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Topical Review

Endocytosis at the synaptic terminal

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Exocytosis of neurotransmitter from a synaptic vesicle is followed by efficient retrieval of its constituent membrane and proteins. Real-time measurements indicate that fast and slow modes of retrieval operate in parallel at a number of presynaptic terminals. Two mechanisms can be distinguished by electron microscopy: clathrin-mediated retrieval of small vesicles and bulk retrieval of large cisternae. Methods that investigate the behaviour of individual vesicles have recently demonstrated a third route of retrieval: the rapid reversal of a pore-like connection between the vesicle and surface ('kiss-and-run'). Key aims for the future are to identify the molecules underlying different mechanisms of endocytosis at the synapse and the signals that select between them.

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Neurons transmit information at chemical synapses by exocytosis of small vesicles laden with neurotransmitter. This event is triggered by calcium influx and can occur at rates of hundreds of Hertz (Katz, 1969). The presynaptic terminal therefore faces a problem: in order to maintain the supply of secretion-competent vesicles and a normal morphology, the excess membrane and vesicle proteins must be recovered. The importance of recycling synaptic vesicles is illustrated by the fruit fly mutant *shibire* that cannot retrieve synaptic vesicles at high temperatures: on warming, the flies become paralysed and fall out of the air.

Exactly how the presynaptic terminal retrieves excess membrane is still a matter of debate. Three basic mechanisms of endocytosis are thought to operate: retrieval of small vesicles coated by clathrin, bulk retrieval of large portions of membrane and fast recapture of vesicles that do not fully collapse into the surface. In this review we examine the evidence for these three routes of endocytosis and ask how far they might account for the physiological properties of endocytosis measured at different synapses. Real-time measurements demonstrate that many synapses contain both fast and slow mechanisms of endocytosis and the relative importance of these different routes depends on how strongly the synapse is stimulated. The molecular mechanisms of fast and slow retrieval are less clear.

Mechanisms of endocytosis at the synaptic terminal

In the early 1970s, two groups used electron microscopy to examine how stimulation altered the ultrastructure of the frog neuromuscular junction (NMJ). Stimulating at 10 Hz for 1 min, Heuser & Reese (1973) observed decreased numbers of synaptic vesicles and the appearance of membranous cisternae emanating from the plasma membrane (Fig. 1*B*). If a rest period was allowed after stimulation, vesicles reappeared, apparently at the expense of the cisternae. Clathrin-coated pits and vesicles were frequently observed, particularly at sites removed from the active zone. In contrast, Ceccarelli *et al.* (1973) observed little change in the ultrastructure of the terminal following stimulation at lower frequency (2 Hz for up to 4 h; Fig. 1*C*). After release, vesicles were recycled fast enough to prevent depletion. These two sets of observations have been widely interpreted as indicating that two mechanisms of vesicle release and retrieval exist at the NMJ: some vesicles collapse fully into the plasma membrane and are then recycled by clathrin-mediated endocytosis (CME) while other vesicles release neurotransmitter *without full collapse* and are then retrieved by a direct and rapid reversal of this process. The latter mechanism has been poetically termed kiss-and-run (Fesce *et al.* 1994).

The idea that endocytosis at the synapse might occur by a simple reversal of the fusion step is attractive because it would provide a rapid and economical way of recycling vesicles. The importance of kiss-and-run has been difficult to assess (Fesce *et al.* 1994), but a leap forward has recently been made by the use of methods that resolve the fusion and retrieval of individual vesicles. Capacitance measurements in pituitary nerve terminals show that microvesicles form a transient connection with the surface membrane in about 5 % of fusion events (Klyachko & Jackson, 2002). Imaging fusion of individual vesicles at the ribbon synapse of retinal bipolar cells also indicates that kiss-and-run is

