Perspectives on Kiss-and-Run: Role in Exocytosis, Endocytosis, and Neurotransmission

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Abstract

Regulated exocytosis and endocytosis are critical to the function of many intercellular networks, particularly the complex neural circuits underlying mammalian behavior. Kiss-and-run (KR) is an unconventional fusion between secretory vesicles and a target membrane that releases intravesicular content through a transient, nanometer-sized fusion pore. The fusing vesicle retains its gross shape, precluding full integration into the planar membrane, and enough molecular components for rapid retrieval, reacidification, and reuse. KR makes judicious use of finite presynaptic resources, and mounting evidence suggests that it influences synaptic information transfer. Here we detail emerging perspectives on KR and its role in neurotransmission. We additionally formulate a restraining force hypothesis as a plausible mechanistic basis for KR and its physiological modulation in small nerve terminals. Clarification of the mechanism and function of KR has bearing on understanding the kinetic transitions underlying SNARE-mediated fusion, interactions between vesicles and their local environment, and the influence of release dynamics on neural information processing.

INTRODUCTION

The Importance of Vesicle Recycling and Fusion Modes

Neurotransmitter release by exocytosis underlies rapid chemical signaling between neurons. Alterations in the amount, location, and kinetics of release have profound consequences for neurophysiological function. Accordingly, synaptic vesicles, the compartments that store transmitter, are subject to exquisite control mechanisms that govern where, when, and how their contents are released. Because vesicle dynamics are of critical importance to synaptic function, deciphering how vesicles are utilized, retrieved, and modulated is crucial to understanding how neurotransmission participates in neural network function.

Synaptic vesicles are tiny lipid-ensheathed structures ~40–50 nm in diameter. Anywhere from dozens to several hundred of them (1) are harbored in a typical ~1-μm presynaptic terminal in the central nervous system (CNS). Vesicles undergo Ca^{2+}-dependent fusion with a target membrane, opening a conduit for the release of transmitter termed the exocytic fusion pore (2, 3). Elegant work by several labs has uncovered the molecular identity of various proteins directly or indirectly involved in vesicle fusion, including synaptobrevin (also known as VAMP), syntaxin and SNAP25 (collectively known as SNARE proteins), complexin, the Muncs, and synaptotagmin (4–6). Additional ultrastructural and biochemical evidence has provided insight into the macromolecular organization of these proteins (7–9); such organization enables them to respond rapidly to Ca^{2+} triggering (10) and to overcome energy barriers inherent in merging together two stable membranous compartments (11).

After exocytosis, vesicles must be recycled in preparation for another round of release. Vesicle recycling is critical to replenishing presynaptic vesicle pools (12, 13), sustaining transmitter release in the face of continuous activity (14), and preserving presynaptic morphological and structural integrity (15). The dynamic capabilities of synapses are determined in part by the number of recycling vesicles and the speed of their recycling. Generally, the number of functionally active vesicles is less than that evident from electron microscopy. This is because synapses contain functionally distinct vesicle pools, including a variable fraction that does not recycle, variously termed reserve or resting pools (1, 16). At some central synapses the fraction of recycling vesicles can be as low as ~15–30% (17; but cf. 18). Recent data from monitoring in vivo recycling at calyceal and neuromuscular junction synapses have further suggested that a mere 1–5% of vesicles are available to recycle during relevant behavioral activity (19). The number of recycling vesicles can sometimes be expanded by cytosolic signaling (20), but even so, limits on the functionally active population place kinetic demands on speedy vesicle recycling to sustain release (21–23).

Possibly relevant to the dynamics of vesicle reutilization, one current view is that exocytosis occurs in at least two distinct ways: (*a*) by full-collapse fusion (FF), wherein the fusion pore rapidly dilates, allowing the vesicle to fully flatten into the planar surface of the target membrane and integrate its lipid and protein content, or (*b*) by kiss-and-run (KR), in which the vesicle releases its contents through a transient, narrow fusion pore while retaining its gross morphological shape. As described below, there are several inherent differences between these two fusion modes, both in the way transmitter is released and in the details of subsequent vesicle retrieval and reuse. Building on the classic work of Heuser & Reese (12), a large body of investigation has firmly established a central role for FF and clathrin-mediated endocytosis (CME) in vesicle cycling. Individual kinetic steps and many molecular components of this cycle have been revealed (24–28). In small terminals of CNS neurons, FF leaves vesicle components on the plasma membrane for an average period of ~15 s (29–31) before vesicle membrane and protein are recaptured by CME at sites outside the active zone.

Much less is certain about KR, the subject of this review. Part of the appeal of KR stems from its possible relevance to multiple issues of interest: how SNAREs, synaptotagmin, and other synaptic proteins operate to facilitate fusion; in what manner transmitter is released; and how vesicles are efficiently retrieved. Although the importance of KR to the operation of nonneuronal systems is now generally accepted (32–34), its relevance in neuronal systems has remained a topic of intense debate. Fortunately, there has been considerable recent progress. In preparing this review, we mainly emphasize work since the last full review from our group, in 2006 (22). Other investigators have published several excellent reviews (35–37), as well as three informative pieces in Annual Reviews journals: one by Sørensen (3) on the fusion machinery and fusion pore, another by Jackson & Chapman (38) on the fusion pore, and a third by Dittman & Ryan (27) on the molecular circuitry of endocytosis. These treatments run the gamut from clear acceptance to frank skepticism about KR. In this article, we do not avoid controversial issues but try to state opposing views clearly and simply before providing our personal interpretation. We attempt to do justice to an extensive literature and to a growing array of new experimental strategies.

Working Definitions of a Kiss-and-Run Event

A range of experimental criteria have been employed to assign individual fusion events to KR or FF. The criteria most commonly used emphasize morphological or biophysical features that can be assayed by various probes of vesicle dynamics (see Appendix).

Vesicle morphology. Ceccarelli and colleagues (13) forwarded their original proposal for transient fusion (putative KR) on the basis of synaptic ultrastructure and labeling experiments undertaken by the use of antibodies against luminal epitopes within the vesicle. They observed preservation of vesicle morphology as a key aspect of what came to be termed KR (39). Indeed, conservation of vesicle shape has remained a cornerstone feature of KR, along with retention of basic functionality (22). Investigators of nonneuronal cells have generally embraced granule morphology as a basic criterion for KR (33, 34, 40). For example, a diffusible fluorescent marker in the cytosol of PC12 cells was continuously excluded from the volume occupied by large dense-core vesicles (LDCVs) undergoing secretion (41), a feature of transient fusion termed cavicapture (42).

Less clear is the extent to which retrieved vesicles keep their membrane constituents such as lipids and proteins (43). Retention of vesicular membrane components shows great variability in neuroendocrine cells (40). During transient fusion events, lipidic probes can be fully discharged into the plasma membrane (44, 45) or retained in the vesicle (46). Likewise, some intrinsic membrane proteins may escape whereas others remain captive, leading some investigators to subdivide KR into subclasses (40). Given the range of possibilities, a simple working definition of KR would include any nonclassical exo-endocytic event that maintains vesicle shape and at least minimal functionality (22).

Vesicle recycling speed. At synapses, exclusion of a cytosolic marker (41) is not suited for demonstrating the morphological retention of a vesicle, because its ~50-nm cavity is well below the optical diffraction limit. This presents a significant hurdle in the study of small synaptic vesicles that has motivated novel microscopy approaches (47) and alternative probe development (see Appendix). Meanwhile, much of the focus has been on a powerful aspect of vesicle recycling by KR in nerve terminals: its speed. Current estimates for the elapsed time between exo- and endocytosis during KR are <1 s. Kinetic distinctions are arguably the most widely applied and functionally relevant criteria for classifying an event as KR (see Appendix and **Figure 1**). In such classifications,

individual records must be assignable to a population of events clearly distinct from those arising from classical vesicle recycling, a sometimes ambiguous distinction (30; but see below).

EMERGING CONCEPTS IN THE STUDY OF PRESYNAPTIC DYNAMICS

New Approaches and Insights

Next, we briefly describe seven novel strategies or insights that have significantly shaped our current perspective on presynaptic dynamics and vesicle fusion modes. Some of these uncover thornier issues that are considered in detail in later sections.

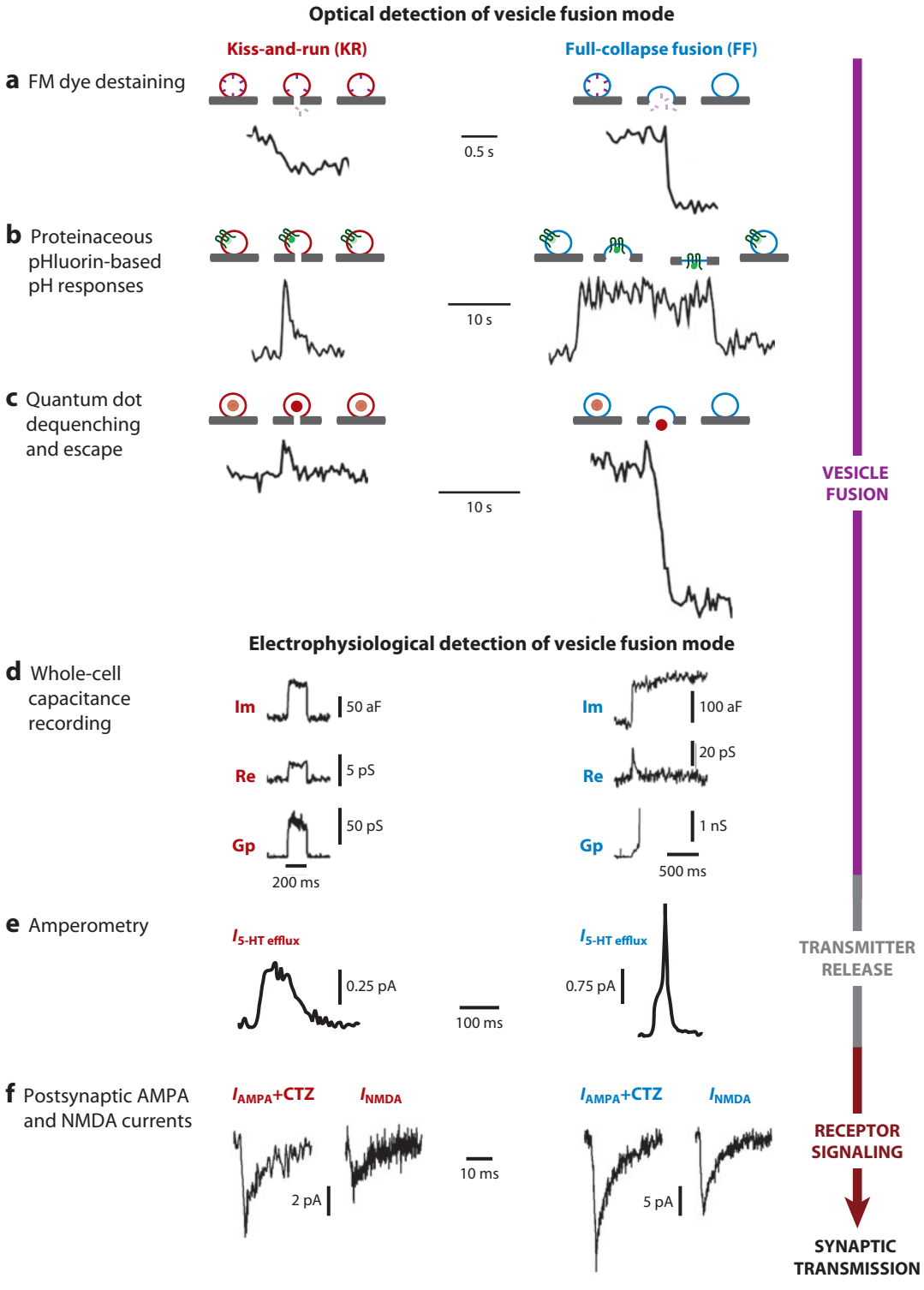
Advent of a set of optical reporters with improved and complementary features. Novel optical reporters of intravesicular pH with better signal and less background, including vGlut1-pHluorin, SypH4X, and quantum dots (Qdots), have improved the ability to track vesicular proteins or individual vesicles as a whole. Their principles, along with other key methods, are described in the Appendix.

An emerging picture of the unitary properties of kiss-and-run events. Using newer indicators, investigators who study KR have arrived at considerable consensus on the kinetics of single KR events (31, 48–50). Estimates of fusion pore open times are of the order of <1 s (48–50), much faster than in many nonneuronal cells. After fusion pore closure, reacidification ensues with τ = ~1–3 s (31, 48, 49). Further study with Qdots suggests that after KR, reuse proceeds with a $t_{1/2}$ < 6 s and can support multiple rounds of KR before FF (49). After they are recaptured, KR vesicles remain within a vesicle diameter of their initial release site before the next fusion (49).

Significant regulation of kiss-and-run prevalence and unitary properties. It has become increasingly clear that KR displays highly variable prevalence, depending on the release probability of the synapse (P_r) (48), intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (50, 51), phosphorylation state (50), frequency of stimulation, history of previous activity, and pool identity of the fusing vesicle (49, 52, 53). Moreover, fusion pore dynamics and retrieval kinetics appear to be heavily modulated (45, 49, 50, 54). All these factors likely have significant consequences for synaptic transmission (55–57).

