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Spontaneous Neurotransmission: An Independent Pathway for Neuronal Signaling?

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Recent findings suggest that spontaneous neurotransmission is a bona fide pathway for interneuronal signaling that operates independent of evoked transmission via distinct presynaptic as well as postsynaptic substrates. This article will examine the role of spontaneous release events in neuronal signaling by focusing on aspects that distinguish this process from evoked neurotransmission, and evaluate the mechanisms that may underlie this segregation.

Spontaneous Neurotransmitter Release is a Ubiquitous Property of Presynaptic Nerve Terminals

Presynaptic nerve terminals are remarkable nanomachines that can release neurotransmitter at a wide range of frequencies. Most neurotransmitter release occurs in response to depolarization of nerve terminals during axonal action potential firing. Presynaptic action potential-driven neurotransmitter release has been extensively studied, and its impact on postsynaptic neurons forms the backbone of our current understanding of electrical and biochemical signaling in the nervous system (1, 2, 63). In contrast, spontaneous neurotransmitter release that typically occurs with a low probability is poorly understood functionally and mechanistically. These spontaneous release events usually correspond to fusion of a single synaptic vesicle (often called "miniature" or "unitary" release), and they take place with a frequency of 0.01–0.02 Hz per release site (24, 25, 50). Spontaneous neurotransmission has been a powerful analytical tool to examine properties of individual synapses and to monitor alterations in the number of functional synapses, difficult parameters to assess using evoked neurotransmission due to simultaneous release of neurotransmitter from multiple synapses. Besides their usefulness as an analytical tool, the question of whether this extremely low-frequency neuronal communication carries information or merely represents "noise" has been debated since its discovery by Bernard Katz and colleagues in the 1950s (21, 17, 40). Studies in the last two decades provide substantial evidence that these spontaneous release events can modulate or drive action potential firing in some central neurons (9, 55, 56, 69), thus supporting the premise that spontaneous neurotransmission can contribute to electrical signaling in neuronal networks (31, 45, 59, 60, 79). An increasing number of studies have also revealed that spontaneous release events trigger biochemical signaling leading to maturation and stability of synaptic networks (48, 80, 83), local dendritic protein synthesis (74), and control postsynaptic responsiveness during homeostatic synaptic plasticity (4, 6, 22, 42, 75). Most surprisingly, these studies have shown specific effects of postsynaptic excitatory receptor blockade or inhibition of neurotransmitter release under resting conditions, which could not be achieved by inhibition of action potential-mediated signaling alone.

Several recent review articles provide insight into presynaptic mechanisms that give rise to spontaneous release, its regulation by Ca^{2+} and neuromodulators (7, 27, 58), as well as its impact on postsynaptic signal transduction underlying homeostatic plasticity (12, 65, 77, 88). In this article, we will examine aspects of spontaneous neurotransmission that distinguish it from evoked neurotransmission. We will focus on questions and hypotheses concerning the role of spontaneous release events in neuronal signaling and the mechanisms that may underlie their segregation from evoked transmission.

Segregation of Spontaneous and Action Potential-Driven Synaptic Signaling

The classical view of spontaneous neurotransmission relies on the assumption that spontaneous release originates from action potential-independent low-probability fusion of the same synaptic vesicle population that gives rise to evoked neurotransmission. Traditionally, evoked and spontaneous forms of fusion are believed to occur at the same location, leading to activation of the same set of postsynaptic receptors. These assumptions are bolstered by observations made in several preparations where characteristics of spontaneous

unitary release events look identical to their evoked counterparts recorded under conditions that favor a very low release probability (e.g., low extracellular Ca^{2+}) or asynchronous release (e.g., substituting Ca^{2+} with Sr^{2+}) (17, 19). Although a number of studies spanning several decades have challenged these classical assumptions (e.g., Refs. 5, 10, 11, 14, 16, 32, 34, 66, 86), they were also re-affirmed by recent work performed on the neuromuscular junction and central synapses (e.g., Refs. 28, 35, 61, 81, 90). Nevertheless, recent findings indicating an independent function for spontaneous release in homeostatic plasticity are becoming increasingly difficult to reconcile with the classical view.

