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Synaptic Vesicle Recycling Through the Lens of Ultrafast Endocytosis

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Abstract

The mechanisms underlying synaptic vesicle endocytosis remain controversial. In the 1970s, Heuser and Reese put forward a hypothesis that clathrin-mediated endocytosis is the predominant vesicle retrieval mechanism. In their seminal papers, another pathway was also described: uncoated large vesicles or cisternae emanating from the plasma membrane 1 s after a single stimulus. This pathway likely represents a recently described ultrafast endocytic pathway that recovers synaptic vesicles during physiological stimuli. Had we known of the existence of ultrafast endocytosis or paid more attention to the cisternae-based uptake pathway, would the experimental results over subsequent years have been interpreted differently? Here, I retrospectively review the literature on synaptic vesicle recycling through the lens of ultrafast endocytosis.

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INTRODUCTION

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The mechanisms underlying synaptic vesicle endocytosis have been hotly debated over the last 50 years. In the 1970s, the so-called golden decade of electron microscopy in neurobiology, two models for synaptic vesicle recycling were proposed: clathrin-mediated endocytosis and kiss-and-run (**Figure 1**; the details of the experimental results are discussed in the **Supplemental Text**). Specifically, Heuser & Reese (1973) demonstrated that synaptic vesicles are retrieved over tens of seconds to minutes via clathrin-mediated endocytosis. In their seminal studies, they also observed bulk uptake of membranes occurring within 1 s of stimulation (Heuser & Reese 1979, Miller & Heuser 1984). However, given that these large uncoated pits did not contain transmembrane proteins (Miller & Heuser 1984), they concluded that clathrin-mediated endocytosis was likely the predominant mechanism for synaptic vesicle retrieval. Around the same time, Ceccarelli and colleagues (Ceccarelli et al. 1973, Torri-Tarelli et al. 1985) put forward another hypothesis: that synaptic vesicles can be endocytosed directly at the active zone by the reversal of the exocytic process. Although no direct experimental evidence was provided, the kiss-and-run model was an attractive alternative to clathrin-mediated endocytosis given that it could account for the kinetics needed to recycle synaptic vesicles to sustain rapid neurotransmission (Fesce et al. 1994). These experiments led to a 50-year battle in the field.

THE WAR OF THE COATS AND THE NUDES

Are endocytic pits coated or nude, and is endocytosis fast or slow? Following the golden decade of electron microscopy in neurobiology, investigations have steadily shifted toward revealing the kinetics of endocytosis and its molecular underpinnings in several genetic model organisms to distinguish between two models of synaptic vesicle recycling: clathrin-mediated endocytosis and kiss-and-run. As evidenced in the classic ultrastructural studies, the clathrin pathway requires tens of seconds for recycling vesicles, whereas the kiss-and-run mechanism can be as rapid as 10 ms but certainly within 1–2 s. Along with electrophysiological methods (capacitance measurements) to measure kinetics, several optical tools have been developed: lipophilic FM dyes, a pH-sensitive green fluorescent protein (pHluorin), and quantum dots (Qdots)—these methods have been reviewed in detail (Kavalali & Jorgensen 2014; S. Watanabe & E.M. Jorgensen, unpublished manuscript). Since many excellent reviews are available (Rizzoli & Jahn 2007, Dittman & Ryan 2009, Saheki & De Camilli 2012, Rizzoli 2014, Kononenko & Haucke 2015, Cousin 2017, Milosevic 2018, Chanaday et al. 2019, Mochida 2022), I simply summarize the findings here.