Figure 1

Multiple methods distinguish between modes of vesicle fusion and recycling. (*a–f*) A gallery of methods distinguish kiss-and-run (KR) (*left*) from full-collapse fusion (FF) (*right*) in various neurosecretory cells, including pyramidal cells of the hippocampus (*a–c, f*), the calyx of Held (*d*), and pancreatic β cells (*e*). The distinction is found along multiple dimensions of synaptic transmission, including fusion itself (*a–d*), transmitter release (*e*), and postsynaptic receptor activation (*f*). Exemplar evidence is based on optical detection (*a–c*) of the differential kinetics and extent of FM1-43 dye release (*a*); retrieval kinetics of synaptophysin tracked by pH-dependent, pHluorin-based SypH4X (*b*); and escape or retention of pH-sensitive quantum dots (*c*)—as depicted in the vesicle diagrams above each optical trace. Fluorescence traces are baseline aligned on the same vertical scale (in arbitrary fluorescence units) for each row (*a–c*). These complement electrophysiological methods (*d–f*) using whole-cell capacitance records with imaginary (Im) and real (Re) components reflecting vesicle size (in aF), retrieval kinetics (in ms), and pore conductance (Gp; in pS) (*d*); amperometric signals indicating different release profiles of serotonin (*e*); and whole-cell measurements of NMDA currents in Mg^{2+}-free solution [to curtail basal Mg^{2+} block of NMDA receptor (NMDAR)] and of AMPA currents in cyclothiazide (CTZ) (to block desensitization) (*f*). Rapidly increasing but detectable Gp (*d*; *right*) and prespike "foot" signals (*e*; *right*) indicate progression to FF through a fusion pore intermediate whose lifetime is often presumed too transient for temporal resolution. See Appendix for further details. Panel *a* reprinted from Reference 51, copyright 2010, The Physiological Society; panels *b* and *c* reprinted from References 49 and 115; panel *d* reprinted from Reference (56) with permission, copyright 2006, Macmillan Publishers; panel *e* reprinted from Reference 97 with permission from Elsevier; and panel *f* reprinted from Reference 57, copyright 2009, The Physiological Society.



Electrical recordings show that full-collapse and kiss-and-run fusion modes drive distinct postsynaptic responses. KR supporters and skeptics alike agree that "perhaps the biggest difficulty with the idea that kiss-and-run is the normal mode of exocytosis during low-frequency stimulation is the lack of electrophysiological evidence to support it" (29). Such evidence has now been provided in a landmark paper by Richards (57), who simultaneously monitored fusion mode with FM dye and recorded the corresponding postsynaptic currents (**Figure 1*f***). These findings are put into a broader conceptual and functional context below.

Superlocalization microscopy reveals that fusion mode choice is predicted largely by prior vesicle dynamics. Single, brightly labeled synaptic vesicles have been tracked in real time with nanometer accuracy (58) in three dimensions to uncover remarkable intra- and interbouton dynamics that likely impact synaptic performance. In fact, a striking relation exists between a vesicle's prior pattern of motion and its ensuing fusion mode. Long-time residents at the ultimate release site are strongly inclined to undergo KR, whereas vesicles traveling from afar are almost sure to fully collapse. Furthermore, fusion close to the central axis of the synapse favors KR, whereas fusion at the periphery is a virtual guarantee of FF. Evidently, the choice of fusion modes is not a purely stochastic event but is strongly affected by previous vesicle dynamics (59).

Fusion pore stability is related to the operation of SNARE proteins. Clues that SNARE-mediated force generation is involved in tilting the balance between KR and FF have come from disparate lines of experiments. First, weakening the mechanical coupling between SNARE complex formation and the membrane anchors in the bilayer dampens the progression from KR to FF (60, 61). Second, studies of SNARE function in reconstituted systems (62) suggest that, whereas one SNARE complex suffices for bilayer fusion (as in KR), three such complexes are needed to prevent the nascent fusion pore from reclosing (as in FF). Third, the stability of the fusion pore can be altered by modifiers of SNARE function, including synaptotagmin (63, 64), complexin (65, 66), and Gβγ (67, 68). Taken together, all these findings fit with the unifying hypothesis proposed below.

Complex presynaptic architecture underlies vesicle coordination and may influence fusion properties. Recent ultrastructural studies have uncovered an elaborate meshwork of filamentous links that connect synaptic vesicles to neighboring structures, including the cytoskeleton, active zone, and other vesicles (69–71), creating vesicle groups averaging approximately four vesicles in size (69). This interconnectedness may have physiological impact on certain forms of synaptic depression (72). Cytoskeletal organization is a potential target of modulation by different degrees of protein phosphorylation and may help determine fusion mode, as we discuss below.

Controversy Arising from Variable Abundance of Kiss-and-Run in Small Nerve Terminals

Disagreement exists regarding the incidence of KR in small nerve terminals, as is evident in discordant results obtained with pHluorin-based probes (**Figure 2**). Some studies suggest a great predominance of KR, at least under certain conditions (48–50, 73), whereas others find little or no indication of it (29, 30, 74). The wide range of findings raises questions about possible differences in experimental procedure and interpretation but also suggests that discrepancies may arise because KR prevalence is highly modulated.

Figure 2*a* illustrates the recycling of pHluorin-tagged vesicle proteins along with the expectation that various fusion modes will result in kinetically distinct optical signals. As **Figure 2*d*** shows, the SypHy signal behaved like a single component, conforming to a kinetic scheme in

which a simple process of vesicle retrieval ($\tau_e \approx 16$ s) was followed by reacidification ($\tau_r = 4$ s) (29). In contrast, using a similar probe, SypH4x, Zhu et al. (31) and our group (75) (**Figure 2*e***) found evidence for two distinct components of vesicle retrieval, one as slow as that in **Figure 2*d*** and the other considerably faster and roughly equal in prevalence (75). The fit of the averaged single-vesicle signals was better with two components, even after appropriate statistical penalties for additional parameters. The same was true for dwell time distributions, representing the time between fusion and the initiation of vesicle reacidification for individual fusion events (31). The signal obtained after acute application of the H^+-pump inhibitor bafilomycin confirmed that a large proportion of vesicles had been retrieved early and had undergone reacidification (31, 75).

In similar pHluorin-based experiments, Balaji & Ryan (30) found a distribution of single-vesicle dwell times that could be fitted with a single exponential ($\tau_e \approx 14$ s; **Figure 2*b***). This finding suggested a single Poisson process and fitted very well with the data of Granseth et al. (29). It would be interesting to explore how such a description meshes with CME, which involves many biochemical steps (76, 77); perhaps a single rate-limiting process dominates its dynamics. Balaji & Ryan (30) went on to speculate that previous evidence in favor of KR was a result of mistakenly taking the briefest bins of the dwell time distribution (<20% of events), with recycling kinetics consistent with putative KR, out of context. This perspective, however, conflicts with at least three lines of evidence: (*a*) Fast KR has been robustly identified at these synapses by using Qdot-based criteria dependent on vesicle morphology and not recycling speed (49), (*b*) averaged single-channel pHluorin records by others contain two clearly separable components (31, 75) (**Figure 2*c***), and (*c*) using the same vGlut1-pHluorin probe, Leitz & Kavalali (50) found distributions of dwell times with multiple components. This includes a large proportion of events (>80%) with dwell times of <200 ms (**Figure 2*c***, arrow), far in excess of what would be expected if retrieval were a single Poisson process. Thus, we believe that the published record contains a genuine difference in data that is more fundamental and puzzling than a mere error of interpretation.

Adding to this, Zhu et al. (31) provided a vivid experimental indication of the existence of multiple modes of retrieval. They found that some membrane proteins of an individual vesicle can subdivide; one subgroup is rapidly retrieved, whereas the remaining proteins undergo conventional slow retrieval. These researchers showed striking records of purported single-vesicle behavior, consisting of two phases of SypH4x fluorescence decay: (*a*) a portion that is redarkened by reacidification of a rapidly retrieved vesicle and (*b*) a remaining signal that dims much later, presumably by CME and reacidification. An acute bafilomycin exposure demonstrated that the rapid component of fluorescence decay is truly associated with reacidification following internalization, similar to that proposed for KR. Further FM dye experiments suggested that two vesicles' worth of membrane were retrieved after supposed single-vesicle fusion. These experiments put forth the possibility of partial loss of protein probe molecules from a vesicle without precluding that vesicle's ability to pinch off from the surface membrane. By extension, the retrieved vesicle would keep part of its complement of intrinsic proteins (31, 49, 50) and trap extracellular quencher (53), extracellular buffer (48, 49), and FM dye (31). The main doubt about this conclusion hinges on the presumption that only one vesicle initially fused (31). An appearance of early and late fluorescence recovery could have conceivably emerged from the fusion of two vesicles on a faster timescale than that at which the signal was sampled. We are, however, persuaded by the original interpretation (31) on the basis of the corroborative data of Zhang et al. (49).

Taken together, these comparisons emphasize the wide variation in apparent KR prevalence, even when KR incidence is studied with similar pHluorin-based probes at cultured hippocampal synapses bathed in 2 mM Ca^{2+}-containing media (**Figure 2*b*,*c***). One must consider the possibility that the disparate findings genuinely reflect an underlying modulation of fusion mode prevalence by physiological factors that vary during and between experiments.

PHYSIOLOGICAL MODULATION OF FUSION MODE PREFERENCE

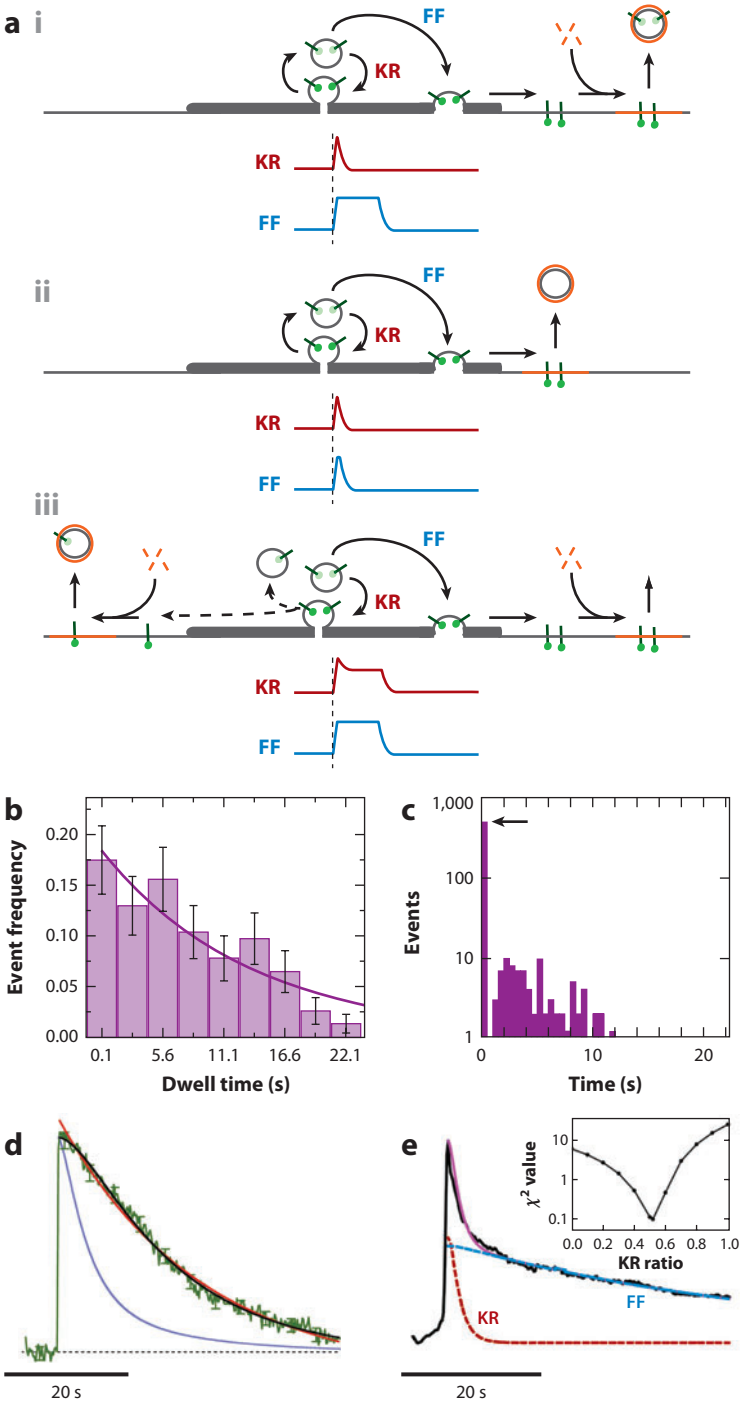
Dependence on Synaptic Release Probability and Calcium

Figure 3 provides a sampler of evidence that the relative incidence of KR is strongly regulated by key factors such as presynaptic P_r, intracellular Ca^{2+} accumulation, impulse frequency, and the previous history of repetitive activity, as schematized in **Figure 3*a***. In early pHluorin experiments, Gandhi & Stevens (48) observed that the pattern of vesicular retrieval varied sharply with P_r, with KR predominant (70% of events) at $P_r = 0.2$ and dropping to ~25% of events at $P_r = 0.42$ (**Figure 3*b*, *i***). In the same spirit, Leitz & Kavalali (50) directly addressed the effect of varying extracellular Ca^{2+} and found a systematic variation in dwell time distributions as extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was elevated from 1 to 8 mM. At 1 mM $[Ca^{2+}]_o$ (close to the physiological level of 1.1 mM), ~87% of events began to decay <0.5 s after vesicle fusion, consistent with KR. At 8 mM $[Ca^{2+}]_o$, the distribution of decay dynamics associated with vesicle retrieval and acidification was much slower, fitting an exponential with $\tau \approx 13$ s (average single-vesicle records in 2 mM and 8 mM Ca^{2+} are depicted in **Figure 3*b*, *ii***). Thus, the gamut of previously reported experimental results was largely recapitulated simply by varying $[Ca^{2+}]_o$ (50).