How can a postsynaptic response element distinguish an incoming unitary release event and trigger a distinct signaling pathway if the two forms of release originate from the same location in a terminal and activate the same population of target receptors? Under physiological conditions, unitary synaptic transmission events that arise from fusion of individual synaptic vesicles are largely similar in terms of their kinetics and receptor activation profiles regardless of whether vesicles were exocytosed spontaneously or in response to an action potential (18, 38, 54, 73, 81, 85). Under high-frequency burst activity, evoked release events summate at individual synapses leading to stronger depolarizations that maximize postsynaptic NMDA receptor activation (20) (see FIGURE 1). Synchronous activation of multiple clustered synaptic inputs can also trigger a local depolarization that leads to NMDA receptor potentiation. Therefore, in the absence of temporal and spatial summation of inputs at the level of an individual synapse, synaptic release events evoked at low frequency are expected to be indistinguishable from spontaneous release events with respect to their receptor activation and ensuing signaling profile.

Recent evidence, however, suggests significant differences between postsynaptic signaling profiles elicited by evoked and spontaneous neurotransmission. Several studies have shown that the timing and mechanism of homeostatic plasticity can be regulated by NMDA and/or AMPA receptor activation at rest in addition to the well characterized effect of action potential blockade (4, 22, 42, 74, 75). For instance, spontaneous neurotransmitter release and subsequent NMDA receptor activation,