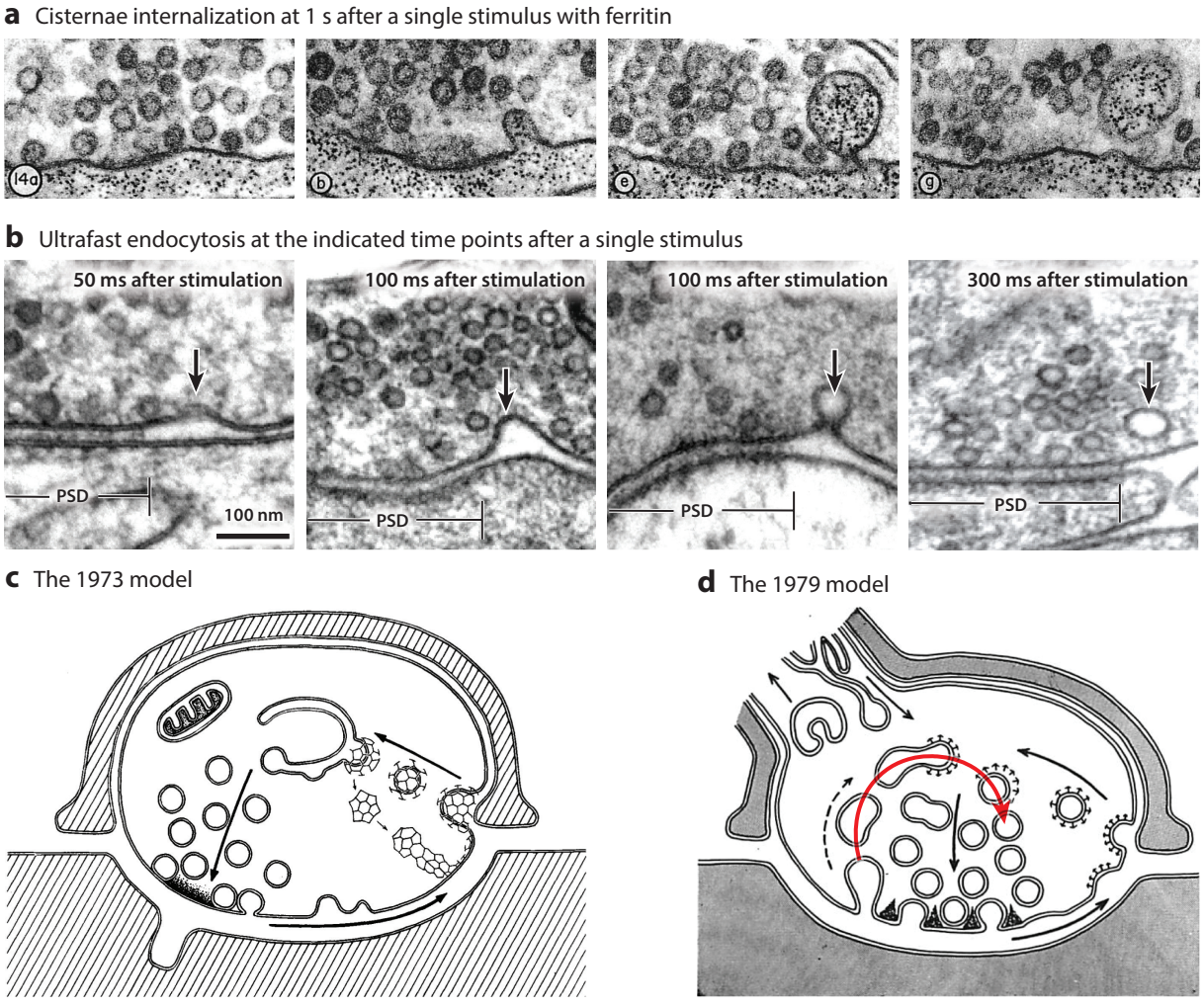

Figure 1

(*a*) Representative images of the rapid cisternae internalization pathway. (*b*) Representative images of ultrafast endocytosis in mouse hippocampal neurons. (*c*) A model diagram showing synaptic vesicle recycling. (*d*) A model diagram showing synaptic vesicle recycling. A red arrow indicates the pathway highlighted in this review as the predominant pathway for synaptic vesicle recycling. Abbreviation: PSD, postsynaptic density. Panels *a* and *d* adapted from Heuser & Reese (1979); copyright 1979 Massachusetts Institute of Technology, all rights reserved. Panel *b* adapted from Watanabe et al. (2013b). Panel *c* adapted with permission from Heuser & Reese (1973).

Both kinetic and molecular studies have produced mixed results but still led to the conclusion that clathrin-mediated endocytosis is the major pathway for synaptic vesicle recycling. Live-cell imaging experiments show rapid internalization of vesicle proteins or fluorescent dyes either with a single exponential component with a time constant of approximately 8–10 s after high-frequency stimulation (Balaji & Ryan 2007, Balaji et al. 2008) or with multiple time constants, the fast component being a few seconds or less and the slower component lasting seconds (Teng et al. 1999, Richards et al. 2000, Harata et al. 2006, Zhu et al. 2009, Richards 2010). The fast component in earlier studies (particularly prior to 2013) is often attributed to kiss-and-run. In contrast to kinetic studies, molecular work has revealed that clathrin and clathrin-associated