Richards (51) monitored exocytotic fusion mode with FM1-43 and intrabouton $[Ca^{2+}]_i$ with the Ca^{2+} probe Oregon green BAPTA-1. A gradual shift from 75% to ~0% KR was observed during stimulus trains at 5 or 10 Hz. These changes ran parallel to an accumulation of intrabouton $[Ca^{2+}]_i$. Thus, an orderly relationship was found between $[Ca^{2+}]_i$ and the predominance of FF

Figure 2

Conflicting results from pHluorin-based probes. (*a*) Recycling during kiss-and-run (KR) (*red*) and full-collapse fusion (FF) (*blue*) assayed by using pHluorin-tagged (*green*) vesicular proteins. (*i*) KR is expected to produce fast fluorescence transients (*red trace*) as single vesicles retain the tagged proteins and recycle through a short-lived fusion pore intermediate. In contrast, FF is expected to produce fluorescence traces (*blue trace*) with a long dwell time (mean >10 s) as single vesicle fusion deposits tagged proteins onto the plasma membrane and the slow accumulation of clathrin subunits (*orange*) and vesicular proteins at extrasynaptic sites leads to recapture of a clathrin-coated vesicle via clathrin-mediated endocytosis (CME). At least two potential alternative scenarios (*ii* and *iii*) may, however, obscure this clear kinetic distinction. (*ii*) Some FF vesicles recaptured via CME may have dwell time kinetics comparable to those of KR recycling, particularly if CME occurs from a clathrin-precoated pit. (*iii*) Some KR vesicles may lose some or all of their pHluorin-tagged proteins during transient lipid continuity, resulting in an apparent slowing of retrieval as lost proteins are recaptured via the classical CME pathway. Regardless of interpretation, clear differences in data do exist from similar pHluorin-based experiments. (*b*,*c*) Dwell time histograms from two different labs using single-vesicle responses of vGlut1-pHluorin-transfected hippocampal CA3-CA1 boutons in 2 mM Ca^{2+}. Experiments resulted in either (*b*) a single exponential distribution (150 events, with <20% of events in the 100-ms bin and an overall mean dwell time of ~14 s), or (*c*) a multicomponent distribution (>80% of 571 events occurring with dwell time <200 ms; indicated by *black arrow*). In panels *d* and *e*, average single-vesicle responses from synaptophysin constructs with one (*d*) or four (*e*) pHluorins (SypHy or SypH4X, respectively) display discordant fluorescence decay kinetics. In panel *d*, fluorescence average of SypHy (*green*) is interpreted as evidence for exclusive recycling via CME; kinetics fit well with a single exponential of $\tau = 22$ s (*red*) but are best described by a fit (*black*) presuming sequential endocytosis (average $\tau_e = \sim 16$ s) and reacidification ($\tau_r = 4$ s). Data are not well fitted by a model that incorporates 80% of vesicles being endocytosed with $\tau_e \sim 1$ s (*blue*). In contrast, the fluorescence traces of SypH4X in panel *e* have a clear multicomponent appearance, well fit (*purple*) by assuming the coexistence of fast KR (*red*; average $\tau_e = \sim 0.5$ s) and slow FF-CME (*blue*; average τ_e >10 s) vesicle recycling in roughly equal proportions. The inset shows that the χ^2 value of the fit falls to a minimum at a KR ratio of 52%. Panel *b* reprinted from Reference 30, copyright 2007, National Academy of Sciences. Panel *c* reproduced from Reference 50 with permission. Panel *d* reprinted from Reference 29 with permission from Elsevier. Panel *e* reprinted from References 49 and 75.



(**Figure 3*b*, *iii***). Richards's (51) data were in accord with earlier data from Harata et al. (53), suggesting a progressive shift toward FF with graded increases in stimulation frequency, although more complex features of frequency modulation are considered below. Like Leitz & Kavalali (50), Richards (51) found that the progressive shift toward FF was abolished by delivery of EGTA as an intracellular Ca^{2+} buffer (**Figure 3*b*, *iii***), suggesting that the tilt toward FF was supported by Ca^{2+} accumulation and possibly diffusional spread. Leitz & Kavalali (50) applied FK506, a specific inhibitor of calcineurin (CaN), and found that it markedly abbreviated fusion pore opening, thus opposing the effect of Ca^{2+} elevation on fusion pore behavior. Whatever the detailed molecular mechanism, the published evidence suggests that both elevation of Ca^{2+} entry (50) and naturally occurring gradations in P_r (48) promote FF at the expense of KR.

Role of Stimulus Frequency

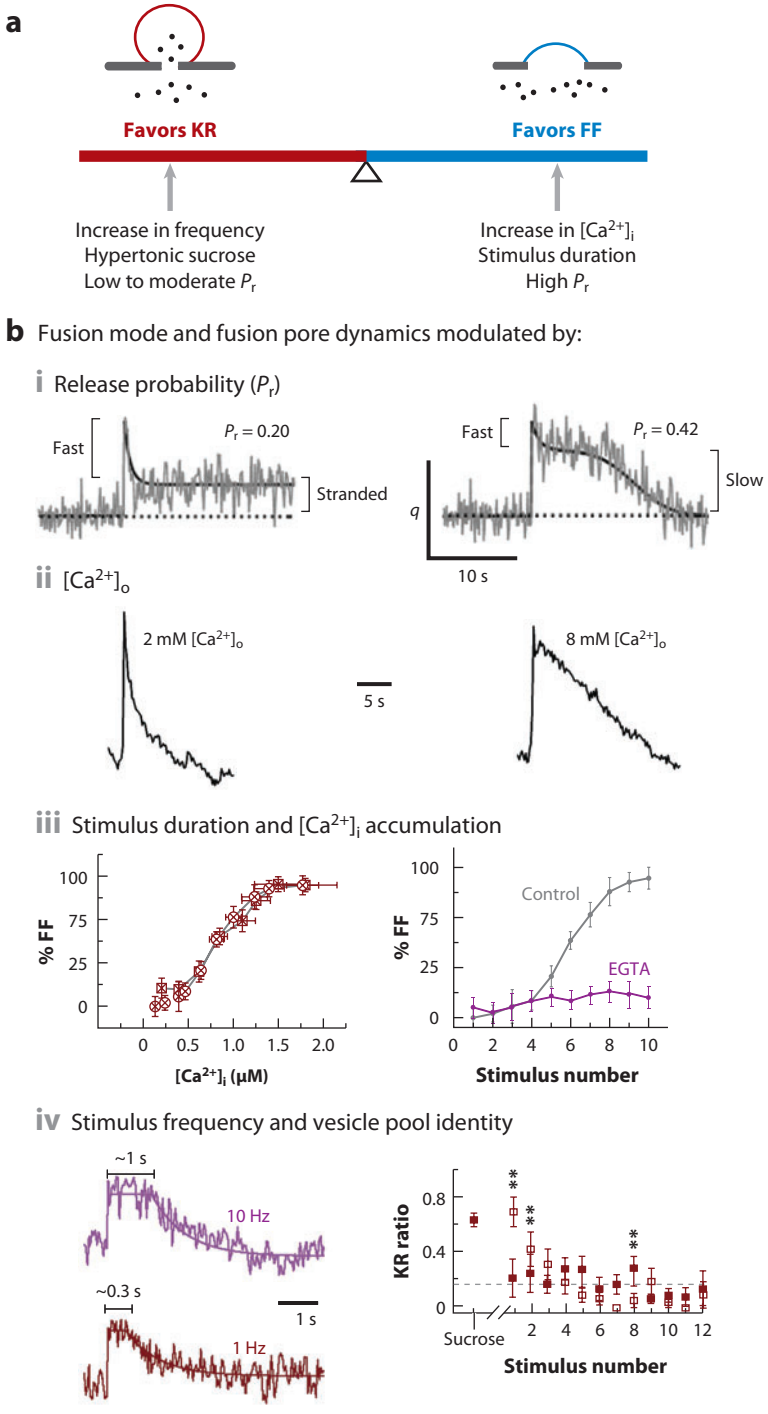
Published observations of the effects of varying stimulus frequency are difficult to interpret, in part because several parameters may be influenced simultaneously. First, increases in frequency may recruit low-P_r synapses that have an increased propensity to fuse by KR (48). This recruitment may contribute to an increased incidence of KR as frequency is increased at hippocampal synapses (49) and at mouse neuromuscular junctions (78). Second, increases in frequencies beyond ~2 Hz cause Ca^{2+} to progressively accumulate and favor FF (51), possibly by cumulative activation of SNARE complexes and engagement of multiple enzymatic mechanisms, including CaN activation (50) and phosphorylation of myosin II by myosin light chain kinase (MLCK) (78). Third, increases in frequency, possibly acting through Ca^{2+}, may modify the dynamics of the fusion pore during individual KR events. By tripling the fusion pore open time (49) (**Figure 3*b*, *iv***) and possibly fusion pore size, elevating frequency may influence the per-event exchange of lipid and protein probes between vesicle and surface membrane, over and above any alteration in the incidence of KR.

Dependence on History of Activity

Progressive changes in the balance between KR and FF unfold over the course of a train of stimuli. Following a rest period, KR dominates early in the train, whereas FF dominates late in the train

Figure 3

Dynamic modulation of fusion mode preference and unitary properties. (*a*) Multiple parameters underlie complex modulation of fusion mode preference, serving to increase the proportion of either kiss-and-run (KR) (*left*) or full-collapse fusion (FF) (*right*) events. (*b*) (*i*) Differences in basal synaptic release probability (P_r) correspond to variable proportions of fast and slow endocytosis in synaptopHluorin-expressing hippocampal boutons. In some cases, fusion leaves stranded vesicle protein on the presynaptic membrane. (*ii*) Elevating extracellular Ca^{2+} from 2 mM to 8 mM prolongs the decay kinetics of average single-vesicle responses of vGlut1-pHluorin-expressing hippocampal boutons. Traces are shown on a normalized vertical scale in arbitrary fluorescence units (not depicted). (*iii*) Correspondingly, a strong correlation exists between intracellular Ca^{2+} concentration and fusion mode (*left*). This correlation is consistent with block by EGTA, a Ca^{2+} chelator, of a progressive shift in fusion mode from KR to FF during 5-Hz stimulation (*right*). (*iv*) Increases in frequency extend fusion pore open time from a mean of ~300 ms at 1 Hz (*left*; *red*) to ~1 s at 10 Hz (*left*; *purple*) in quantum dot–loaded single vesicles in hippocampal terminals. Stimulation at 0.1 Hz leads to a progressive increase in the proportion of fusion events that proceed into FF (*right*; *open squares*). This shift is nullified by preapplication of hypertonic sucrose (*right*; *filled squares*) that discharges readily releasable pool (RRP) vesicles, suggesting that predominant loss of KR-favoring RRP vesicles underlies a fusion mode shift at low-frequency (i.e., 0.1-Hz) stimulation. *i* reprinted with permission from Reference 48, copyright 2003, Macmillan Publishers; *ii* calculated from data generously provided by Leitz & Kavalali (50); *iii* reprinted from Reference 51, copyright 2010, The Physiological Society; *iv* reprinted from Reference 49.



(**Figure 3*b*, *iii*** and ***iv***, right panels). Multiple approaches converge on the same basic finding but also lead to differing explanations. One hypothesis focuses on the accumulation of cytoplasmic Ca^{2+} and its responsiveness to intracellular Ca^{2+} buffering with EGTA (**Figure 3*b*, *iii***). Another hypothesis focuses on the vesicle's pool of origin, in particular, membership in the readily releasable pool (RRP). Zhang et al. (49) found progressive changes in fusion mode preference over the course of a train of 12 stimuli at 0.1 Hz, a frequency too low to generate Ca^{2+} accumulation. One possibility is that the train first depletes those vesicles that were long-time residents in the RRP; these vesicles are most prone to KR (59). In fact, discharge of the RRP with hypertonic sucrose eliminated the excess of KR (49) (**Figure 3*b*, *iv***). Regardless of the underlying mechanism, the prevalence of KR following a long rest aligns well with the natural patterns of spikes in vivo. Firing patterns in hippocampal neurons consist mostly of long periods of low activity punctuated by short bursts of high-frequency discharge, perhaps a dozen spikes at ~30 Hz (99, 100).

Altogether, there is ample evidence that the incidence of KR is not fixed but varies greatly with experimental conditions. KR is sometimes dominant and sometimes minor, depending on the biological system, P_r, pattern of firing, and degree of Ca^{2+} accumulation. Loosely speaking, the extremes of behavior observed in studies of modulation encompass the patterns evident in published reports that support or throw doubt on the existence of KR. We do not claim that this summary provides a complete explanation of why results with protein probes appear so variable. Perhaps additional factors, including the level of modulatory tone from G protein–coupled receptors (GPCRs), should be considered. Such additional factors are discussed below.

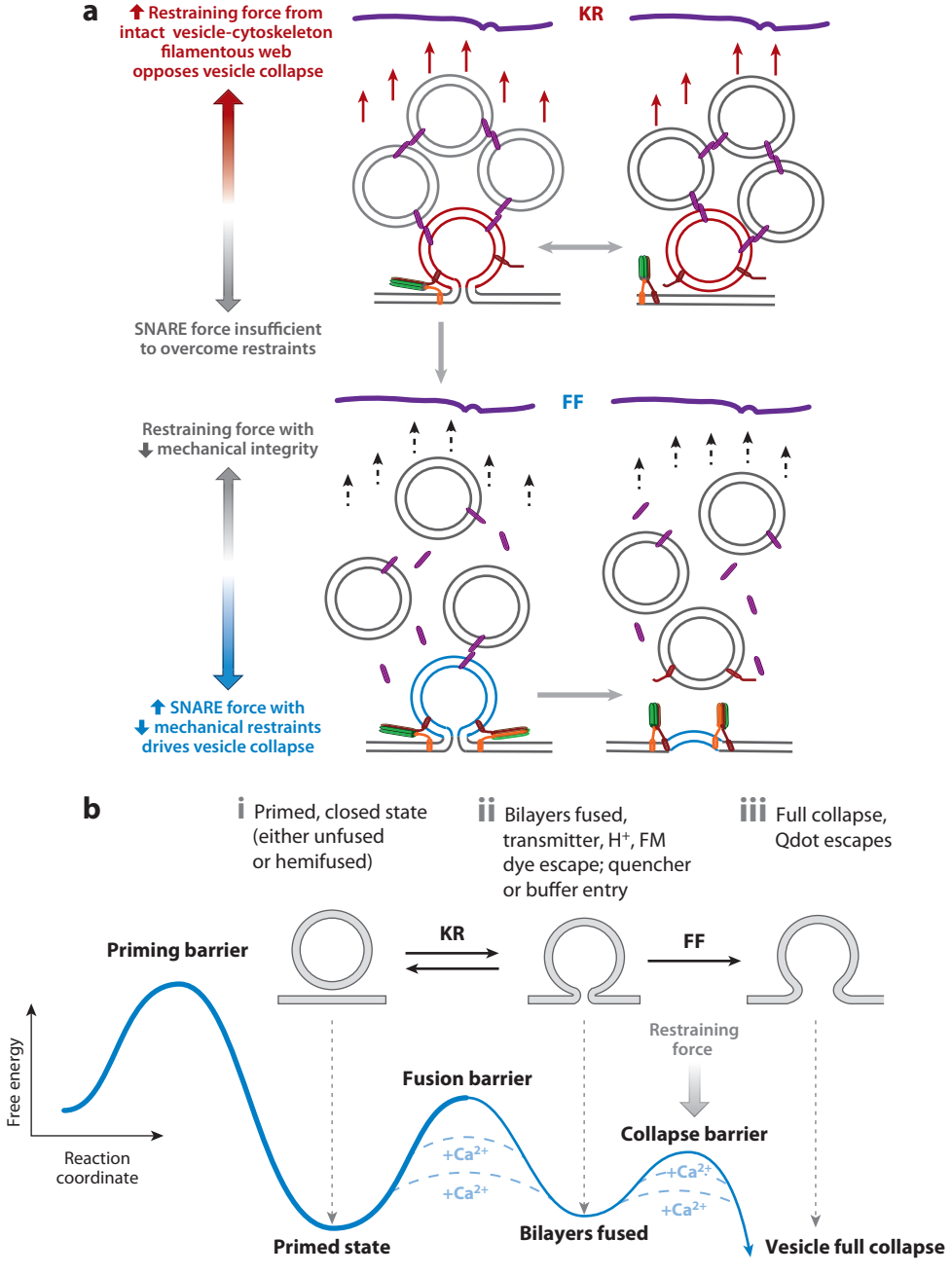
PUTATIVE MOLECULAR MECHANISMS OF KISS-AND-RUN AND ITS MODULATION

The impressive body of knowledge about the molecular apparatus of CME (see review in Reference 79) sets up expectations for comparable clarity about the mechanism of KR. In the