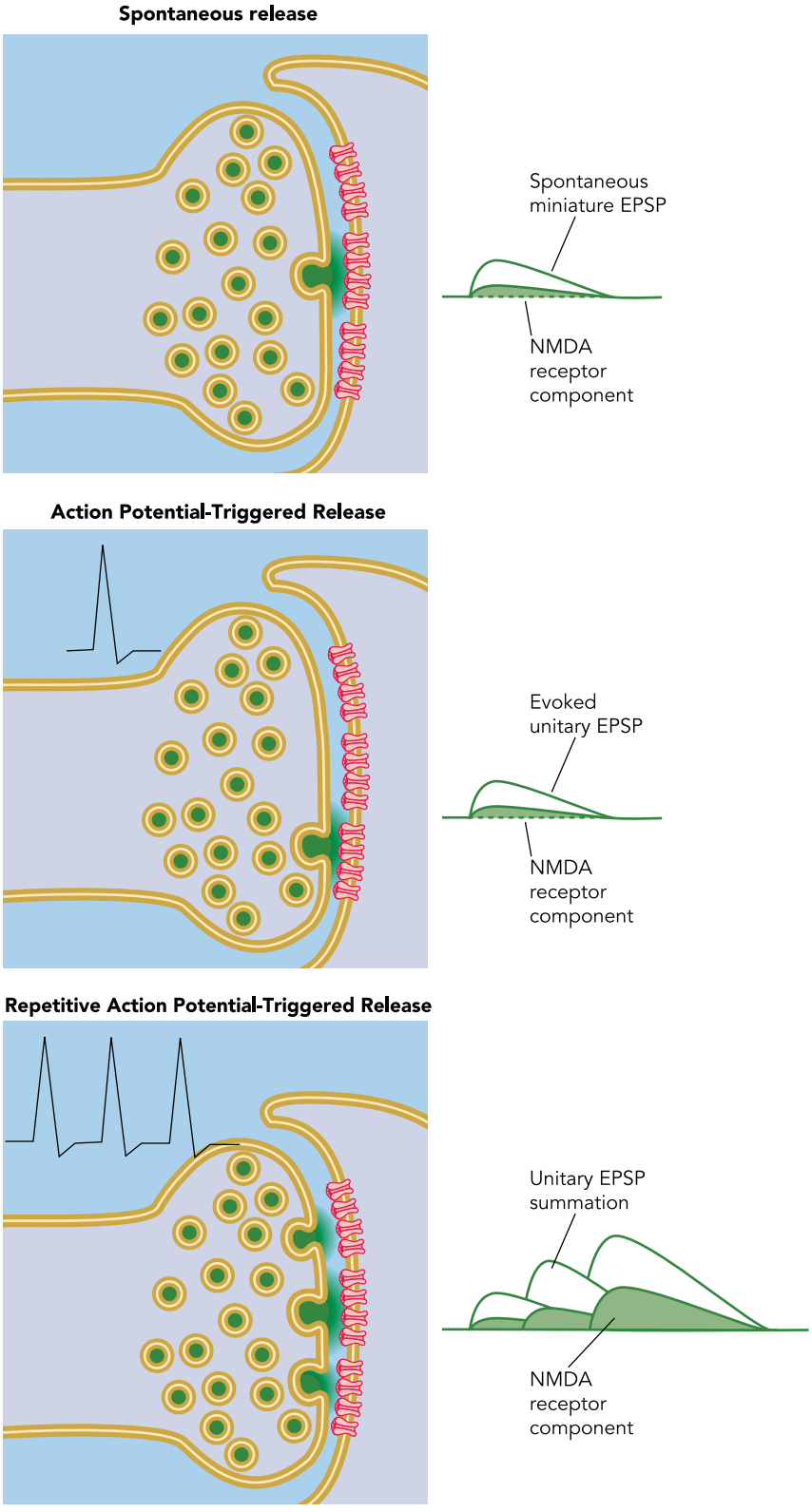

FIGURE 1. Distinguishing evoked and spontaneous fusion events at individual synapses
Excitatory postsynaptic potentials originating from spontaneous or presynaptic action potential-triggered fusion of a single synaptic vesicle containing the excitatory neurotransmitter glutamate are largely indistinguishable. Under both circumstances, only a small fraction of the current is carried by NMDA receptors (20). The impact of evoked release can only be distinguished during high-frequency bursts of presynaptic action potentials leading to temporal and spatial summation of events at individual postsynaptic sites and maximize postsynaptic NMDA receptor activation. Therefore, differences in signaling mediated by action potential-triggered and spontaneous neurotransmitter release are difficult to accommodate within a model in which the two forms of release originate from the same location in a terminal and activate the same population of target receptors.

rather than evoked release, specifically suppresses dendritic protein translation machinery by promoting phosphorylation and inactivation of eukaryotic elongation factor-2 (eEF2), a critical catalytic factor for ribosomal translocation during protein synthesis (76). This suppression of protein translation stabilizes postsynaptic sensitivity to released neurotransmitters by maintaining subunit composition glutamate receptors (74, 75). These findings raise the question of how signaling by spontaneous miniature excitatory postsynaptic currents (EPSCs) mechanistically differs from those triggered by evoked release. If the two forms of neurotransmission activate different signaling cascades, then how can a postsynaptic neuron tell the difference between a quantal event driven by action potential and one that occurs spontaneously?

In a recent study, our group presented a set of findings that may help resolve this conundrum. We took advantage of MK-801, a high-affinity use-dependent open channel blocker of NMDA receptors and found that in high-density or autaptic hippocampal cultures, as well as hippocampal slices, use-dependent block of spontaneous NMDA receptor-mediated miniature excitatory postsynaptic currents (NMDA-mEPSCs) and evoked NMDA-dependent excitatory postsynaptic currents (NMDA-eEPSCs) were largely independent. In these experiments, MK-801 application at rest caused rapid reduction of NMDA-mEPSCs within minutes, but this block did not significantly hinder receptor activation in response to subsequent evoked release (FIGURE 2). We could also demonstrate that MK-801 block of NMDA-eEPSCs has minimal effect on subsequent NMDA-mEPSCs detected on the same cell. Furthermore, once NMDA receptors that are activated by both evoked and spontaneous release were blocked, NMDA-mEPSCs showed significant recovery at rest without concomitant recovery of NMDA-eEPSCs. Taken together, these findings suggest that evoked and spontaneous forms of glutamate release activate largely nonoverlapping populations of NMDA receptors. In retrospect, these rather surprising results are in agreement with two key earlier observations. First, application of MK-801 in the absence of stimulation for up to 15 min results in minimal block of subsequent evoked NMDA responses, which supports the strict use dependence of MK-801 action (31, 37, 64, 68). Second, spontaneous release typically occurs with a rate in the order of 0.01 Hz per release site (25, 50, 66). Collectively, these earlier findings predict that a 10-min application of MK-801 at rest should provide more than sufficient time to diminish NMDA receptor activity triggered by spontaneous release without hindering subsequent NMDA-eEPSCs, which is indeed the case as indicated by our laboratory's recent experiments (5).

There are several scenarios that can accommodate the difference between spontaneous and evoked synaptic vesicle fusion pathways at the microscopic level and help explain these findings (see FIGURE 3). Arguably, the simplest scenario is the possibility that spontaneous and evoked fusion events originate from different synapses, thus they target distinct postsynaptic sites and activate different receptors. However, studies in hippocampal synapses monitoring uptake and release of fluorescent markers as well as trafficking of fluorescently tagged synaptic vesicle proteins have documented substantial co-localization of spontaneous and evoked synaptic vesicle recycling in individual synaptic boutons (5, 11, 23, 28, 50, 61, 66). The same studies have also shown that the sizes of the vesicle pools labeled with spontaneous vs. evoked uptake of fluorescent probes in a single synaptic terminal are strongly correlated (23, 28, 50, 61, 66). Furthermore,