proteins are essential for synaptic vesicle recycling (Shupliakov et al. 1997; Nonet et al. 1999; Schmidt et al. 1999; Verstreken et al. 2002, 2003; Koh et al. 2004; Marie et al. 2004; Anggono et al. 2006; Diril et al. 2006; Granseth et al. 2006; Ferguson et al. 2007; Hayashi et al. 2008; Jakobsson et al. 2008; Kim & Ryan 2009; Milosevic et al. 2011; Raimondi et al. 2011; Armbruster et al. 2013; Gu et al. 2013). Interestingly, studies of the vesicle fission protein Dynamin GTPase (Antonny et al. 2016) show mixed results. In *Drosophila*, a temperature-sensitive mutant of Dynamin shows endocytic pits stalled on the plasma membrane upon exposure to the restrictive temperature; almost no pits are coated (Koenig & Ikeda 1989, 1996). Similar phonotypes are observed in a *Caenorhabditis elegans* Dynamin temperature-sensitive mutant (Kittelmann et al. 2013, Watanabe et al. 2013a). By contrast, in mouse neurons lacking Dynamin 1 alone or both Dynamin 1 and 3 (two brain-enriched isoforms of Dynamin), clathrin-coated pits are stalled on a large membrane involution, which is continuous with the plasma membrane (Ferguson et al. 2007, Hayashi et al. 2008, Raimondi et al. 2011) [although abundance of such structures is questionable, given our recent studies (Imoto et al. 2022, 2024)—see below]. Nude pits observed in these studies do not likely represent kiss-and-run intermediates because these pits are either larger than single vesicles or located outside of the active zone. Although the existence of kiss-and-run is unequivocal, particularly for secretion of dense-core vesicles and secretory granules (Alvarez de Toledo et al. 1993, Albillos et al. 1997, Alés et al. 1999, He et al. 2006, Chiang et al. 2014, Ge et al. 2022), its contribution to the synaptic vesicle cycle remains unclear. Altogether, these results have suggested that endocytosis may be both clathrin dependent and clathrin independent, but given the ostensible requirement for clathrin machinery, clathrin-mediated endocytosis has been thought to be the predominant mechanism for synaptic vesicle recycling. However, whether this endocytic pathway is fast enough to sustain synaptic transmission remains the major criticism.

ULTRAFAST ENDOCYTOSIS: REDISCOVERY OF CISTERNAE INTERNALIZATION?

In an attempt to resolve the discrepancy in the literature, we decided to revisit the classic freeze-slamming experiments. Instead of *Xenopus* neuromuscular junctions, we used genetic model organisms: *C. elegans* and *Mus musculus* (mouse). To stimulate neurons in intact worms, we expressed in their cholinergic motor neurons a variant of Channelrhodopsin-2 (ChR) (Nagel et al. 2003), a light-sensitive cation channel that allows depolarization of membranes and eventual synaptic transmission upon blue light illumination. To couple light stimulation with freezing of animals, we modified a specimen carrier of a high-pressure freezer to create a light path and controlled flashes of LED with a custom-built computer program (Watanabe et al. 2013a, 2014). We delivered a single flash of light lasting 20 ms and froze the animals at defined time points after stimulation, ranging from 20 ms to 10 s to capture potential kiss-and-run-like fusion events and clathrin-coated pits on the plasma membrane (Watanabe et al. 2013a). As in frog neuromuscular junctions, all the fusion intermediates captured show a wide opening at their base, indicative of a full-collapse fusion event. However, no clathrin-coated pits were observed during the first 10 s of stimulation. Instead, endocytosis occurred lateral to the active zone, next to a dense projection or adherens junction. These endocytic events were initiated as soon as 30 ms after stimulation and completed by 1 second after. Because of these kinetics, this endocytic event was named ultrafast endocytosis. Endocytic pits lack apparent electron-dense coats, indicating that this pathway is clathrin independent. Around the same time, Kittelmann et al. (2013) performed similar experiments in *C. elegans* with 30-s light stimulation and found endocytic vesicles appearing near dense projections, confirming that the first step of synaptic vesicle recycling is likely bulk membrane uptake. Our experiments in cultured mouse hippocampal neurons show essentially the same

results: Clathrin-independent ultrafast endocytosis occurs lateral to an active zone (Watanabe et al. 2013b) (**Figure 1*b***). Ultrafast endocytosis typically completes within 100 ms but can last over approximately 1 s following a single stimulus. Endocytic vesicles are delivered to endosomes, and synaptic vesicles form from these endosomes in a clathrin-dependent manner (Watanabe et al. 2014). In fact, in clathrin knockdown experiments, ultrafast endocytosis occurs normally, but regeneration of synaptic vesicles from endosomes is perturbed (Watanabe et al. 2014). Based on these factors, ultrafast endocytosis is likely analogous to the bulk membrane uptake observed after a single stimulus in Heuser and Reese's experiments (see the red arrow in **Figure 1*d***).