Figure 4

Multiple energy barriers to membrane fusion: a restraining force hypothesis. (*a*) A mechanistic hypothesis for fusion mode preference balances restraining forces and SNARE-driven, fusion-promoting forces, incorporating several lines of evidence (see text for more details). Kiss-and-run (KR) (*top*) is highly prevalent under conditions whereby filamentous links between vesicles (*purple*) and possibly cytoskeletal elements (not shown) provide a strong restraint against full-collapse fusion (FF) and assembly of the SNARE complex [from synaptobrevin (*brown*), syntaxin (*orange*), and SNAP-25 (*green*) subunits] yields a minimal number of effective SNAREs driving fusion. Fusion during KR is terminated as the SNARE transmembrane domains transition from a *trans* orientation to a *cis* orientation, causing a dissipation of the force driving the vesicle and plasma membranes together. Alternatively, engaging more SNAREs during the transient fusion pore opening may drive the vesicle toward FF (even with intact restraining forces). The likelihood of vesicle FF (*bottom*) is, however, maximal under conditions that favor both an increased number of effective SNARE complexes, providing a cumulative increase in fusion-promoting force, and minimal restraining forces such as those which might occur by activity-dependent dissociation of filamentous links (as shown). For clarity, no more than two SNAREs are shown for vesicles closest to the fusion site. Transitions to FF may often be too fast to observe the intermediate, fusion pore states (*left*). (*b*) (*Top*) Relationship between vesicle states and signals from probes of individual fusion events. Three vesicle states are depicted as corresponding to local minima in an energy profile. A vesicle transitions from (*i*) a nonreleasable, primed state to (*ii*) a fusion pore intermediate state toward (*iii*) a final, irreversible FF state. KR represents reversible transitions between states *i* and *ii*. Qdot denotes quantum dot. (*Bottom*) Forward transitions along the reaction coordinate are influenced by Ca^{2+}, which lowers the energy barrier for bilayer fusion (and thus for pore formation) but also eases the transition to FF, likely with differences in Ca^{2+} dependence. Restraining forces heighten the barrier between bilayer fusion (pore formation) and FF. Vesicle cartoons in panel *b* are adapted from Reference 116, and the first two energy barriers at the bottom are depicted as in Reference 3.

absence of broadly accepted genetic evidence (80, 81), our current understanding of KR falls far short of this high bar. Nonetheless, we piece together here a working hypothesis as a scaffold to synthesize disparate, yet increasingly compelling data. The hypothesis is based on several components but hinges on the idea that zippering of individual SNARE complexes contributes brief pulses of fusion-promoting force that may suffice only to generate KR or summate to exceed a further threshold to achieve FF (60–62) (schematized in **Figure 4**).



Direct Participation of SNAREs in the Choice of Fusion Mode?

Recent studies have tested for the involvement of SNARE complexes in fusion pore regulation by varying their number or strength. In an artificial system for reconstitution of membrane-membrane fusion, Shi et al. (62) concluded that only one SNARE complex is needed to cause lipidic continuity but that three or more are required to cause rapid dilation of the fusion pore and to drive FF. This conclusion is consistent with sequential energy barriers separating a basal, unfused vesicle state from initial fusion and hampering that intermediate from subsequently progressing to FF (**Figure 4**). Controversy about the minimal number of SNARE proteins required to drive fusion in living cells (82–84) may stem from the wide variety of assay systems that may access different states (**Figure 4**), the degree of synchrony in SNARE complex recruitment, or the presence or absence of unique counterforces acting against fusion (**Figure 4**).

A complementary intervention to assess SNARE function has been to attenuate the elementary force-generating units by molecular manipulation of the individual SNAREs themselves. Multiple laboratories have inserted linkers of variable length between the transmembrane anchor of the R-SNARE synaptobrevin II and its SNARE complex–forming motif and have observed striking changes in the extent or pattern of fusion (60, 61; see also References 85 and 86). In adrenal chromaffin cells, expansion of the fusion pore was slowed in a systematic fashion, as detected by carbon fiber amperometry and cell capacitance (60, 61). The influence of SNAREs outlasts the initiation of exocytosis and appears to extend for tens of milliseconds after the fusion pore is first established. The finding of attenuated fluctuations in release rate preceding the synchronous spike of release (60) corroborated the idea that individual SNARE complexes make weaker contributions but that compensation is provided by recruitment of additional SNARE complexes. The delay in large fast events and their weakening (60, 61) could be interpreted as reflecting imperfect compensation and the time needed for extra complexes to zipper. The important conclusion is that SNARE mechanics are implicated in the choice between KR and FF.

Taken together, these studies suggest a scenario wherein KR is simply the reversal of membrane continuity (we term it defusion by analogy to deactivation, the reversal of ion channel activation) when the force threshold for FF is not reached (**Figure 4**). The threshold would be determined by the interplay between the regenerative recruitment of SNARE-mediated force and some kind of restraining force that opposes vesicle opening. There is a loose analogy to the activation of voltage-dependent Na^+ channels, favoring spike generation. Spike threshold arises from the interplay between regenerative increases in the recruitment of Na^+ channels and the negative feedback effect of K^+ channels, driving the Na^+ channels to deactivate.

The force of SNARE complexes is generated by the release of energy associated with formation of the classical SNARE bundle (87), a zippering mechanism that extends to the helical transmembrane regions (88). The force contributed by an individual complex continues so long as the transmembrane domain of the R-SNARE remains in the vesicular membrane, but this force must dissipate as that domain finds its way into the plasma membrane (where it must go before the complex can ultimately be unraveled by the ATPase NSF). Thus, each individual SNARE complex may undergo a stereotyped, sequential zippering that proceeds to completion (89), but the vesicle as a whole need not fuse irreversibly. Recruitment of SNARE complexes is thought to be highly regenerative because of their structural arrangement with the fusion regulator complexin. The threshold for the transition to FF would be reached when the force pulses were temporally synchronous but not when they were sufficiently displaced in time to not coincide and summate. Cycles of fusion and "defusion" could repeat, presumably generating the phenomenon of flickering that is seen in membrane capacitance and amperometric signals.

The SNARE machinery and its modulators are only one side of the balance of forces. By themselves, they would not explain why lipid continuity does not automatically result in FF.

We propose that the needed restraint is provided by an opposing force that is generated by yet another mechanical interaction with the vesicle undergoing exocytosis. Cytoskeletal elements and vesicle-vesicle links may be considered as cell biological components that could oppose SNARE-generated force and thus favor defusion (see Reference 79 for discussion of other possibilities). There is compelling evidence that in endocrine and other nonneuronal cells, an intact cytoskeleton, including actin, is critical for the retention of granules. Myosin II activity also comes into play, possibly by promoting prolonged opening of fusion pores; the myosin II inhibitors (−) blebbistatin and ML-9 promote fusion pore closure and decrease fusion pore lifetimes. Although we do not have a clear physical picture of how myosin II might work, it is clear that its impact on fusion pore properties is further influenced by MLCK and myristoylated alanine-rich C-kinase substrate (MARCKS), both modulators of myosin (90).

Possible Restraints Opposing Full-Collapse Fusion of Synaptic Vesicles

To extend our working hypothesis, we postulate that in small nerve terminals, links between neighboring synaptic vesicles serve as a key restraining force. Modern methods of microscopy use rapid freezing to bypass glutaraldehyde fixation and uncover an abundance of cytoskeletal links between adjacent vesicles (69–71, 91, 92). On average, each vesicle is linked to one or two of its neighbors (69). Intervesicular links may perform two complementary functions. First, when a vesicle undergoes FF, some of the energy released by the collapse may be used to bring the next vesicle into favorable proximity to the plasma membrane. Second, the same intervesicular links may exert a restraining force to oppose collapse of the membrane-proximal vesicle (**Figure 4**). Thus, the intervesicular links may enhance the efficiency of vesicle utilization, by favoring KR and thus preserving the fusing vesicle for later reuse, while also readying the next vesicle in line if FF occurs.

This hypothesis provides a plausible explanation for recent findings about the dependence of fusion modes on vesicle position (59). Experiments using 3-D microscopy of Qdot-loaded vesicles revealed that KR was strongly favored if fusion took place near the center of the synapse after a stationary dwell time of ~30 s or more (59). Vesicles residing in the RRP for tens of seconds would have the opportunity to become deeply embedded in a network of interlinked vesicles; newly retrieved or freshly recruited vesicles would be less enmeshed. Likewise, vesicles positioned near the center of the presynapse, where vesicles are concentrated, would engage in more neighborly interactions than would those at the outer edges of the active zone.

Our proposed conceptualization of KR provides an alternative to the idea that FF is impeded by a proteinaceous fusion pore (38). In that case, the molecular structure that precludes vesicle collapse also provides the critical barrier against intermixing of vesicle and plasma membrane components. Thus, KR must be accompanied by an enforced segregation of membrane proteins. The hypothesis of a proteinaceous fusion pore faces three challenges. First, although mutational analysis supports or is at least consistent with the idea that the Q-SNARE syntaxin can form staves of a barrel-like fusion pore (93), there is no indication that this is also the case for the primary R-SNARE for central transmission, synaptobrevin. Second, as few as two R-SNAREs appear sufficient to drive fusion (84), whereas a minimum of three staves are needed to form a barrel-like aqueous channel (2). Third, the proteinaceous fusion pore hypothesis calls for a sharp bend in both R-SNARE and Q-SNARE, even after SNARE complex formation, but full-length synaptobrevin takes the form of a straight helix in the full-blown complex (88).

In contrast, the restraining force hypothesis does not yoke together maintenance of vesicle shape and retention of membrane components (**Figure 4**), thus allowing for transient membrane continuity and lateral diffusion of both lipids and intrinsic membrane proteins. Accordingly, protein (e.g., synaptopHluorin) or lipid (e.g., FM dyes) probes could undergo partial loss (31) or even full escape (29, 30), whereas the vesicle maintains its shape because of the restraining force. The

extent of probe escape would be highly variable, depending on (*a*) the degree to which the probe is bound or free to diffuse, (*b*) the speed of lateral diffusion from vesicular to surface membrane, and (*c*) the duration of the transient membrane continuity.

The restraining force hypothesis reduces the disparity between neuronal and nonneuronal findings and is a shift away from past attempts to distinguish between $KR_{fusion\ pore}$ in small nerve terminals and $KR_{cavicapture}$ in endocrine cells (22). A major difference would be removed if lipid bilayers truly merged in both systems. Some remaining distinctions may lie in the nature of the opposition to full flattening (endocrine granules are not obviously tied to each other in the same way as synaptic vesicles) and the event that closes off the possibility of FF (defusion for small vesicles and a dynamin-based pinch for granules).

Modulation of Fusion Modes Revisited

The interplay between restraining mechanisms and the tendency to collapse provides a basis for modulation of fusion modes. Most, if not all, forms of modulation would operate by modification of either side of the balance, affecting the overall force exerted by the fusion machinery or the elements resisting FF. On both sides of this balance (push to FF; pull back to KR), a further distinction can be made between (*a*) core players in the generation of forces (e.g., SNARE complexes, complexin; filamentous links), (*b*) direct or indirect regulators of those core players (Ca^{2+}, synaptotagmin, G protein subunits; CaN, MLCK, myosin II), and (*c*) bystander elements that spare profusional and restraining forces but otherwise influence the redistribution of membrane molecules while fusional continuity exists [synaptophysin and possibly cholesterol (94)]. Some physiological manipulations such as raising cytoplasmic Ca^{2+} or stimulus frequency may exert multiple, counterpoised effects. Therefore, it may not be surprising that their impact is complicated and hard to decipher.

Complexin binds to SNAREs and can almost be considered as an honorary SNARE protein. Effects of wild-type and mutant complexins (66) fit with data obtained with synaptobrevin, whereby molecular extension favors KR, presumably through loss of mechanical advantage. An et al. (66) found that a truncated version of complexin, containing only the SNARE complex–binding region, persisted at fusion sites for seconds, causing fusion to tilt from FF to KR and the release of transmitter and lipid probes to shift from full to partial. Although the authors interpret the observations in terms of a proteinaceous fusion pore (2), another possibility is that complexin alters the lifetime or duty cycle of a SNARE-driven, lipidic fusion pore.