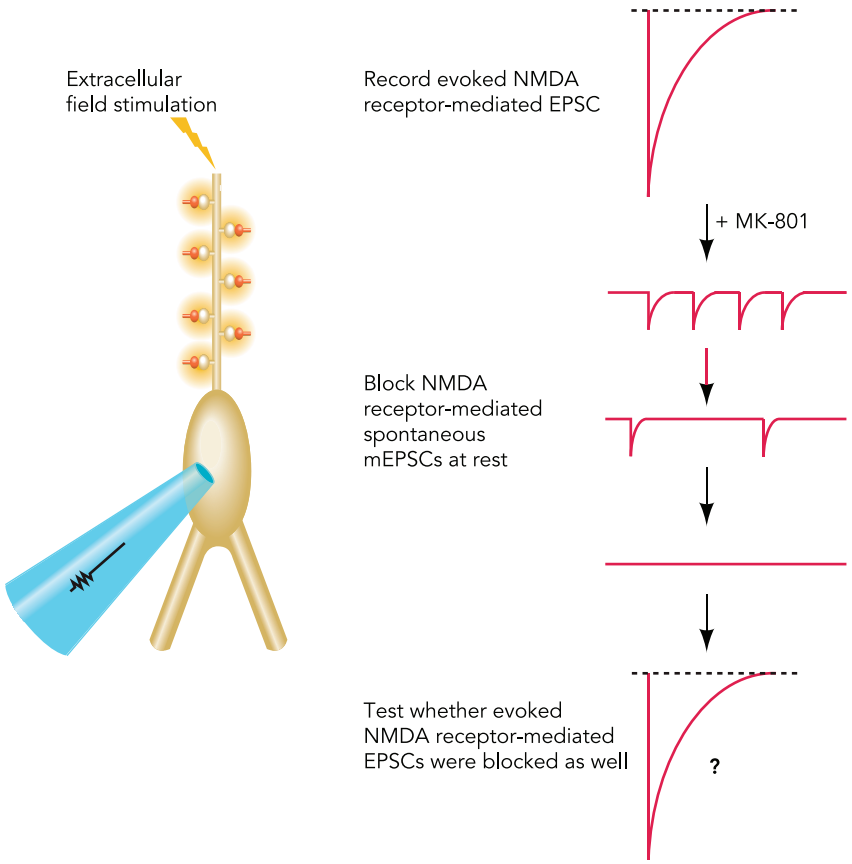

FIGURE 2. **Assessing the cross-talk between NMDA receptors activated in response to evoked vs. spontaneous fusion events**
In these experiments, application of MK-801, a high-affinity use-dependent open channel blocker of NMDA receptors under resting conditions caused rapid reduction of NMDA-mEPSCs within minutes, but this block did not significantly hinder receptor activation in response to subsequent evoked release. We performed these experiments using whole cell voltage-clamp recordings in high density as well as in autaptic hippocampal cultures, in addition to hippocampal slices. This result suggests that spontaneous NMDA receptor-mediated miniature excitatory postsynaptic currents (NMDA-mEPSCs) and evoked NMDA-dependent excitatory postsynaptic currents (NMDA-eEPSCs) are largely independent.

optical analysis of the synaptic vesicle protein synaptophysin tagged with superecliptic pHluorin (synaptophysin-pHluorin) (97) showed that a large majority of synapses (~80%) were capable of both evoked and spontaneous release, although the kinetics of the two forms of release did not show correlation in a given synapse (5). A study in the frog neuromuscular junction found that the level of spontaneous release is relatively uniform across active zones and that the location of spontaneous release corresponded well with the sites of evoked release, although the propensity of evoked release varied widely among active zones (95). These findings support the premise that spontaneous and evoked release have substantial overlap in their sites of origin, but they may not possess significant correlation with respect to their kinetics. Therefore, complete segregation of spontaneous and evoked neurotransmitter release into different synapses seems a rather unlikely possibility.