Is ultrafast endocytosis an artifact of ChR-based stimulation or elevated Ca^{2+} concentration (4 mM) in the external solution, in line with the assumed effect on terminals by 4-AP put forward by Heuser and Reese? To address this issue fully, we have recently revisited the original experiment but with electrical stimulation and the physiological concentration of Ca^{2+} (1.2 mM) (Ogunmowo et al. 2023). We found that ultrafast endocytosis is induced under this condition; the only difference was the size of endocytic vesicles—approximately two vesicles' as opposed to four vesicles' worth of membrane was internalized, likely reflecting the reduced number of synaptic vesicles exocytosed under this condition. There was no accumulation of clathrin-coated pits in these terminals, again suggesting that ultrafast endocytosis is likely the major pathway.

Interestingly, we have rarely observed clathrin-coated pits on the plasma membrane under any experimental conditions we have tried. This observation is not due to the stimulation we applied (a single stimulus), because even after 100 pulses given at 20 Hz in the presence of a Dynamin inhibitor, which in principle traps all endocytic pits at the plasma membrane, coated pits did not accumulate on the plasma membrane (Watanabe et al. 2013b). Even in neurons lacking the two major isoforms of Dynamin, Dynamin 1 and 3, clathrin-coated pits are rare (Imoto et al. 2022). Notably, only a few synapses show the large membrane involutions with clathrin-coated vesicles observed in earlier studies. Thus, under mild stimulation conditions, clathrin-mediated endocytosis may not participate in synaptic vesicle recycling.

Since the publication of our work, several groups have tested our model and concluded that synaptic vesicle endocytosis is clathrin independent in mammalian central synapses. Delvendahl et al. (2016) used capacitance measurements in cerebellar mossy fiber boutons to monitor membrane flux and found that endocytosis is rapid and independent of clathrin (Delvendahl et al. 2016). Haucke and colleagues (Kononenko et al. 2014, Soykan et al. 2017) performed pHluorin assays with molecular perturbation or rapid acid infusion to quench the pHluorin-attached vesicle proteins left on the surface and demonstrated that endocytosis is AP-2 and clathrin independent but actin dependent. pHluorin imaging with single action potentials also showed ultrafast internalization of vesicle proteins (Chanaday & Kavalali 2018, Myeong et al. 2024). It is important to note that clathrin-independent ultrafast and fast endocytosis slows down as the number of applied stimuli increases (Soykan et al. 2017), likely reflecting the time it takes to retrieve excess membranes and proteins after multiple rounds of exocytosis. Nonetheless, these results suggest that endocytosis is clathrin independent, and synaptic vesicles are regenerated from endosomes. This two-step process separates the slow clathrin-dependent protein sorting and vesicle regeneration from the most rate-limiting steps of synaptic transmission: the clearance of release sites and restoration of the plasma membrane surface area (and, thereby, surface tension). Thus, this pathway enables continuous fusion events at synapses (Watanabe & Boucrot 2017).

MECHANISMS OF ULTRAFAST ENDOCYTOSIS

We recently wrote a review article summarizing the mechanisms of ultrafast endocytosis (for details, please refer to Imoto & Watanabe 2025). Our current working model is that the initial

invagination is generated through mechanical force, and this curvature is recognized and further bent or stabilized by membrane curvature proteins and scaffolding proteins. One notable feature of ultrafast endocytosis is that the size of endocytic pits and vesicles reflects the total amount of membrane exocytosed at a given time. In the original experiment, we used 4 mM Ca^{2+} in the external solution to boost release probability. Under this condition, we observed the fusion of three to four vesicles following a single optogenetic stimulus and large endocytic vesicles of approximately 80 nm in diameter, which corresponds to approximately three to four vesicles' worth of membranes (Watanabe et al. 2013b). In a recent paper, we performed similar experiments but with 1.2 mM Ca^{2+} and a single electrical pulse and found that approximately two vesicles fuse (Kusick et al. 2020), and correspondingly, endocytic vesicle size is smaller (~60 nm) (Ogunmowo et al. 2023). This compensatory nature is likely because exocytosis and endocytosis are coupled by mechanical stress: Exocytic vesicles compress the active zone membrane laterally against the actin-enriched stiff membrane in the periactive zone, resulting in membrane buckling (Ogunmowo et al. 2023). Thus, the size of the membrane invagination depends on how many vesicles have fused. Once the initial membrane curvature forms, endocytic proteins that were prerecruited to the endocytic zone via biomolecular condensation respond and mature nascent pits into endocytic vesicles. This condensation process requires brain-specific isoforms of proteins Syndapin 1 and Dynamin 1xA (Imoto et al. 2022). Through its extended C terminus, Dynamin 1xA interacts with another endocytic protein, Endophilin A1, an essential protein in ultrafast endocytosis, and primes it for ultrafast endocytosis (Watanabe et al. 2018, Imoto et al. 2024). Because of this prerecruitment, endocytosis can complete within 50 ms (Imoto et al. 2022).