Going beyond the core fusion machinery, G protein $\beta\gamma$ subunits bind to the SNARE complex and exert a counter-FF influence in much the same way as synaptobrevin extension and truncated complexin. This mechanism was demonstrated by Chen et al. (95) in chromaffin cells and by Gerachshenko et al. (67) at lamprey synapses, another reassuring parallel between nonneuronal and neuronal fusion. The synaptic work demonstrated that the modulation by $G\beta\gamma$ involves SNAP-25 (direct binding of its C terminus by $G\beta\gamma$) rather than synaptobrevin, but the general theme of SNARE protein modulation held true.

FUNCTIONAL IMPACT OF KISS-AND-RUN: VESICLE ECONOMY OR RELEASE DYNAMICS?

Vesicle fusion and recycling by KR may have a number of important consequences for the quantity and/or quality of information conveyed by secretory activity. In this section we consider two rationales for KR. One possibility is that KR conserves transmitter-containing vessels for further reuse and conserves energy that would be required to recreate them after FF (**Figure 5**). A second idea is that KR allows the same organelle to signal in two different ways (**Figure 6**).

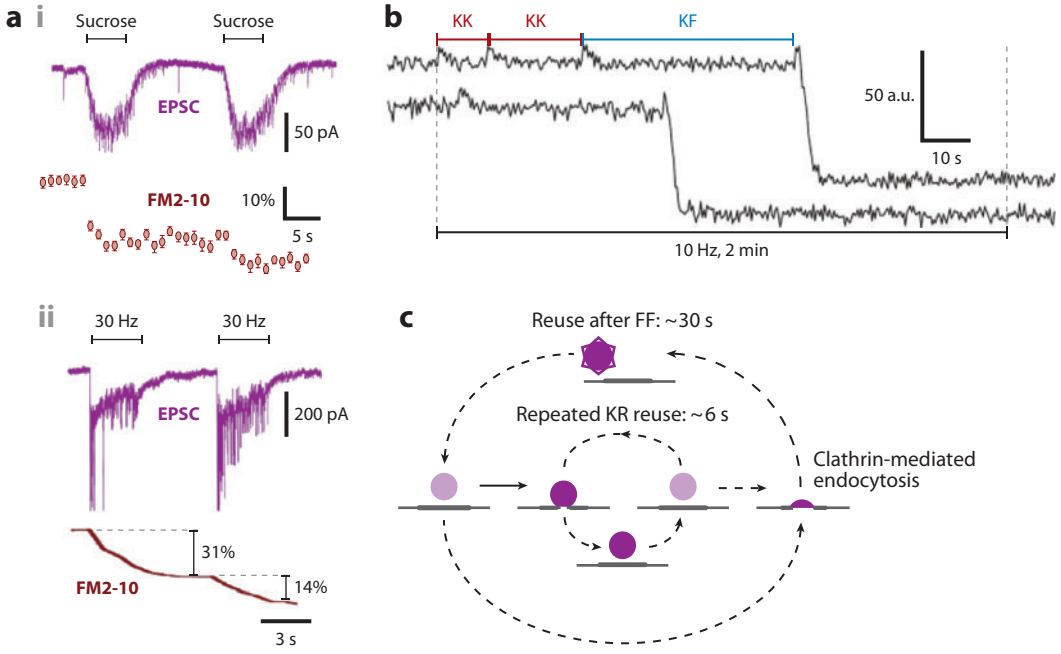


Figure 5

Kiss-and-run (KR) enhances vesicle recycling and reuse during neurotransmission. (*a*) Presynaptic boutons loaded with FM2-10, an FM dye with relatively fast membrane dissociation kinetics, were imaged. Investigators simultaneously monitored the postsynaptic response to (*i*) hypertonic sucrose or to (*ii*) 3 s of 30-Hz stimulation to trigger transmitter release from the readily releasable pool. Rapid reuse of vesicles that have discharged their FM2-10 content underlies maintenance of the postsynaptic response to repeat stimulus pairs with minimal subsequent destaining. EPSC denotes excitatory postsynaptic current. (*b*) Direct monitoring of single-vesicle reuse during 10-Hz stimulation with quantum dots shows three repetitions of KR followed by full-collapse fusion (FF) (*top trace*) or a single KR before FF (*bottom trace*). KK refers to two sequential KR fusion events by the same single vesicle, whereas KF refers to a KR event followed by FF. (*c*) The dynamics of single synaptic vesicles during various stages of the synaptic vesicle cycle indicate that KR (*inside circle*) on average provides a significant kinetic advantage over FF (*outside loop*) during vesicle recycling and reuse. During this cycle, vesicles are either acidified and filled with transmitter (*light purple*) or empty at neutral pH (*dark purple*). Panel *a* reprinted from Reference 52 with permission from Elsevier; panel *b* reprinted from Reference 49.

Functional Impact of Kiss-and-Run in Nonneuronal Cells

KR has a relatively clear functional rationale in the case of LDCVs in endocrine cells. Use of KR is warranted because it (*a*) supports postfusional regulation of the extent of release without changes in the number of spikes, thus allowing an extra degree of modulatory control, and (*b*) allows differential release of multiple transmitter substances, thus expanding the sophistication of secretory signaling. LDCVs often contain more than one species of cargo molecule, ranging from small organic compounds to large peptides or proteins. For example, in adrenal chromaffin cells, the primary neuroendocrine output of the sympathetic nervous system, single LDCVs contain both catecholamines and neuropeptides. Catecholamines are selectively released via KR at basal firing rates. Stress-mediated sympathetic activation leads to increased catecholamine release and recruits neuropeptide secretion. Fulop et al. (96) demonstrated that this differential transmitter release is accomplished through an activity-dependent dilation of the granule fusion pore. Likewise, in β cells of the endocrine pancreas, LDCVs copackage ATP, serotonin (5-HT), and insulin. Opening of small, transient KR fusion pores, detected amperometrically, appeared coincident with individual 5-HT and ATP release events, measured with exogenously expressed

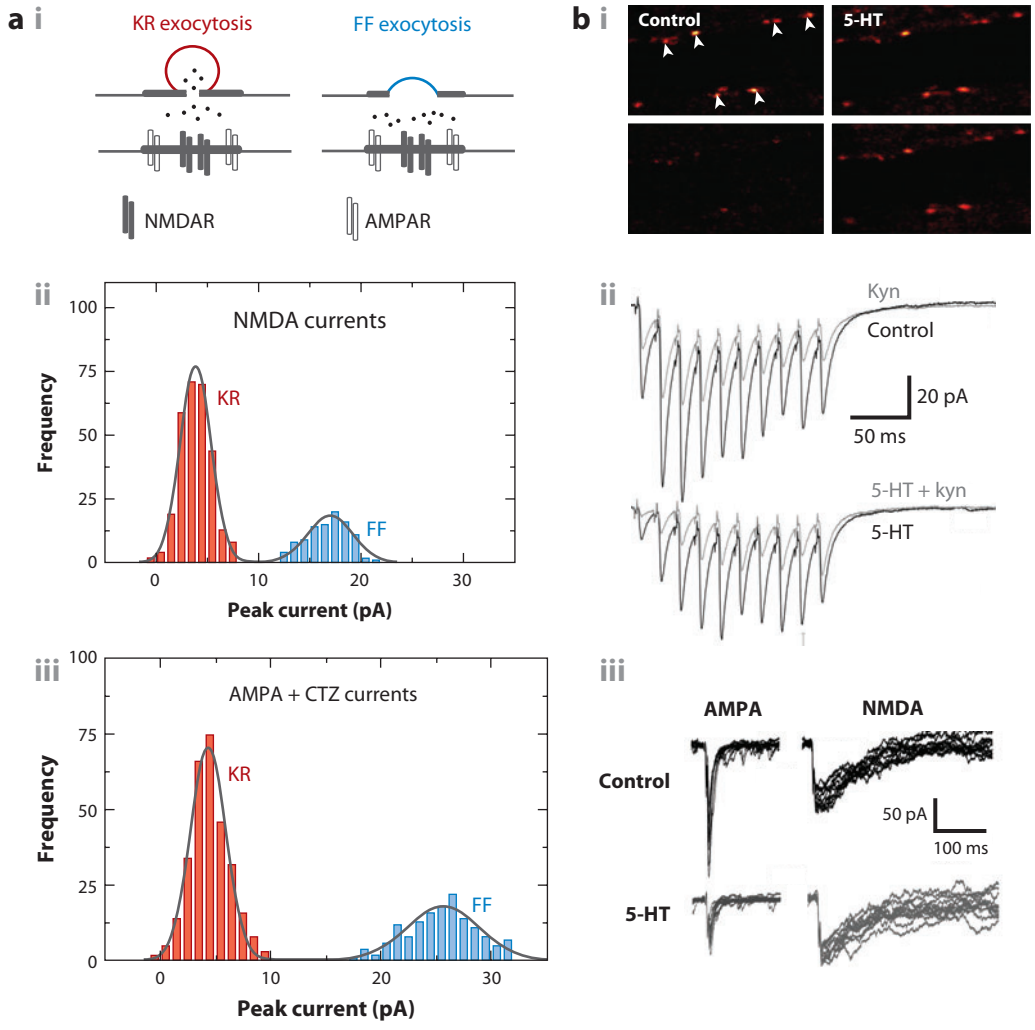


Figure 6

Fusion mode influences transmitter release and postsynaptic signaling. (*a*) (*i*) The possible arrangement of postsynaptic receptors and cleft glutamate profiles during kiss-and-run (KR) or full-collapse fusion (FF). Fusion mode impacts AMPA and NMDA responses in (*a*, *ii* and *iii*) hippocampal and (*b*) lamprey reticulospinal synapses. In panel *a*, fusion mode was classified by using FM dye as depicted in **Figure 1**; separable postsynaptic responses during KR (*red*) and FF (*blue*) are plotted as amplitude histograms. AMPA responses were enlarged by the use of cyclothiazide (CTZ) to block desensitization. (*b*) (*i*) Application of 5-HT impairs FM dye destaining by recruiting G$\beta\gamma$ and shifting the dominant fusion mode to KR during 1-Hz stimulation in lamprey reticulospinal synapses. (*ii*) Stimulation for 200 ms at 50 Hz causes a progressive increase in $[glu]_{cleft}$ in 5-HT-treated synapses, but not in control synapses, mirroring the activity-dependent shift in fusion modes from KR to FF. $[glu]_{cleft}$ was probed by using the low-affinity glutamate receptor (GluR) antagonist kynurenate (kyn; *gray traces*), which elicited uniform inhibition of excitatory postsynaptic currents (EPSCs) in control synapses (*top*, *black trace*) but progressively weaker inhibition in 5-HT-treated synapses (*bottom*, *black trace*), as would be expected if KR gradually gave way to FF over the course of the train. (*iii*) 5-HT treatment significantly dampened AMPA receptor (AMPAR) but not NMDA receptor (NMDAR) currents, consistent with the preferential signaling of KR exocytosis to NMDARs observed in the hippocampal experiments in panel *a*. Panel *a* (*ii* and *iii*) reprinted from Reference 57, copyright 2009, The Physiological Society. Panel *b* reprinted from Reference 113 with permission.

ATP-sensitive channels. Once again, KR of LDCVs allowed the release of small transmitter molecules while likely retaining the larger insulin peptide (97).

On the basis of these and many other examples, KR has clear functional advantages in endocrine cells. By using KR to release small neurotransmitters exclusively, secretory granules economize in multiple ways, gaining (*a*) savings of the functionality of the granule for repeated exocytosis of small neurotransmitter, (*b*) savings of the potential for releasing a large protein cargo upon subsequent demand; and (*c*) savings of the energetic cost of regenerating the granule de novo. When the switch to FF occurs, granules are expended, and the burden of recreating them is tallied, but the functional gain is a recruitment of an additional form of peptidergic communication (33). These conclusions are not only compelling but also noncontroversial.

Neuronal Synapses: Kiss-and-Run and Vesicular Reuse as a Facet of Presynaptic Economy

The teleology of KR at synapses is worth reconsidering in light of new perspectives on molecular mechanisms. According to the restraining force hypothesis, KR would be flexible enough to allow a small minority of the ~60 synaptobrevin molecules per vesicle (98) to escape the plasma membrane while leaving many unused ones in the vesicle, for the sake of later interactions with fresh SNAP-25 and syntaxin molecules. In energetic terms, expelling a few used-up R-SNAREs following a KR event would be more efficient than discharging all 60 every time a vesicle fused. Reversal of membrane fusion (defusion), seen as vesicle retrieval, would help maintain vesicle number/availability. By the time vesicles recovered their availability for another bout of release ($t_{1/2} \approx 6$ s according to Qdot experiments; see **Figure 5*b*,*c***), reacidification (and, by inference, pH gradient–coupled glutamate refilling) would be complete (48, 49) and permit rapid recovery of neurotransmission during sequential stimuli (52) (**Figure 5*a***, ***i*** and ***ii***). The immediate discharge of spent SNARE complexes would maximize the time available for disentangling them through the action of the ATPase NSF.

The observation that KR is prevalent at the beginning of action potential trains but gives way to FF with sustained firing (49, 51) (**Figure 3*b***, ***iii*** and ***iv***) sets limits on the importance of KR for vesicle economy. On the one hand, the dynamics are well suited to the firing patterns of hippocampal pyramidal cells recorded in vivo, consisting of bursts of action potentials at high frequency, separated by prolonged periods of quiescence (99–101). On the other hand, KR and reuse can help preserve neurotransmission during the onset of high-frequency firing (14) but must eventually give way to FF as rapid spiking continues.

Parallel Mechanisms for Efficient Vesicle Retrieval During Clathrin-Mediated Endocytosis

According to conventional wisdom, "clathrin-mediated endocytosis involves invaginating membrane, recruiting and assembling the clathrin coat, pinching off and finally disassembling the coat, overall lasting about 30 seconds to 1 min" (102; see also Reference 103) (**Figure 5*c***). However, newer evidence suggests additional adaptations that may work alongside KR for the sake of vesicle economy. Some newly found features of CME may contribute, as discussed here.

Faster speed of membrane internalization. There has been a progressive revision of the estimated speed of CME, which has been described with simple exponentially distributed kinetics (30) and as being highly regulated on a cell-wide basis, with average time constants ranging from 5.5 s to 38.9 s (104).