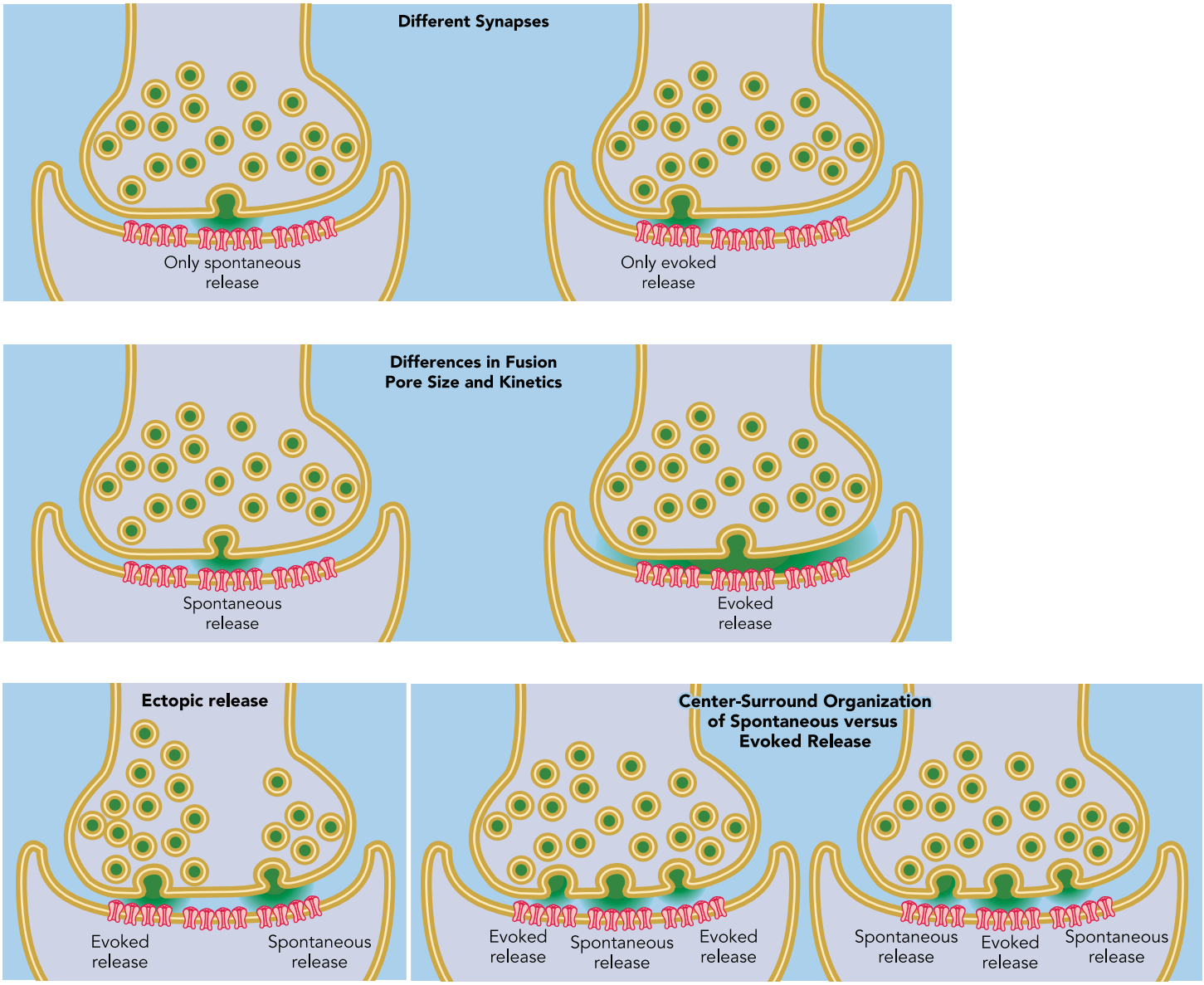

FIGURE 3. **Four non-mutually exclusive scenarios for the structural origin of spontaneous neurotransmission**
Different synapses: According to this model, some synapses may have a strong propensity for spontaneous fusion, whereas others may preferentially release neurotransmitter in response to action potentials. This model is not necessarily mutually exclusive with the "center-surround" model. Small nerve terminals (<0.2 μm^2) may preferentially fit into this category. *Difference in fusion pore size or kinetics*: The dichotomy between MK-801 block of NMDA-eEPSCs and NMDA-mEPSCs may also be accounted for by potential differences in fusion pore kinetics or glutamate release profile of spontaneous and evoked fusion events. For instance, in a given synapse, evoked fusion events may reach a higher fraction of receptors, whereas spontaneous fusion events may activate only a small number of receptors, although the two receptor populations overlap. *Ectopic release*: Spontaneous synaptic vesicle fusion may occur away from the active zone or the main release site, thus releasing neurotransmitter ectopically. *Center-surround organization of spontaneous vs. evoked release*: According to this model, spontaneous and evoked fusion events occur at distinct locations within a synaptic terminal. In particular, terminals larger than 0.2 μm^2 can accommodate the two forms of release within their geometry (5). In this model, spontaneous fusion events may either predominantly occur at the center of the terminal or at the periphery as long as the distance between the two fusion events allow postsynaptic signaling with minimal overlap (see Ref. 5 for a detailed discussion).