Clathrin adaptor proteins also participate in ultrafast endocytosis independently of their role in endosomal budding. At synapses, a pool of vesicle proteins from previous rounds of exocytosis is present on the plasma membrane, awaiting retrieval via the next rounds of exocytosis and endocytosis. This pool is termed a readily retrievable pool (Hua et al. 2011). During synaptic activity, there is a substantial exchange of proteins from this pool with recently exocytosed ones (Soykan et al. 2017). The clathrin adaptor proteins seem to confine this pool at the plasma membrane within the periactive zone. In the absence of AP-2 or AP180, synaptic vesicle proteins are more widely distributed along the plasma membrane and cannot be efficiently internalized (Gimber et al. 2015), suggesting that they are involved in cargo capturing at the plasma membrane. Another adaptor, Epsin1, is involved in organizing F-actin in the periactive zone (Ogunmowo et al. 2023). When its expression is knocked down, F-actin no longer forms a ring-like organization, and consequently, the initiation of ultrafast endocytosis fails, similarly to when F-actin is perturbed with Latrunculin (Watanabe et al. 2013b). These adaptor functions do not require clathrin. Thus, many molecular players that are known to function in clathrin-mediated endocytosis seem to be repurposed at synapses for fast and ultrafast synaptic vesicle recycling.

LITERATURE REVIEW THROUGH THE LENS OF ULTRAFAST ENDOCYTOSIS

Given the discovery of ultrafast endocytosis and its mechanism, experimental results over the years may require reinterpretation. In this section, I discuss the retrieval mechanism literature through the lens of the ultrafast endocytosis model.

Clathrin-Mediated Endocytosis

The role of clathrin in synaptic vesicle endocytosis is hotly debated. Certainly, several model systems, including, but not limited to, frog neuromuscular junctions and lamprey reticulospinal neurons, display coated pits after prolonged intense stimulation (Gan & Watanabe 2018).

Similarly, many ultrastructural studies show coated pits in many model systems, but in almost all cases, neurons were either chemically fixed at low temperature (often nonphysiological) or intensely stimulated (e.g., with high K^+, prolonged high-frequency stimulation). For example, the phenotypes of *C. elegans* mutants lacking synaptojanin are slightly different when they are fixed by ice-cold fixatives (Harris et al. 2000) or maintained at physiological temperature prior to being high-pressure frozen (Jospin et al. 2007, Kittelmann et al. 2013): Clathrin-coated pits are stuck on the plasma membrane only when the animals are preserved by the former method. Similarly, when cultured mouse hippocampal neurons are incubated at room temperature for 5 min prior to experiments, synaptic vesicles are retrieved through clathrin-mediated endocytosis due to the failure of ultrafast endocytosis (Watanabe et al. 2014). Considering these findings, I performed a literature search in PubMed with the search term "synaptic vesicle endocytosis electron microscopy." **Supplemental Table 1** and **Figure 2** summarize the results from the search, showing the positive correlation between clathrin-coated pits and nonphysiological temperatures. Our computational modeling also predicts the failure of ultrafast endocytosis when the membrane stiffness changes (e.g., due to temperature change) or the number of fusion events is too low in a sufficiently large active zone (Ogunmowo et al. 2023).

Supplemental Material >

On the opposite side of the spectrum, when neurons are challenged with intense high-frequency stimulation, more vesicle proteins are exposed to the surface and may require the clathrin pathway to be retrieved. However, activity-dependent bulk endocytosis would then kick in and internalize excess membranes and proteins (Cousin 2009). In fact, even with 500 stimuli at 10 Hz or 200 stimuli at 40 Hz, pHluorin signals decay normally in neurons with reduced clathrin expression at the physiological temperature (Soykan et al. 2017; however, see Wei et al. 2024), suggesting that endocytosis is clathrin independent even under these stimulation conditions. Thus, whether clathrin is necessary at the plasma membrane remains unclear. Although the number of clathrin-coated pits does not increase even after intense stimulation, they are present in resting synaptic terminals (more so in postsynaptic terminals than in presynaptic boutons).

When does clathrin-mediated endocytosis operate, and what purposes does it serve? Like in any other cell types, clathrin-mediated endocytosis is needed for homeostasis of membrane proteins. A plethora of proteins are on the surface of synapses, and their internalization would require clathrin and its associated proteins. Similarly, G protein–coupled receptors are also likely internalized via clathrin-mediated endocytosis. A recent study from Jullié et al. (2020) shows that μ-opioid receptors are internalized at presynaptic terminals upon application of the agonist and that their endocytic route is distinct from that of synaptic vesicles. Although not tested directly, the involvement of β-arrestin (Haberstock-Debic et al. 2005, Eichel et al. 2018) likely suggests that these receptors are internalized via clathrin-mediated endocytosis.

