/2	Suppression of asynchronous release	65, 67, 68, 104, 135, 213
Complexin	SNARE clamp to limit asynchronous release	54, 136, 180
Synaptotagmin 7	Ca^{2+} sensor for asynchronous release	144, 145, 216
Doc2	Ca^{2+} sensor for asynchronous release	148, 149, 150
RIM	Enhancement of asynchronous release	43, 46, 47
P2×2	ATP-gated activation of Ca^{2+}-permeable receptor as Ca^{2+} source for asynchronous release	155
TRPV1	Ligand-gated activation of Ca^{2+}-permeable receptor as Ca^{2+} source for asynchronous release	156
Presynaptic voltage-gated Ca^{2+} channels	Asynchronous Ca^{2+} current for asynchronous release	157
VAMP4	Vesicular SNARE for asynchronous release	53, 58, 163
Synaptobrevin 2	Vesicular SNARE for asynchronous release	53, 58, 163
Syntaxin-1	Target membrane SNARE for asynchronous release	60, 61
SNAP-25	Target membrane SNARE for asynchronous release	59
Munc18-1	S/M protein required for asynchronous release	15, 62
Synapsin 2	Desynchronization of release	165
SAP97	Postsynaptic protein to promote asynchronous release via N-cadherin	166

Abbreviations: RIM, Rab3-interacting molecule; S/M, Sec-1/Munc18; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

[a]References are supportive in respect to the proposed function (*blue*), provide some evidence against it (*brown*), or deliver additional mechanistic insight (*black*).

Table 2

Proteins involved in spontaneous neurotransmitter release

Protein	Proposed effect	References[a]
Complexin	SNARE clamp to prevent spontaneous fusion	51, 53, 178, 179, 180, 181
Voltage-gated Ca^{2+} channels	Ca^{2+} source for spontaneous release	183, 187, 188, 190, 192, 194, 195
TRPV1	Tonic activation of Ca^{2+}-permeable receptor as Ca^{2+} source for spontaneous release	189
Ryanodine receptor	Intracellular source of Ca^{2+} for spontaneous release	191
Doc2	Ca^{2+} sensor for spontaneous fusion	149, 150
Synaptotagmin 1/2	Ca^{2+} sensor for spontaneous fusion	67, 186, 214
Synaptotagmin 1/2	Clamp to limit spontaneous fusion	65, 68, 72, 135, 213, 214, 215
Vti1a	Vesicular SNARE for spontaneous fusion	58, 204, 210, 211
VAMP7	Vesicular SNARE for spontaneous fusion	58, 204, 205, 210, 211
Synaptobrevin 2	Vesicular SNARE for spontaneous fusion	58, 204, 205, 210, 211
SNAP-25	Target membrane SNARE for spontaneous fusion	59, 212
Syntaxin-1	Target membrane SNARE for spontaneous fusion	60, 61
Munc18-1	S/M protein required for spontaneous fusion	15, 62

Abbreviations: S/M, Sec-1/Munc18; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

[a]References are supportive in respect to the proposed function (*blue*), provide some evidence against it (*brown*), or deliver additional mechanistic insight (*black*).