Nevertheless, it is difficult to completely exclude the premise that some spontaneous and evoked fusion events may occur at different synapses. Indeed, immature synaptic boutons typically favor spontaneous release and fail to respond to action potential stimulation (49, 70, 84, 92), which raises the possibility that a population of nascent synapses in an otherwise mature synaptic network may selectively sustain spontaneous release. Recent optical imaging results from our group indicate that a sizable fraction of synapses (~20%) support spontaneous or evoked transmission at the expense of the other (5). Interestingly, this analysis also revealed a set of presynaptic terminals that support action potential-driven release with negligible concurrent spontaneous vesicle exocytosis. The prevalence of these types of synapses is hard to ascertain due to an inherent bias associated with optical analysis where identification of functional synaptic boutons by fluorescent puncta selection typically favors large synapses over small ones. Therefore, it is likely that this fraction is higher than our estimates. The rapidly expanding repertoire of super resolution optical techniques will be important for future studies to address this question with better accuracy (91).

The dichotomy between MK-801 block of NMDA-eEPSCs and NMDA-mEPSCs may also be accounted for by potential differences in fusion pore kinetics or glutamate release profile of spontaneous and evoked fusion events. For instance, in a given synapse, evoked fusion events may reach a higher fraction of receptors, whereas spontaneous fusion events may activate only a small number of receptors, although the two receptor populations overlap. This possibility, however, contradicts several earlier observations. Both forms of fusion have been shown to equally stimulate AMPA receptors despite potential differences in their fusion pore kinetics (73, 82). AMPA receptors possess a substantially lower affinity for glutamate than NMDA receptors, and their activation by both spontaneous and evoked release suggests rapid unloading of vesicular glutamate under both circumstances (62). In addition, both forms of vesicle trafficking can be tracked with styryl dyes (e.g., FM1-43 dyes) as well as large probes such as antibodies to synaptic vesicle proteins or horseradish peroxidase (23, 66), arguing against the involvement of a narrow fusion pore hindering glutamate release.

A third scenario suggests that spontaneous fusion events may occur ectopically (46, 13), outside the active zones, as proposed by some earlier work (14, 10). This scenario is consistent with a recent study in retinal bipolar cell presynaptic terminals that took advantage of the high optical resolution provided by total internal reflection fluorescence microscopy. In this elegant study, Zenisek showed that spontaneous fusion events were largely excluded from synaptic ribbons, which comprised the preferential site for evoked fusion (96). Independent MK-801 sensitivity of evoked and spontaneous neurotransmission may partly be consistent with this possibility as long as this "ectopic" release occurs at discrete spots and activates a clustered set of adjacent receptors. The fact that the kinetics of spontaneous and evoked quantal events match under most circumstances (18, 38, 73, 81, 85) makes a diffuse form of off-target ectopic release an unlikely option to account for differences in NMDA receptor activation by evoked and spontaneous neurotransmitter release. The compartmentalization of evoked and spontaneous fusion sites may occur within a single synapse, presumably in the vicinity of a given active zone, thus activating receptors in different subdomains of the postsynaptic density. This idea agrees with quantitative estimates suggesting medium to large ($>0.2\ \mu m^2$) synapses can accommodate independent signaling via spontaneous and evoked release with some geometric constraints (5). However, as indicated above, small synapses ($<0.2\ \mu m^2$) may preferentially maintain spontaneous or evoked release and contribute to the dichotomy between NMDA-eEPSCs and NMDA-mEPSCs. Studies using two-photon imaging of Ca^{2+} transients induced by single vesicle fusion events revealed that unitary release activates only a small fraction of available NMDA receptors (52). Therefore, there is sufficient latitude for non-overlapping activation of NMDA receptors within a single synapse by evoked and spontaneous release events.

In summary, there are multiple scenarios that may explain the segregation of spontaneous and evoked synaptic signaling. These include accommodation of the two release forms within the same synapse, possibly maintained via a separate pool of vesicles, which may recycle independently (11, 23, 66). This scenario implies that synaptic vesicles that maintain spontaneous and evoked neurotransmission may possess certain molecular distinctions or possibly distinct molecular tags that segregate their trafficking and function (11, 23, 34, 66). In some cases, spontaneous synaptic vesicle fusion may occur away from the active zone, thus releasing neurotransmitter ectopically (96). Finally, some synapses may have a strong propensity for spontaneous fusion, whereas others may preferentially release neurotransmitter in response to action potentials. Although existing optical imaging data supports the first two possibilities, electrophysiological findings that show differential activation of NMDA receptors as well as developmental profiles of spontaneous and evoked release makes the third scenario a viable alternative. The relative prevalence of these distinct forms of synaptic

compartmentalization requires future experiments, which should undoubtedly reveal differences among distinct synapse populations as well as in a particular synapse during its lifetime.