pHluorin signals following single stimuli display a fluorescence decay with different kinetics at both physiological and nonphysiological temperatures; some do not return to the baseline, suggesting failure of retrieval (Balaji & Ryan 2007, Chanaday & Kavalali 2018). These proteins may be eventually internalized by clathrin-mediated endocytosis. Yet, the most perplexing aspect of most pHluorin data is that fluorescence decays with a single exponential component, indicating that one biological process likely determines the kinetics (Balaji & Ryan 2007). Since reacidification of vesicles initiates immediately after endocytic vesicles are severed from the plasma membrane, this biological process is likely at the fission step, which is mediated by the GTPase Dynamin. Consistently, when the mutant form of Dynamin 1 that cannot form condensates is expressed in neurons lacking Dynamin 1, the kinetics of membrane internalization measured by time-resolved electron microscopy slows down to the same degree as the kinetics of protein internalization measured by pHluorin (Imoto et al. 2022, 2024), suggesting that this step underlies the rate-limiting step. However, the ultrafast endocytosis model suggests that there is another fusion and fission

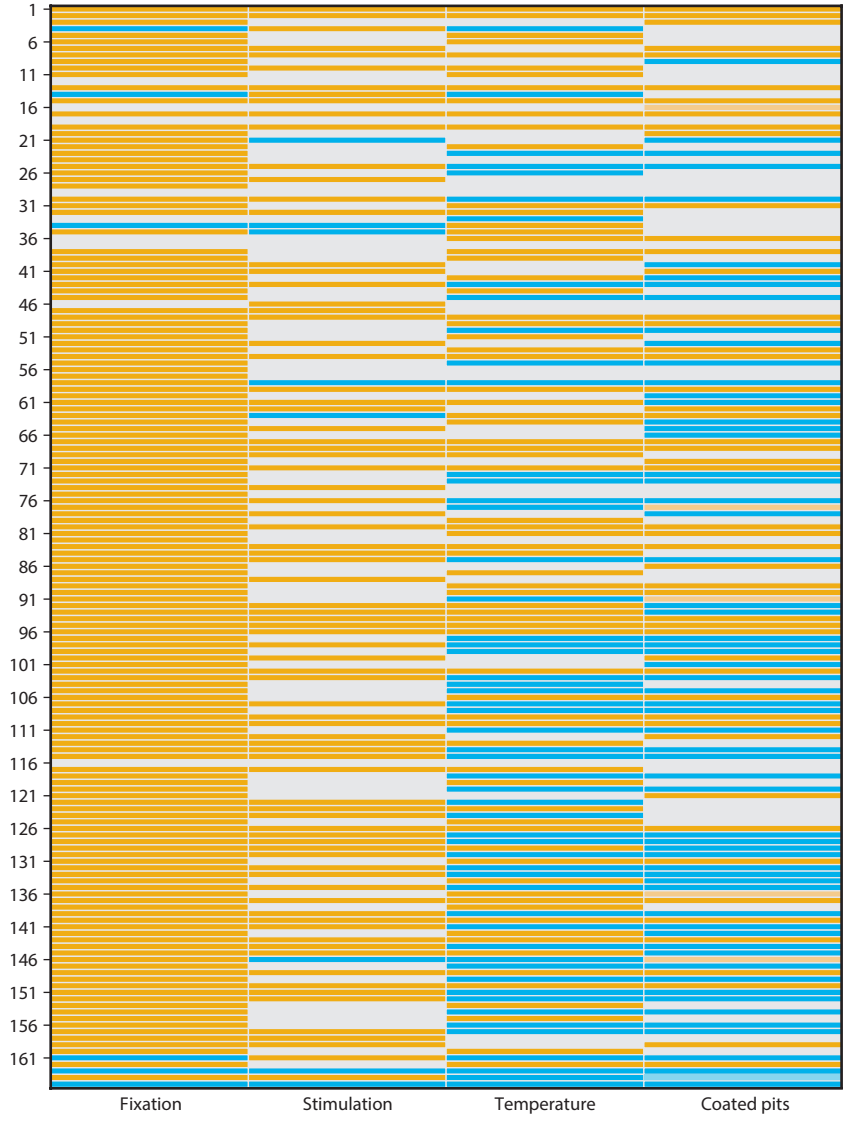

Figure 2

A heat map showing the results of a literature search on PubMed using the search term "synaptic vesicle endocytosis electron microscopy." The number on the *y*-axis corresponds to the assigned number for each article in **Supplemental Table 1**. The fixation column indicates whether specimens are chemically fixed (*yellow*) or cryo-preserved (*blue*). The stimulation column indicates whether a strong (*yellow*; e.g., high-frequency stimulation, prolonged duration, or elevated potassium) or weak (*blue*; e.g., single) stimulus is applied to the specimens. The temperature column indicates whether specimens are processed at nonphysiological (*yellow*) or physiological temperatures (*blue*). The coated pits column indicates whether coated pits are observed (*yellow*) or not (*blue*). In all columns, gray indicates that the values are unclear or not specified. The details are found in **Supplemental Table 1**. Notably, the colors of the temperature column often match with those of the coated pits column.