More efficient recycling of sets of vesicle proteins after full-collapse fusion. For example, in chromaffin cells, the vesicle membrane protein dopamine-β-hydroxylase remains clustered on the plasma membrane with other specific granule markers even after FF, thus supporting the selective sorting of granule membrane components (105). In neurons, clusters of synaptotagmin persist long after FF has taken place (106). It would be interesting to delineate the scaffolding that keeps such protein components together. Synaptophysin has been proposed as a possible vesicle organizer (107), and its interaction with synaptobrevin may be a useful way of conserving or rebuilding a proper complement of vesicle proteins (98).

Use of a readily retrievable pool. One way to speed up CME recycling is to use a readily retrievable pool, vesicles resident on the membrane surface in a clathrin-precoated state, ready to respond to stimulation with a rapid wave of vesicle pinching off (see also References 31, 48, and 108). Little is known about the speed of coat disassembly in nerve terminals and the subsequent steps needed to prepare the retrieved vesicle for fusion (103). Such information is needed to understand just how efficient FF plus CME might be. Whether or not its dynamics match those of KR plus reuse (**Figure 5*c***), CME-based recycling provides the chance to rebuild a vesicle with a fresh complement of molecular constituents; such rebuilding is ultimately critical for maintained synaptic performance (22).

Neuronal Synapses: Does Kiss-and-Run Influence Information Flow During Neurotransmission?

Perhaps the most exciting aspect of the KR phenomenon is that it may generate an analog form of synaptic signaling that is fundamentally different than the binary nature of classical quantal transmission (109). By allowing further modulatory control beyond the instant of fusion (postfusional regulation), KR may continuously vary the signaling impact of the fusion event. As discussed above, this holds true for LDCVs that harbor two kinds of signaling molecules within a single vessel and that release either one or both molecules with KR or FF (96, 97). There is growing evidence that small vesicles in neurons can also be deployed to communicate in a nonbinary manner, even when restricted to a single type of neurotransmitter. In this case, graded signaling can be achieved by using KR to control the dynamics of neurotransmitter release; two lines of signaling may be mobilized by driving multiple types of postsynaptic receptor.

The popular notion about KR is that it allows partial release of neurotransmitter. However, there is only a slim likelihood of partial release according to current data, because even for the smallest fusion pore opening, the vesicle will be drained of transmitter within tens of milliseconds, long before the fusion pore closes (48, 49). A more likely mechanism involves regulation of transmitter efflux kinetics, which plays upon kinetic features of postsynaptic receptors. As an example, consider AMPA- and NMDA-type glutamate receptors (AMPARs and NMDARs, respectively) during KR and FF at excitatory synapses, as schematized in **Figure 6*a*** (***i***). Both classes of glutamate receptors respond to neurotransmitter by undergoing activation and desensitization. As a result, their peak conductance is highly sensitive to the dynamics of glutamate concentration in the synaptic cleft. AMPARs respond optimally to a sharp spike of $[glu]_{cleft}$ but desensitize in response to a ramp of concentration. NMDARs desensitize less and are more tolerant of a slow rise in $[glu]_{cleft}$ (110).

There is general agreement between computational and experimental approaches to understanding the relationship between $[glu]_{cleft}$ and synaptic communication. Realistic models must reckon with (*a*) fusion pore kinetics, (*b*) location of KR and FF in reference to the synaptic cleft and clusters of receptors, (*c*) transmitter diffusion, and (*d*) postsynaptic receptor kinetics. An early

model focused on KR fusion at the center of the synapse (55) but was soon extended to the more general case of nonconcentric locations (109, 111, 112). All the models emphasize the importance of the pattern of vesicle opening and focus increasingly on local microdomains of $[glu]_{cleft}$. The finding that KR is restricted largely to the most central region of the synaptic cleft (59), where NMDARs are presumably most concentrated (**Figure 6*a*, *i***), raises interesting questions for NMDAR-driven synaptic plasticity. The great predominance of FF over KR at more peripheral locations (59), mirroring the ratio of AMPAR/NMDAR densities, may help hasten the rise and fall of fast postsynaptic events.

Evidence for an impact of kiss-and-run on neuronal signaling. Richards (57) used the distinct optical signals arising from FM destaining (**Figure 1*a***) to sort out electrophysiological events arising from the two fusion modes (**Figure 1*f***). The correlation between full and partial destaining and miniature excitatory postsynaptic currents was made possible by creating a small area of Ca^{2+}-containing solution amid a nominally Ca^{2+}-free milieu. AMPAR-mediated synaptic currents accompanying full destaining of FM dye were up to tenfold greater than those associated with slow, partial destaining. Similarly, NMDA currents during partial destaining events were on average approximately fourfold lower in peak amplitude than those recorded during full destaining (**Figure 6*a*, *ii***). Block of AMPAR desensitization with cyclothiazide increased the AMPAR currents during putative KR events, consistent with a role for desensitization in curbing the AMPAR response (**Figure 6*a*, *iii***). All these observations were consistent with the theoretical predictions of modeling the release of transmitter via KR and the impact of various $[glu]_{cleft}$ waveforms on postsynaptic receptors (55, 109). The postsynaptic receptor response reflects the first few milliseconds of fusion pore opening, and not the closing of the fusion pore hundreds of milliseconds later. In contrast, the dynamics and net amount of FM dye loss likely mirror the entire history of fusion pore opening. According to the restraining force hypothesis, both the initial flickers and the overall duration of the fusion pore opening will be controlled by the same delicate balance between restraining force and SNARE-mediated progression toward FF.

Role of kiss-and-run in intact systems. In a fresh approach to vesicle fusion modes, Alford and colleagues (113) focused on the role of KR in synaptic transmission in the lamprey spinal cord, a classical system for the study of neural network activity and its relation to behavior. 5-HT treatment of glutamatergic synapses causes a shift in fusion modes, appearing to favor KR at the expense of FF. This neuromodulation is mediated by the mobilization of $\beta\gamma$ G protein subunits, which bind to the SNARE protein SNAP-25 (67), compete with binding of synaptotagmin (78), and thereby hamper the operation of the fusion machinery. At an appropriate [5-HT], the quantal amplitude was diminished without any change in P_r, reminiscent of the effects of synaptobrevin extension in chromaffin cells (60, 61). Involvement of KR was shown by retention of FM1-43 within vesicles after 5-HT application (**Figure 6*b*, *i***), with verification of dye retention by uptake of sulforhodamine as quencher. By making paired electrophysiological recordings in the presence and absence of low-affinity antagonists, Gerachshenko et al. (113) demonstrated that activation of the presynaptic GPCR lowered the peak synaptic $[glu]_{cleft}$, causing a differential inhibition of synaptic currents mediated by NMDARs and AMPARs. AMPAR-mediated synaptic responses were substantially reduced, whereas NMDAR-mediated components of neurotransmission remained largely intact (**Figure 6*b*, *iii***). The 5-HT-mediated inhibition displayed Ca^{2+} sensitivity, as would be expected if $G\beta\gamma$ binding to the SNARE complex could be displaced by Ca^{2+}-bound synaptotagmin. When Ca^{2+} piled up presynaptically during bouts of activity, the presynaptic inhibition mediated by 5-HT/$G\beta\gamma$ was relieved, leading to a frequency-dependent increase in $[glu]_{cleft}$ (**Figure 6*b*, *ii***). The switch in fusion modes had a significant impact on network activity within the

whole lamprey spinal cord and behavioral consequences for fictive swimming behavior (113). This work advances the field by shifting the emphasis from single synaptic events to a more naturalistic system wherein the impact of a key neuromodulator of fusion properties can be studied in the context of a physiological circuit.

CONCLUDING REMARKS

Multiple approaches indicate that KR exists at small nerve terminals of central neurons (**Figure 1**), just as it does in neuroendocrine systems. We believe that the wide variations in the quantitative contribution of KR events in hippocampal neurons (**Figure 2**), a source of controversy among various laboratories, reflect bona fide differences in experimental data and will ultimately be traced to variations in modulatory state. There is ample evidence that the prevalence of KR relative to classical FF can be strongly influenced by physiologically important parameters such as P_r, Ca^{2+} accumulation, vesicle pool of origin (**Figure 3**), and degree of presynaptic inhibition (**Figure 6*b***). It would therefore not be surprising to find that subtle differences in experimental conditions, such as the ambient level of GPCR activation, tilted some experiments toward KR and other experiments toward FF. Even if the outcome is not so simple, it will be fruitful to pursue the regulation of fusion mode prevalence as a basic aspect of synaptic performance. It remains to be seen whether the main advantage of KR lies in the economy of vesicle recycling and reuse (**Figure 5**) or in providing differential effects on AMPAR- and NMDAR-mediated transmission (**Figure 6*a***, ***ii*** and ***iii***, and **Figure 6*b***, ***iii***).

Fusion pore formation, fusion of membrane bilayers, and morphological full flattening are conceptually distinct phenomena that are too complex to be lumped under the simple term of fusion. However, an exciting convergence of evidence from model systems and nonsynaptic preparations suggests that KR can arise from a reversal of membrane fusion (defusion). If this case were to hold at synapses, KR would be all the more interesting because it would offer insights into the workings of the synaptic exocytic machinery itself, with SNAREs driving two separate steps (**Figure 4**).

The term KR was first coined in a 1994 article titled, "Neurotransmitter Release: Fusion or Kiss-and-Run?" (39). The "or" in this title highlights a long-standing ambiguity about KR that would dissipate if it proved to be bona fide lipidic membrane fusion that was held back from collapse by molecular anchors consisting of a cytoskeletal leash, an intervesicular leash, or both (**Figure 4**).

Finally, GABAergic signaling has received far less attention with regard to fusion modes than has glutamatergic transmission. KR occurs at inhibitory synapses (59), and many of the same kinetic considerations about postsynaptic GABA receptors may hold (114). This topic is ripe for study.

APPENDIX: AN EXPANDING TOOLBOX PROBES MULTIPLE ASPECTS OF VESICLE DYNAMICS

Evolving strategies assay distinct features of vesicle recycling and transmitter release in distinguishing between fusion modes. Here we briefly outline basic concepts and insights derived from these methods.

Lipophilic FM Dye Destaining

These membrane-impermeant dyes, synthesized by Mao and colleagues (117), were first used by Betz & Bewick (118) to study vesicle recycling at the neuromuscular junction. The best-known compound is FM1-43, but redshifted (FM4-64) and brighter (SGC5) (119) variants expand the versatility of this approach. FM reversibly binds membranes, causing >100-fold-higher quantum

yield, and stains active terminals by endocytic uptake into recycling vesicles. When exocytosis is triggered in dye-free medium, vesicle turnover corresponds to fluorescence decay as FM dye escapes fusing vesicles. Differential extent and rate of FM1-43 release from single synaptic vesicles have been used to differentiate between KR and FF (73, 120) (**Figure 1*a***) and remain a useful strategy, as illustrated in a recent study of the Ca^{2+} control of fusion mode preference (51). It remains unclear whether dye escapes via membrane dissociation and aqueous permeation through the fusion pore (121) or via lipidic diffusion from the vesicle to the plasma membrane (44, 45).

Genetically Encoded pHluorin-Based Probes

Pioneered by Miesenböck et al. (122), pHluorin is a GFP-based, genetically encoded pH sensor that was initially used to monitor vesicle turnover by fusing it to the luminal aspect of synaptobrevin (synaptopHluorin). pHluorin is dim in basally acidified vesicles but brightly fluorescent as H^+ ions escape during vesicle fusion. Following fusion pore closure or CME, reacidification ensues and fluorescence redims. Recent improvements for resolving single-vesicle events have relied on tagging pHluorin to the vesicular glutamate transporter vGlut1-pHluorin, which is rarely left on the presynaptic surface (123), or on incorporating four pHluorin molecules onto a single synaptophysin, SypH4X (31). Spectrally shifted probes like mOrange2 (124) and pHTomato (125) operate on principles similar to those of pHluorin but provide expanded utility in multiplex optical analysis of synaptic function. pHluorin transients may be fast (<1 s), consistent with KR, or slow (>10 s), consistent with CME after FF (**Figure 1*b***). Interpretation of the pHluorin signal is sometimes complicated by kinetic overlap between KR and a fast CME component (30), issues germane to overexpression of modified proteins in an endogenous background (106), the possibility that a fusion-retrieval pair results from nonidentical vesicles (126), and the potential departure of pHluorin-tagged proteins from a vesicle undergoing transient KR fusion (see text for more details).

Single Nanoparticles (Quantum Dots)

Our group has developed a novel application of Qdots—bright, semiconductor-based nanoparticles—to track vesicle dynamics (49, 59, 127). Qdots enter recycling vesicles in a 1:1 stoichiometry without perturbing presynaptic function as assayed with the more conventional indicators above or synaptic currents (49, 127). The Qdot size (~15 nm diameter) ensures escape from a vesicle only during FF (127), and its fluorescence is pH dependent enough to provide a transient, resolvable signal during KR (49). Qdots thus provide distinct optical signals specific to KR or FF (see two exemplar traces in **Figure 1*c***) and have proven useful for (*a*) reporting the preservation of the morphological integrity of the fusing vesicle, which is different in principle from the retention or departure of vesicular membrane constituents; (*b*) showing a vesicle's requisite ability to trap extracellular pH buffer and fluorescence quencher; (*c*) revealing that the fusion pore open time is modulated; and (*d*) indicating that KR prevalence changes with ongoing stimulation in a manner dependent on the RRP.

Whole-Cell Capacitance Recording

Whole-cell or cell-attached measures of capacitance reflect membrane surface area, which increases upon fusion. Neher and colleagues (128, 129) used capacitance recordings decades ago to study granule fusion. Unlike the optical methods, which may assay vesicle dynamics within the bouton, capacitance focuses on what occurs at the plasma membrane, offering excellent time resolution and direct assessment of fusion pore properties without resorting to exogenous probes (34, 130, 131). The capacitance signal does not, however, inherently distinguish one vesicle from

another, which is an obstacle for tracking vesicle identity and reuse at the single-vesicle level. Wu's group (56) pioneered capacitance recordings in large calyceal synapses, where single fusion events were recorded as rapid and transient increases in membrane capacitance with a quantifiable fusion pore conductance, consistent with KR, or as persistent capacitance elevations lasting several seconds with fusion pore conductance too high to measure, consistent with FF (**Figure 1*d***).