Differential Presynaptic Regulation of Spontaneous and Evoked Neurotransmission

In addition to segregation of postsynaptic receptors and downstream signal transduction pathways between spontaneous and evoked forms of fusion, a large body of work has uncovered surprising distinctions in presynaptic regulation between the two forms of neurotransmitter release by a number of pathways. Selective targeting of evoked release by neuromodulators is not surprising, since inhibition of neuronal action potential firing or presynaptic voltage-gated Ca^{2+} influx can easily achieve this outcome via well established pathways without interfering with spontaneous release. However, some signal transduction pathways selectively impact spontaneous neurotransmission or alter the two forms of release in opposite manner. For instance, in rat cerebellar slices, activation of presynaptic group II metabotropic glutamate receptors selectively inhibits spontaneous but not Ca^{2+}-dependent evoked release machinery (26). In contrast, immature visual cortical neurons show a specific enhancement of spontaneous mEPSCs in response to BDNF application, whereas the same treatment leaves evoked neurotransmission largely unaffected (78). Furthermore, in hippocampal neurons, inhibition of DNA methyltransferases, key enzymes that methylate DNA and regulate gene expression in cells, results in a selective activity-dependent decrease in the frequency of miniature EPSCs, which in turn impacts neuronal excitability and network activity (51). Along the same lines, certain nitric oxide-related species inhibit evoked neurotransmission but enhance spontaneous mEPSCs (57), and neuronal cholesterol depletion or inhibition of cholesterol synthesis causes a similar increase in the rate of spontaneous transmission, although it depletes most vesicles that carry out evoked neurotransmission or impairs their fusion efficiency (87, 94). Finally, chronic induction of endoplasmic reticulum stress causes an increase in paired pulse depression consistent with a small (~20%) increase in neurotransmitter release probability but at the same time gives rise to a dramatic fourfold increase in spontaneous excitatory transmission (53). These seemingly disparate results share a common premise where the signal transduction pathways in question impact spontaneous and evoked vesicle fusion differentially and, in some cases (e.g., NO species, cholesterol depletion), in the opposite direction.

Although the elucidation of aspects of presynaptic machinery responsible for this dichotomy in regulation of the two forms of neurotransmitter release remains incomplete, recent studies have provided significant new leads.

Structure-function analysis of neuronal SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) including plasma membrane associated SNAP-25 and vesicular synaptobrevin 2 (also called VAMP2), which together with syntaxin1 comprise the core synaptic vesicle fusion machinery (39, 71), revealed three key differences between molecular interactions that give rise to spontaneous and evoked fusion. First, loss of SNAP-25 and synaptobrevin 2 in central neurons largely abolishes Ca^{2+}-dependent evoked release but diminishes most but not all spontaneous release (8, 67, 86), suggesting a role for alternate SNAREs in mediating low levels of spontaneous release. Second, in synaptobrevin 2-deficient synapses, spontaneous release could be rescued by expression of a synaptobrevin 2 construct with insertion of 12 residues between the SNARE motif and transmembrane region, whereas the same construct was not able to restore action potential evoked release (15), indicating the physical constraints on SNARE complex assembly are less stringent for spontaneous release. Finally, in neurons obtained from SNAP-25-null mice, expression of SNAP-25 mutants destabilizing the COOH-terminal end of the SNARE bundle did not rescue spontaneous neurotransmitter release but largely restored evoked release probability. In contrast, destabilizing the middle or deleting the NH_2-terminal end of the SNARE bundle potentiated the propensities of both spontaneous and evoked fusion. Interestingly, both manipulations had a more dramatic effect on spontaneous release compared with evoked neurotransmission (89). Taken together, these three observation suggest that, although both forms of fusion by and large utilize the same molecular machinery, they rely on distinct molecular interactions of the same components for normal function.

In addition to differences in basic synaptic vesicle fusion machinery, Ca^{2+}-dependent regulation of spontaneous release rate may also require diverse molecular players compared with evoked release. Spontaneous synaptic vesicle fusion can be regulated by extracellular Ca^{2+} as well as fluctuations in intracellular calcium (3, 36, 43). In contrast to the highly cooperative Ca^{2+} dependence of evoked transmission, spontaneous neurotransmission displays close to linear Ca^{2+} dependence (44, 72). Spontaneously recycling synaptic vesicles can be labeled with antibodies against the lumenal domain of synaptotagmin1 (66), but specific Ca^{2+} binding residues within synaptotagmin 1, which supports Ca^{2+} dependence of spontaneous

release, differ from the key residues that determine cooperativity of evoked release (93). In addition, loss of synaptotagmin 1 may remove a fusion clamp on spontaneous release or instead may recruit an alternate Ca^{2+} sensor with distinct Ca^{2+} dependence profile such as doc2b (29). Ca^{2+} dependence and fusion propensity of spontaneous release can also be modified by other synaptic vesicle proteins such as synaptotagmin 12 (47).