Supplemental Material >

event at endosomes and that the final step of reacidification may not occur until synaptic vesicles are regenerated from the endosomes; fluorescence does not come back to the baseline in mutants that cannot uncoat clathrin (Watanabe et al. 2018). Thus, this additional step should be reflected in the fluorescence decay. In addition, most vesicle proteins are on the surface and readily retrievable, but vesicular glutamate transporter 1 (vGlut1) molecules do not seem to be on the surface (Balaji & Ryan 2007). Thus, recently exocytosed vGlut1 molecules must travel to the endocytic zone and be internalized via ultrafast endocytosis if this is the only endocytic pathway. Certainly, there is a reserve of vGlut1 in intracellular organelles (e.g., endosomes) that can be sorted into synaptic vesicles, but surface vGlut1 must also be internalized after stimulation. Thus, an additional mechanism such as clathrin-mediated endocytosis is plausible. It would be interesting to investigate whether there is an interplay between ultrafast endocytosis and clathrin-mediated endocytosis and whether the prevalence of clathrin-coated pits increases under certain stimulation conditions in mammalian central synapses at physiological conditions.

Clathrin-mediated endocytosis is also needed after intense neuronal stimulation for retrieval of nonsynaptic vesicle proteins. Recent studies have revealed that synaptic vesicles may act as reservoirs of transmembrane proteins not directly involved in neurotransmitter loading and secretion such as glucose transporter 4 (Glut4) and autophagy-related protein ATG9 (Yang et al. 2022). Under energy stress, glucose transporter Glut4 is mobilized from vesicles to the plasma membrane in an AMPK-dependent manner. Simultaneous measurement of exocytosis and endocytosis kinetics of vGlut1 and Glut4 shows that vGlut1 internalizes rapidly with a tau of approximately 8–10 s, while Glut4 requires approximately 60 s (Ashrafi et al. 2017)—a timescale consistent with clathrin-mediated endocytosis. However, the requirement for clathrin-mediated endocytosis has not been tested in this case. Similarly, recent studies have revealed that autophagy-related protein ATG9 is present on a subset of synaptic vesicles and potentially undergoes activity-dependent exocytosis and endocytosis at synapses (Yang et al. 2022, Olivas et al. 2023). Whether this process is related to neurotransmission is questionable, but defects in endocytosis result in the failure of synaptic autophagy induction, suggesting that ATG9 trafficking is essential for synaptic functions. Given that ATG9 colocalizes with clathrin heavy chain in synaptojanin-null mutants, Yang et al. (2022) conclude that ATG9 retrieval may be via clathrin-mediated endocytosis. However, endosomal resolution rather than endocytosis itself is more disrupted in this mutant, and thus, further studies are necessary to define the role of clathrin-mediated endocytosis in this process. Nonetheless, these results indicate that clathrin-mediated endocytosis may be activated when a subset of synaptic vesicles are utilized.

Kiss-and-Run

Several lines of evidence for kiss-and-run come from kinetic studies. Several investigators have demonstrated that synaptic vesicle recycling occurs with two time constants following high-frequency stimulation, with the faster component typically attributed to kiss-and-run (He & Wu 2007). While it is difficult to interpret pHluorin data since this assay relies on the reacidification of endocytic vesicles, this fast component may have been due to ultrafast endocytosis. Similarly, capacitance measurements in several systems (the calyx of Held, goldfish retinal bipolar cells, cerebellar mossy fiber boutons, etc.) also show a fast component (von Gersdorff & Matthews 1994, He et al. 2006, Delvendahl et al. 2016). These types of data are typically used as evidence for kiss-and-run, but a recent study premised on molecular perturbation indicates that this pathway likely represents a form of clathrin-independent endocytosis analogous to ultrafast endocytosis given its molecular requirements (actin dependent and clathrin independent) and temperature sensitivity (ultrafast endocytosis fails at room temperature) (Delvendahl et al. 2016). Likewise, in goldfish bipolar cells, exocytosis accompanies cell surface expansion and rapid lipid mixing, suggesting that

almost all vesicles undergo full-collapse fusion, and these fusion events are followed by fast endocytosis (von Gersdorff & Matthews 1994). Thus, this pathway likely represents clathrin-independent ultrafast endocytosis, a possibility noted in the earlier study by von Gersdorff & Matthews (1994).