Amperometry

In amperometric recordings, a carbon fiber is used as an electrochemical detector of oxidizable molecules, such as biogenic amines or ATP, that are released after vesicle fusion. First applied to catecholamine release from chromaffin cells (132), the method has been extended to 5-HT release (97, 133, 134) and to dopaminergic neurotransmission (135). Recordings in nonneuronal cells, like the pancreatic β cell, have detected rapid, high amplitude amperometric spike signals corresponding to FF and slow release of transmitter through a narrow KR fusion pore; such signals were seen as a so-called foot signal that either preceded a spike or stood alone (97, 133) (**Figure 1*e***). In a well-known neuronal application, Sulzer and colleagues (135) detected dopamine release from ventral midbrain nerve terminals during individual fusion events. The striking pattern of events suggested release from a fusion pore that flickers open and closes once (a simple event) or several times in succession (a complex event), suggesting individual or multiple KR events.

SUMMARY POINTS

1. Kiss-and-run (KR) is an unconventional mode of vesicle fusion and recycling identified in nonneuronal secretory cells and certain nerve terminals as a complement to the classical mode of full-collapse fusion (FF).
2. Evidence for KR comes from multiple approaches to monitoring vesicle dynamics; such approaches report various aspects of vesicle recycling. New experiments combine multiple approaches to simultaneously monitor fusion mode and upstream or downstream signaling.
3. The prevalence of KR is highly variable and dynamically controlled by a variety of physiological inputs. This dynamic modulation provides perspective on conflicting observations, particularly in small central nerve terminals.
4. Fusion pore size and dynamics are similarly under physiological regulation. The molecular composition of a fusion pore is still debated, but we favor the hypothesis that the fusion pore is SNARE driven and largely lipid lined.
5. The choice between KR and FF is not made stochastically at the moment of fusion but is markedly influenced by a vesicle's behavior prior to fusion.
6. The existence of KR may be explained by a restraining force hypothesis that pits forces favoring fusion of vesicle and plasma membranes against those that restrain the vesicle from fully collapsing.
7. Two main functional rationales for KR have been illuminated by recent experiments: (*a*) the vesicle economy or rapid reuse hypothesis, based on the improved efficiency of vesicle recycling and reuse observed during KR, and (*b*) the signaling hypothesis, based on distinct postsynaptic responses measured during KR-mediated release events.

8. KR potentially influences multiple aspects of presynaptic function. Fusion mode modulation may constitute a vitally tunable feature that contributes to multiple forms of synaptic plasticity that are important for neural circuit function.

FUTURE ISSUES

1. The dynamic restraints impeding FF at the molecular level need to be understood.
2. The balance between KR and FF should be manipulated through pharmacological and genetic modification.
3. The consequences for physiology and ultimately CNS information processing should be assessed.
4. The vesicle cycle should be studied in its entirety by kinetic analysis of individual steps in retrieval and reuse, both after KR and after classical clathrin-mediated endocytosis.

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.

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LITERATURE CITED

1. Rizzoli SO, Betz WJ. 2005. Synaptic vesicle pools. *Nat. Rev. Neurosci.* 6(1):57–69
2. Jackson MB, Chapman ER. 2008. The fusion pores of Ca^{2+}-triggered exocytosis. *Nat. Struct. Mol. Biol.* 15(7):684–89
3. Sørensen JB. 2009. Conflicting views on the membrane fusion machinery and the fusion pore. *Annu. Rev. Cell Dev. Biol.* 25:513–37
4. Jahn R, Scheller RH. 2006. SNAREs—engines for membrane fusion. *Nat. Rev. Mol. Cell Biol.* 7(9):631–43
5. Südhof TC, Rothman JE. 2009. Membrane fusion: grappling with SNARE and SM proteins. *Science* 323(5913):474–77
6. Südhof TC, Rizo J. 2011. Synaptic vesicle exocytosis. *Cold Spring Harb. Perspect. Biol.* 3(12):a005637
7. Brunger AT, et al. 2009. Single-molecule studies of the neuronal SNARE fusion machinery. *Annu. Rev. Biochem.* 78:903–28

8. Fernandez-Busnadiego R, Schrod N, Kochovski Z, Asano S, Vanhecke D, et al. 2011. Insights into the molecular organization of the neuron by cryo-electron tomography. *J. Electron. Microsc.* 60(Suppl. 1):137–48
9. Südhof TC. 2012. The presynaptic active zone. *Neuron* 75(1):11–25
10. Schneggenburger R, Neher E. 2000. Intracellular calcium dependence of transmitter release rates at a fast central synapse. *Nature* 406(6798):889–93
11. Chernomordik LV, Kozlov MM. 2008. Mechanics of membrane fusion. *Nat. Struct. Mol. Biol.* 15(7):675–83
12. Heuser JE, Reese TS. 1973. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. Cell Biol.* 57(2):315–44
13. Ceccarelli B, Hurlbut WP, Mauro A. 1973. Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. *J. Cell Biol.* 57(2):499–524
14. Zhou Q, Petersen CC, Nicoll RA. 2000. Effects of reduced vesicular filling on synaptic transmission in rat hippocampal neurones. *J. Physiol.* 525(Part 1):195–206
15. Bittner GD, Kennedy D. 1970. Quantitative aspects of transmitter release. *J. Cell Biol.* 47(3):585–92
16. Alabi AA, Tsien RW. 2012. Synaptic vesicle pools and dynamics. *Cold Spring Harb. Perspect. Biol.* 4(8):a013680
17. Harata N, Ryan TA, Smith SJ, Buchanan J, Tsien RW. 2001. Visualizing recycling synaptic vesicles in hippocampal neurons by FM 1-43 photoconversion. *Proc. Natl. Acad. Sci. USA* 98(22):12748–53
18. Ikeda K, Bekkers JM. 2009. Counting the number of releasable synaptic vesicles in a presynaptic terminal. *Proc. Natl. Acad. Sci. USA* 106(8):2945–50
19. Denker A, Bethani I, Kröhnert K, Körber C, Horstmann H, et al. 2011. A small pool of vesicles maintains synaptic activity in vivo. *Proc. Natl. Acad. Sci. USA* 108(41):17177–82
20. Kim SH, Ryan TA. 2010. CDK5 serves as a major control point in neurotransmitter release. *Neuron* 67(5):797–809
21. Harata N, Pyle JL, Aravanis AM, Mozhayeva M, Kavalali ET, Tsien RW. 2001. Limited numbers of recycling vesicles in small CNS nerve terminals: implications for neural signaling and vesicular cycling. *Trends Neurosci.* 24(11):637–43
22. Harata NC, Aravanis AM, Tsien RW. 2006. Kiss-and-run and full-collapse fusion as modes of exo-endocytosis in neurosecretion. *J. Neurochem.* 97(6):1546–70
23. Kavalali ET. 2006. Synaptic vesicle reuse and its implications. *Neuroscientist* 12(1):57–66
24. Murthy VN, De Camilli P. 2003. Cell biology of the presynaptic terminal. *Annu. Rev. Neurosci.* 26:701–28
25. Fernandez-Alfonso T, Ryan TA. 2006. The efficiency of the synaptic vesicle cycle at central nervous system synapses. *Trends Cell Biol.* 16(8):413–20
26. Jung N, Haucke V. 2007. Clathrin-mediated endocytosis at synapses. *Traffic* 8(9):1129–36
27. Dittman J, Ryan TA. 2009. Molecular circuitry of endocytosis at nerve terminals. *Annu. Rev. Cell Dev. Biol.* 25:133–60
28. Royle SJ, Lagnado L. 2010. Clathrin-mediated endocytosis at the synaptic terminal: bridging the gap between physiology and molecules. *Traffic* 11(12):1489–97
29. Granseth B, Odermatt B, Royle SJ, Lagnado L. 2006. Clathrin-mediated endocytosis is the dominant mechanism of vesicle retrieval at hippocampal synapses. *Neuron* 51(6):773–86
30. Balaji J, Ryan TA. 2007. Single-vesicle imaging reveals that synaptic vesicle exocytosis and endocytosis are coupled by a single stochastic mode. *Proc. Natl. Acad. Sci. USA* 104(51):20576–81
31. Zhu Y, Xu J, Heinemann SF. 2009. Two pathways of synaptic vesicle retrieval revealed by single-vesicle imaging. *Neuron* 61(3):397–411
32. Ryan TA. 2003. Kiss-and-run, fuse-pinch-and-linger, fuse-and-collapse: the life and times of a neurosecretory granule. *Proc. Natl. Acad. Sci. USA* 100(5):2171–73
33. Jorgačevski J, Kreft M, Vardjan N, Zorec R. 2012. Fusion pore regulation in peptidergic vesicles. *Cell Calcium* 52(3–4):270–76
34. Lindau M, Alvarez de Toledo G. 2003. The fusion pore. *Biochim. Biophys. Acta* 1641(2–3):167–73
35. Kavalali ET. 2007. Multiple vesicle recycling pathways in central synapses and their impact on neurotransmission. *J. Physiol.* 585(Part 3):669–79

36. Smith SM, Renden R, von Gersdorff H. 2008. Synaptic vesicle endocytosis: fast and slow modes of membrane retrieval. *Trends Neurosci.* 31(11):559–68
37. Lindau M. 2012. High resolution electrophysiological techniques for the study of calcium-activated exocytosis. *Biochim. Biophys. Acta* 1820(8):1234–42
38. Jackson MB, Chapman ER. 2006. Fusion pores and fusion machines in Ca^{2+}-triggered exocytosis. *Annu. Rev. Biophys. Biomol. Struct.* 35:135–60
39. Fesce R, Grohovaz F, Valtorta F, Meldolesi J. 1994. Neurotransmitter release: fusion or "kiss-and-run"? *Trends Cell Biol.* 4(1):1–4
40. Rutter GA, Tsuboi T. 2004. Kiss and run exocytosis of dense core secretory vesicles. *Neuroreport* 15(1):79–81
41. Taraska JW, Perrais D, Ohara-Imaizumi M, Nagamatsu S, Almers W. 2003. Secretory granules are recaptured largely intact after stimulated exocytosis in cultured endocrine cells. *Proc. Natl. Acad. Sci. USA* 100(4):2070–75
42. Henkel AW, Almers W. 1996. Fast steps in exocytosis and endocytosis studied by capacitance measurements in endocrine cells. *Curr. Opin. Neurobiol.* 6(3):350–57
43. An S, Zenisek D. 2004. Regulation of exocytosis in neurons and neuroendocrine cells. *Curr. Opin. Neurobiol.* 14(5):522–30
44. Taraska JW, Almers W. 2004. Bilayers merge even when exocytosis is transient. *Proc. Natl. Acad. Sci. USA* 101(23):8780–85
45. Takahashi N, Kishimoto T, Nemoto T, Kadowaki T, Kasai H. 2002. Fusion pore dynamics and insulin granule exocytosis in the pancreatic islet. *Science* 297(5585):1349–52
46. Thorn P, Fogarty KE, Parker I. 2004. Zymogen granule exocytosis is characterized by long fusion pore openings and preservation of vesicle lipid identity. *Proc. Natl. Acad. Sci. USA* 101(17):6774–79
47. Toomre D, Bewersdorf J. 2010. A new wave of cellular imaging. *Annu. Rev. Cell Dev. Biol.* 26:285–314
48. Gandhi SP, Stevens CF. 2003. Three modes of synaptic vesicular recycling revealed by single-vesicle imaging. *Nature* 423(6940):607–13
49. Zhang Q, Li Y, Tsien RW. 2009. The dynamic control of kiss-and-run and vesicular reuse probed with single nanoparticles. *Science* 323(5920):1448–53
50. Leitz J, Kavalali ET. 2011. Ca^{2+} influx slows single synaptic vesicle endocytosis. *J. Neurosci.* 31(45):16318–26
51. Richards DA. 2010. Regulation of exocytic mode in hippocampal neurons by intra-bouton calcium concentration. *J. Physiol.* 588(Part 24):4927–36
52. Pyle JL, Kavalali ET, Piedras-Rentería ES, Tsien RW. 2000. Rapid reuse of readily releasable pool vesicles at hippocampal synapses. *Neuron* 28(1):221–31
53. Harata NC, Choi S, Pyle JL, Aravanis AM, Tsien RW. 2006. Frequency-dependent kinetics and prevalence of kiss-and-run and reuse at hippocampal synapses studied with novel quenching methods. *Neuron* 49(2):243–56
54. Fulop T, Smith C. 2006. Physiological stimulation regulates the exocytic mode through calcium activation of protein kinase C in mouse chromaffin cells. *Biochem. J.* 399(1):111–19
55. Choi S, Klingauf J, Tsien RW. 2003. Fusion pore modulation as a presynaptic mechanism contributing to expression of long-term potentiation. *Philos. Trans. R. Soc. Lond. Ser. B* 358(1432):695–705
56. He L, Wu X-S, Mohan R, Wu L-G. 2006. Two modes of fusion pore opening revealed by cell-attached recordings at a synapse. *Nature* 444(7115):102–5
57. Richards DA. 2009. Vesicular release mode shapes the postsynaptic response at hippocampal synapses. *J. Physiol.* 587(Part 21):5073–80
58. Peng A, Rotman Z, Deng P-Y, Klyachko VA. 2012. Differential motion dynamics of synaptic vesicles undergoing spontaneous and activity-evoked endocytosis. *Neuron* 73(6):1108–15
59. Park H, Li Y, Tsien RW. 2012. Influence of synaptic vesicle position on release probability and exocytotic fusion mode. *Science* 335(6074):1362–66
60. Kesavan J, Borisovska M, Bruns D. 2007. v-SNARE actions during Ca^{2+}-triggered exocytosis. *Cell* 131(2):351–63
61. Bretou M, Anne C, Darchen F. 2008. A fast mode of membrane fusion dependent on tight SNARE zippering. *J. Neurosci.* 28(34):8470–76