Arguably, the most provocative proposals on the segregation of evoked and spontaneous release originated from analysis of vesicle populations within individual synapses that recycle in the presence or absence of action potential firing. These studies suggest that the two vesicle populations do not overlap (Refs. 23, 41, 66; but also see Refs. 28, 35, 61, 90), which supports distinctions not only in fusion mechanisms but also in overall identity of vesicles that sustain the two forms of release (see FIGURE 4). Moreover, acute application of dynasore, a reversible inhibitor of essential endocytic protein dynamin, showed that evoked synchronous and asynchronous release originate from the same vesicle pool that recycles rapidly in a dynamin-dependent manner, whereas a distinct vesicle pool sustains spontaneous release independent of dynamin activation (11). These findings imply that the distinct identities of spontaneous and evoked recycling vesicles are not perturbed on exocytosis-endocytosis. This premise is consistent with the prevalence of synapses that only support spontaneous neurotransmission and spontaneous synaptic vesicle recycling at early stages of synapse maturation (49, 70, 84, 92). Interestingly, purified synaptic vesicles show an intrinsic tendency for unregulated constitutive fusion (33), suggesting that evoked regulated fusion constitutes a gain-of-function that is attained gradually during synapse maturation. Accordingly, mature synapses may also contain a population of these "immature" vesicles that are unable to respond to brief action potential stimulation but fuse and recycle constitutively (49).

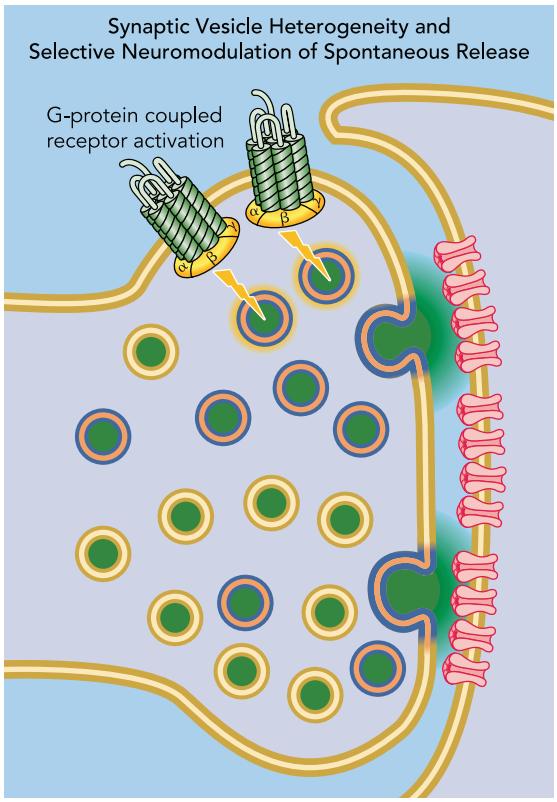

FIGURE 4. **Synaptic vesicle heterogeneity within individual synapses**
The figure depicts a model that can account for the selective regulation of spontaneous neurotransmission by some signal transduction pathways. This model suggests that spontaneous and evoked fusion may occur in the same synapses but may be carried out via a separate pool of vesicles, which may recycle independently (see Refs. 11, 23, 66). Moreover, vesicles that recycle spontaneously (red vesicles) may have an intrinsic molecular difference (possibly possess a unique effector; see Ref. 47) that renders them selectively vulnerable to certain signal transduction pathways (depicted by the red receptor; see Ref. 26).

Compartmentalization of Distinct Forms of Neurotransmission

Our understanding of the mechanisms that maintain spontaneous synaptic transmission are only beginning to be elucidated. There is much work to be done to uncover the role of spontaneous fusion events in neuronal signaling and homeostasis. However, it is interesting to note that, from an engineering point of view, multichannel parallel signaling is a common feature of most communication networks. These auxiliary communication channels typically serve essential logistical functions to ensure error correction, maintenance, and connectivity of the primary information transfer channel. Therefore, it is plausible to expect that by taking advantage of spontaneous neurotransmission, the nervous system incorporates such an auxiliary signaling network that functions to maintain synaptic homeostasis and synaptic connectivity within a sufficiently large dynamic range for reliable information transfer and storage. Testing this premise requires the development of novel approaches. The identification of distinct molecular markers associated with spontaneous synaptic vesicle recycling may in turn enable selective manipulation of spontaneous neurotransmission and help us elucidate its role in neuronal signaling. ■

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