Another line of evidence for kiss-and-run is based on the quantum dye uptake and release assay. In a series of papers by Zhang et al. (2007, 2009), Qdots with a diameter of approximately 15 nm were first loaded into synaptic vesicles and subsequently unloaded by neuronal stimulation (Zhang et al. 2007, 2009). They first compared the kinetics of Qdot unloading with lipophilic FM dye unloading. FM dyes would be released faster than Qdots if kiss-and-run is prevalent since FM dyes are small enough to be released from the fusion pore but Qdots are not. Indeed, the release of Qdots was slower (Zhang et al. 2007). In the subsequent paper, they took advantage of the fact that the fluorescence of Qdots is pH dependent: approximately 15% brighter in neutral pH (extracellular) than in acidic pH (vesicle lumen). They observed full collapse events where a transient increase in fluorescence (presumably as the fusion pore opens) is followed by permanent loss of signals due to the release of the particles. However, they also observed events where Qdots are retained in vesicles: a transient increase in fluorescence signals followed by a rapid decay to the baseline with a time constant of 0.69 s (Zhang et al. 2009). These vesicles can be reused as fast as 10 s, a similar time course to vesicles recycled through ultrafast endocytosis. Although these data are strong and convincing, two major limitations are discussed in the field (Dittman & Ryan 2009). First, the Qdot may be a physical hinderance to the fusion pore dilation, promoting kiss-and-run. Second, Qdots may not be efficiently cleared out of the synaptic cleft, which is about 12–20 nm wide, allowing reuptake through a fast endocytic mechanism such as ultrafast endocytosis. Interestingly, fusion events occurring near the center of a synapse, which would impose the furthest distance for Qdots to travel out of the cleft, tend to show the kiss-and-run signature (reuptake of Qdots). It would be important to revisit these Qdot experiments with a technique such as time-resolved electron microscopy.

The physical hinderance raises an important issue on the biophysics of synaptic vesicle fusion. Unlike dense-core vesicles or secretory granules that undergo kiss-and-run, synaptic vesicles are much smaller, with a diameter of approximately 30–40 nm. Lipids are highly curved in such vesicles, and thus, the membrane is under high tension. When such vesicles fuse with the planar membrane, the tension is released and vesicles collapse immediately. In fact, the theoretical calculation suggests that it only takes a millisecond or less (Stephens et al. 2017). In contrast to the simulations, our time-resolved electron microscopy analysis shows that vesicle collapse requires approximately 10 ms (Kusick et al. 2020). This slowing is likely due to the presence of transmembrane proteins; their diffusion is likely hindered by the steep curvature around the fusion pore. Interestingly, alpha-synuclein promotes dilation of a fusion pore as dense-core vesicles fuse (Logan et al. 2017), suggesting that this process may require external force even for vesicles with a relatively small diameter (~60–80 nm). Whether such a mechanism is needed for synaptic vesicle fusion is unclear, but if so, under certain circumstances—like when the active zone membrane becomes stiffer due to the accumulation of vesicle proteins—kiss-and-run-like fusion events may be more prevalent. Further investigations are warranted.

CONCLUSIONS AND OUTLOOK

Over the last decade, accumulating evidence suggests that the initial step of synaptic vesicle recycling is clathrin independent and that clathrin-mediated processes have additional roles at synapses besides generating new synaptic vesicles from endosomes. If more focus was placed on the cisternae pathway, the data accumulated over the years may have been interpreted differently. As discussed above, the ultrafast endocytosis model (rapid clathrin-independent endocytosis

followed by endosomal sorting) at a glance resolves the discrepancy in the literature. However, many questions remain:

1. Are vesicle membranes and proteins internalized altogether? Or is there an additional route for synaptic vesicle protein internalization? Is there a contribution from clathrin-mediated endocytosis or kiss-and-run?
2. Ultrafast endocytosis, clathrin-independent fast endocytosis, and activity-dependent bulk endocytosis have been described, but are these three forms of clathrin-independent endocytosis related (or even the same)?
3. Are endocytic vesicles always sent to endosomes, or can they be budded directly into synaptic vesicles? And what proteins are required for the process?
4. How can clathrin bud vesicles from endosomes in a few seconds? Are clathrin and its associated proteins preaccumulated on endosomes or endocytic vesicles?
5. Is there a cell type– or organism-specific difference in the modes of synaptic vesicle recycling?
6. Many mutations associated with neurodegeneration are found on proteins regulating synaptic vesicle recycling, but how do these proteins normally regulate synaptic membrane trafficking?

Addressing these questions would require further refinement and development of technologies and approaches but will be fundamental to advance our understanding of synaptic biology. It is my hope that the field comes to an agreement on the mechanisms of synaptic vesicle recycling in near future.

DISCLOSURE STATEMENT

The author is 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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