62. Shi L, Shen QT, Kiel A, Wang J, Wang HW, et al. 2012. SNARE proteins: one to fuse and three to keep the nascent fusion pore open. *Science* 335(6074):1355–59
63. Wang CT, Lu JC, Bai J, Chang PY, Martin TF, et al. 2003. Different domains of synaptotagmin control the choice between kiss-and-run and full fusion. *Nature* 424(6951):943–47
64. Segovia M, Alés E, Montes MA, Bonifas I, Jemal I, et al. 2010. Push-and-pull regulation of the fusion pore by synaptotagmin-7. *Proc. Natl. Acad. Sci. USA* 107(44):19032–37
65. Archer DA, Graham ME, Burgoyne RD. 2002. Complexin regulates the closure of the fusion pore during regulated vesicle exocytosis. *J. Biol. Chem.* 277(21):18249–52
66. An SJ, Grabner CP, Zenisek D. 2010. Real-time visualization of complexin during single exocytic events. *Nat. Neurosci.* 13(5):577–83
67. Gerachshenko T, Blackmer T, Yoon EJ, Bartleson C, Hamm HE, Alford S. 2005. Gβγ acts at the C terminus of SNAP-25 to mediate presynaptic inhibition. *Nat. Neurosci.* 8(5):597–605
68. Blackmer T, Larsen EC, Bartleson C, Kowalchyk JA, Yoon EJ, et al. 2005. G protein βγ directly regulates SNARE protein fusion machinery for secretory granule exocytosis. *Nat. Neurosci.* 8(4):421–25
69. Siksou L, Rostaing P, Lechaire JP, Boudier T, Ohtsuka T, et al. 2007. Three-dimensional architecture of presynaptic terminal cytomatrix. *J. Neurosci.* 27(26):6868–77
70. Fernandez-Busnadiego R, Zuber B, Maurer UE, Cyrklaff M, Baumeister W, Lucic V. 2010. Quantitative analysis of the native presynaptic cytomatrix by cryoelectron tomography. *J. Cell Biol.* 188(1):145–56
71. Harris KM, Weinberg RJ. 2012. Ultrastructure of synapses in the mammalian brain. *Cold Spring Harb. Perspect. Biol.* 4(5):a005587
72. Gabriel T, García-Pérez E, Mahfooz K, Goñi J, Martínez-Turrillas R, et al. 2011. A new kinetic framework for synaptic vesicle trafficking tested in synapsin knock-outs. *J. Neurosci.* 31(32):11563–77
73. Richards DA, Bai J, Chapman ER. 2005. Two modes of exocytosis at hippocampal synapses revealed by rate of FM1-43 efflux from individual vesicles. *J. Cell Biol.* 168(6):929–39
74. Chen X, Barg S, Almers W. 2008. Release of the styryl dyes from single synaptic vesicles in hippocampal neurons. *J. Neurosci.* 28(8):1894–903
75. Zhang Q, Li Y, Tsien RW. 2009. Response to comment on "the dynamic control of kiss-and-run and vesicular reuse probed with single nanoparticles." *Science* 325(5947):1499
76. De Camilli P, Takei K. 1996. Molecular mechanisms in synaptic vesicle endocytosis and recycling. *Neuron* 16(3):481–86
77. Mueller VJ, Wienisch M, Nehring RB, Klingauf J. 2004. Monitoring clathrin-mediated endocytosis during synaptic activity. *J. Neurosci.* 24(8):2004–12
78. Maeno-Hikichi Y, Polo-Parada L, Kastanenka KV, Landmesser LT. 2011. Frequency-dependent modes of synaptic vesicle endocytosis and exocytosis at adult mouse neuromuscular junctions. *J. Neurosci.* 31(3):1093–105
79. Saheki Y, De Camilli P. 2012. Synaptic vesicle endocytosis. *Cold Spring Harb. Perspect. Biol.* 4(9):a005645
80. Verstreken P, Koh TW, Schulze KL, Zhai RG, Hiesinger PR, et al. 2003. Synaptojanin is recruited by endophilin to promote synaptic vesicle uncoating. *Neuron* 40(4):733–48
81. Dickman DK, Horne JA, Meinertzhagen IA, Schwarz TL. 2005. A slowed classical pathway rather than kiss-and-run mediates endocytosis at synapses lacking synaptojanin and endophilin. *Cell* 123(3):521–33
82. van den Bogaart G, et al. 2010. One SNARE complex is sufficient for membrane fusion. *Nat. Struct. Mol. Biol.* 17(3):358–64
83. Mohrmann R, de Wit H, Verhage M, Neher E, Sørensen JB. 2010. Fast vesicle fusion in living cells requires at least three SNARE complexes. *Science* 330(6003):502–5
84. Sinha R, Ahmed S, Jahn R, Klingauf J. 2011. Two synaptobrevin molecules are sufficient for vesicle fusion in central nervous system synapses. *Proc. Natl. Acad. Sci. USA* 108(34):14318–23
85. McNew JA, Weber T, Parlati F, Johnston RJ, Melia TJ, et al. 2000. Close is not enough: SNARE-dependent membrane fusion requires an active mechanism that transduces force to membrane anchors. *J. Cell Biol.* 150(1):105–17
86. Deák F, Shin OH, Kavalali ET, Südhof TC. 2006. Structural determinants of synaptobrevin 2 function in synaptic vesicle fusion. *J. Neurosci.* 26(25):6668–76
87. Sutton RB, Fasshauer D, Jahn R, Brunger AT. 1998. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature* 395(6700):347–53

88. Stein A, Weber G, Wahl MC, Jahn R. 2009. Helical extension of the neuronal SNARE complex into the membrane. *Nature* 460(7254):525–28
89. Gao Y, Zorman S, Gundersen G, Xi Z, Ma L, et al. 2012. Single reconstituted neuronal SNARE complexes zipper in three distinct stages. *Science* 337(6100):1340–43
90. Doreian BW, Fulop TG, Meklemburg RL, Smith CB. 2009. Cortical F-actin, the exocytic mode, and neuropeptide release in mouse chromaffin cells is regulated by myristoylated alanine-rich C-kinase substrate and myosin II. *Mol. Biol. Cell* 20(13):3142–54
91. Wesseling JF, Lo DC. 2002. Limit on the role of activity in controlling the release-ready supply of synaptic vesicles. *J. Neurosci.* 22(22):9708–20
92. Szule JA, Harlow ML, Jung JH, De-Miguel FF, Marshall RM, McMahan UJ. 2012. Regulation of synaptic vesicle docking by different classes of macromolecules in active zone material. *PLoS ONE* 7(3):e33333
93. Han X, Jackson MB. 2006. Structural transitions in the synaptic SNARE complex during Ca^{2+}-triggered exocytosis. *J. Cell Biol.* 172(2):281–93
94. Rituper B, Flašker A, Guček A, Chowdhury HH, Zorec R. 2012. Cholesterol and regulated exocytosis: a requirement for unitary exocytotic events. *Cell Calcium* 52(3–4):250–58
95. Chen XK, Wang LC, Zhou Y, Cai Q, Prakriya M, et al. 2005. Activation of GPCRs modulates quantal size in chromaffin cells through $G\beta\gamma$ and PKC. *Nat. Neurosci.* 8(9):1160–68
96. Fulop T, Radabaugh S, Smith C. 2005. Activity-dependent differential transmitter release in mouse adrenal chromaffin cells. *J. Neurosci.* 25(32):7324–32
97. MacDonald PE, Braun M, Galvanovskis J, Rorsman P. 2006. Release of small transmitters through kiss-and-run fusion pores in rat pancreatic β cells. *Cell Metab.* 4(4):283–90
98. Takamori S, Holt M, Stenius K, Lemke EA, Grønborg M, et al. 2006. Molecular anatomy of a trafficking organelle. *Cell* 127(4):831–46
99. Fenton AA, Muller RU. 1998. Place cell discharge is extremely variable during individual passes of the rat through the firing field. *Proc. Natl. Acad. Sci. USA* 95(6):3182–87
100. Wilson MA, McNaughton BL. 1993. Dynamics of the hippocampal ensemble code for space. *Science* 261(5124):1055–58
101. Aravanis AM, Pyle JL, Harata NC, Tsien RW. 2003. Imaging single synaptic vesicles undergoing repeated fusion events: kissing, running, and kissing again. *Neuropharmacology* 45(6):797–813
102. Rizzoli SO, Jahn R. 2007. Kiss-and-run, collapse and "readily retrievable" vesicles. *Traffic* 8(9):1137–44
103. Ryan TA, Reuter H, Wendland B, Schweizer FE, Tsien RW, Smith SJ. 1993. The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. *Neuron* 11(4):713–24
104. Armbruster M, Ryan TA. 2011. Synaptic vesicle retrieval time is a cell-wide rather than individual-synapse property. *Nat. Neurosci.* 14(7):824–26
105. Ceridono M, et al. 2011. Selective recapture of secretory granule components after full collapse exocytosis in neuroendocrine chromaffin cells. *Traffic* 12(1):72–88
106. Opazo F, Punge A, Bückers J, Hoopmann P, Kastrup L, et al. 2010. Limited intermixing of synaptic vesicle components upon vesicle recycling. *Traffic* 11(6):800–12
107. Cousin MA. 2000. Synaptic vesicle endocytosis: Calcium works overtime in the nerve terminal. *Mol. Neurobiol.* 22(1–3):115–28
108. Hua Y, Sinha R, Thiel CS, Schmidt R, Hüve J, et al. 2011. A readily retrievable pool of synaptic vesicles. *Nat. Neurosci.* 14(7):833–39
109. Lisman JE, Raghavachari S, Tsien RW. 2007. The sequence of events that underlie quantal transmission at central glutamatergic synapses. *Nat. Rev. Neurosci.* 8(8):597–609
110. Liu G. 2003. Presynaptic control of quantal size: kinetic mechanisms and implications for synaptic transmission and plasticity. *Curr. Opin. Neurobiol.* 13(3):324–31
111. Franks KM, Stevens CF, Sejnowski TJ. 2003. Independent sources of quantal variability at single glutamatergic synapses. *J. Neurosci.* 23(8):3186–95
112. Uteshev VV, Pennefather PS. 1997. Analytical description of the activation of multi-state receptors by continuous neurotransmitter signals at brain synapses. *Biophys. J.* 72(3):1127–34
113. Gerachshenko T, Schwartz E, Bleckert A, Photowala H, Seymour A, Alford S. 2009. Presynaptic G-protein-coupled receptors dynamically modify vesicle fusion, synaptic cleft glutamate concentrations, and motor behavior. *J. Neurosci.* 29(33):10221–33

114. Scimemi A, Beato M. 2009. Determining the neurotransmitter concentration profile at active synapses. *Mol. Neurobiol.* 40(3):289–306
115. Granseth B, Odermatt B, Royle SJ, Lagnado L. 2009. Comment on " the dynamic control of kiss-and-run and vesicular reuse probed with single nanoparticles." *Science* 325(5947):1499; author reply 1499
116. Coorssen JR, Zorec R. 2012. Regulated exocytosis per partes. *Cell Calcium* 52(3–4):191–95
117. Betz WJ, Mao F, Bewick GS. 1992. Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. *J Neurosci.* 12(2):363–75
118. Betz WJ, Bewick GS. 1992. Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. *Science* 255(5041):200–3
119. Wu Y, Yeh FL, Mao F, Chapman ER. 2009. Biophysical characterization of styryl dye-membrane interactions. *Biophys. J.* 97(1):101–9
120. Aravanis AM, Pyle JL, Tsien RW. 2003. Single synaptic vesicles fusing transiently and successively without loss of identity. *Nature* 423(6940):643–47
121. Wu Y, Ma L, Cheley S, Bayley H, Cui Q, Chapman ER. 2011. Permeation of styryl dyes through nanometer-scale pores in membranes. *Biochemistry* 50(35):7493–502
122. Miesenböck G, De Angelis DA, Rothman JE. 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* 394(6689):192–95
123. Voglmaier SM, Kam K, Yang H, Fortin DL, Hua Z, et al. 2006. Distinct endocytic pathways control the rate and extent of synaptic vesicle protein recycling. *Neuron* 51(1):71–84
124. Shaner NC, Lin MZ, McKeown MR, Steinbach PA, Hazelwood KL, et al. 2008. Improving the photostability of bright monomeric orange and red fluorescent proteins. *Nat. Methods* 5(6):545–51
125. Li Y, Tsien RW. 2012. pHTomato, a red, genetically encoded indicator that enables multiplex interrogation of synaptic activity. *Nat. Neurosci.* 15(7):1047–53
126. Wienisch M, Klingauf J. 2006. Vesicular proteins exocytosed and subsequently retrieved by compensatory endocytosis are nonidentical. *Nat. Neurosci.* 9(8):1019–27
127. Zhang Q, Cao YQ, Tsien RW. 2007. Quantum dots provide an optical signal specific to full collapse fusion of synaptic vesicles. *Proc. Natl. Acad. Sci. USA* 104(45):17843–48
128. Neher E, Marty A. 1982. Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proc. Natl. Acad. Sci. USA* 79(21):6712–16
129. Fernandez JM, Neher E, Gomperts BD. 1984. Capacitance measurements reveal stepwise fusion events in degranulating mast cells. *Nature* 312(5993):453–55
130. Elhamdani A, Azizi F, Artalejo CR. 2006. Double patch clamp reveals that transient fusion (kiss-and-run) is a major mechanism of secretion in calf adrenal chromaffin cells: High calcium shifts the mechanism from kiss-and-run to complete fusion. *J. Neurosci.* 26(11):3030–36
131. Klyachko VA, Jackson MB. 2002. Capacitance steps and fusion pores of small and large-dense-core vesicles in nerve terminals. *Nature* 418(6893):89–92
132. Wightman RM, Jankowski JA, Kennedy RT, Kawagoe KT, Schroeder TJ, et al. 1991. Temporally resolved catecholamine spikes correspond to single vesicle release from individual chromaffin cells. *Proc. Natl. Acad. Sci. USA* 88(23):10754–58
133. Chow RH, von Ruden L, Neher E. 1992. Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cells. *Nature* 356(6364):60–63
134. Bruns D, Jahn R. 1995. Real-time measurement of transmitter release from single synaptic vesicles. *Nature* 377(6544):62–65
135. Staal RG, Mosharov EV, Sulzer D. 2004. Dopamine neurons release transmitter via a flickering fusion pore. *Nat. Neurosci.* 7(4):341–46

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