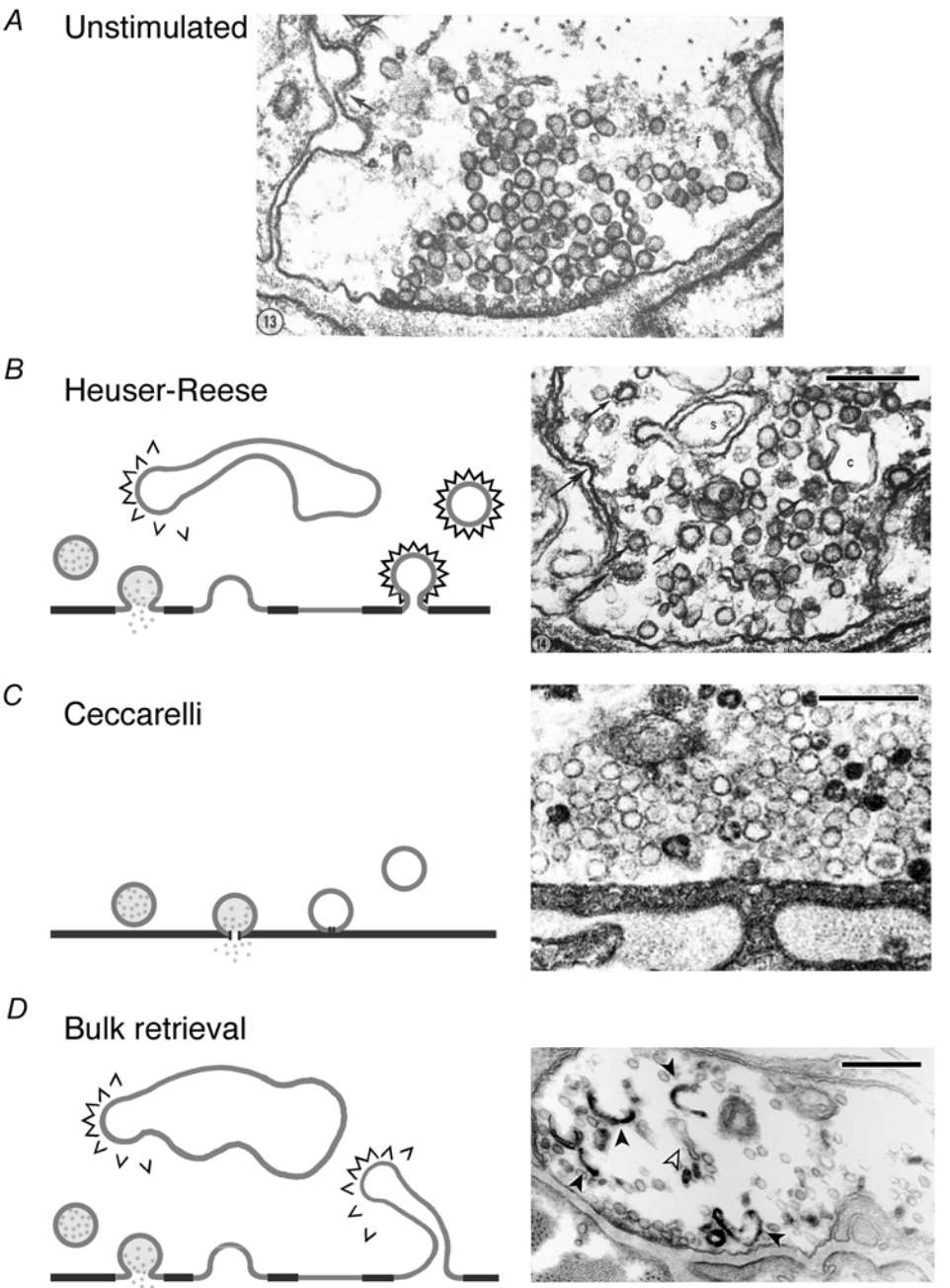

Figure 1. Models of synaptic vesicle retrieval at the frog NMJ

A, electron micrograph of the frog NMJ at rest. *B*, Heuser-Reese model for retrieval. Diagram (left) shows that vesicles fully collapse into the plasma membrane and are retrieved by CME. Coated vesicles may also form from cisternae. Electron micrograph (right) of frog NMJ following stimulation at 10 Hz for 1 min. Coated pits and vesicles (arrowed) and cisternae (c) are seen in the terminal. *C*, Ceccarelli model for retrieval. Diagram (left) shows a vesicle releasing neurotransmitter through a transient fusion pore by 'kiss-and-run'; the vesicle is retrieved at the same site. Micrograph (right) of a terminal stimulated at 2 Hz for 2 h showing an absence of clathrin-coated vesicles and cisternae at low stimulation frequency. *D*, bulk membrane retrieval. In this model (left) large areas of membrane are internalised following complete vesicle collapse. Coated vesicles may bud from membrane invaginations or from large, internalised cisternae. Micrograph (right) shows internalised cisternae that are empty (open arrows) or filled with photoconverted FM1-43 (filled arrows). Scale bars, 250 nm in all cases. Figures reproduced from Heuser & Reese (1973), Ceccarelli *et al.* (1973) and Richards *et al.* (2000) with permission from the Rockefeller University Press and Elsevier Science/Cell Press.

Table 1. Comparison of the kinetics of endocytosis at different presynaptic terminals

Preparation	Endo τ_{fast} (s)	Endo τ_{slow} (s)	Effect of increased stimulation	Effect of increased $[Ca^{2+}]_i$
Goldfish bipolar cells	~1 [a,b]	10 [b,c]	More endocytosis by slow mode. No effect on rates [c]	$[Ca^{2+}]_i$ stimulates fast mode [c]
Mouse inner hair cells	0.3 [e]	7.5 [d] 15 [e]	—	Fast mode seen in more cells. No effect on rates [d]
Frog NMJ	20 [f]	~5 min [f]	Slows rate	No effect of $[Ca^{2+}]_i$ [g]. $[Ca^{2+}]_i$ may inhibit fast endocytosis [f]
Rat hippocampal neurons	6 [h] 1 [i] 0.4–0.9 [l]	20–60 [j] 4–90 [k] 8–>21 [l]	Complex (see text)	Complex (see text)
Mouse calyx of Held	0.1 [m]	~14 [m]	Slows rate	No effect
Rat posterior pituitary terminals	0.3 [n]	>2 [n]	—	—

References: [a] von Gersdorff & Matthews, 1994; [b] Neves & Lagnado, 1999; [c] Neves *et al.*, 2001; [d] Moser & Beutner, 2000; [e] Beutner *et al.*, 2001; [f] Richards *et al.*, 2000; [g] Wu & Betz, 1996; [h] Klingauf *et al.*, 1998; [i] Pyle *et al.*, 2000; [j] Ryan & Smith, 1995; [k] Sankaranarayanan & Ryan, 2000; [l] Gandhi & Stevens, 2003; [m] Sun & Wu, 2002; [n] Klyachko & Jackson, 2002.

negligible under conditions where large numbers of vesicles are released (Zenisek *et al.* 2002). In hippocampal synapses, however, kiss-and-run accounts for a majority of release events when the release probability is low (Gandhi & Stevens, 2003).

A third mechanism of endocytosis, involving the formation of deep membrane infoldings, has also been consistently observed in the frog NMJ (Richards *et al.* 2000), as well as lamprey reticulospinal synapses (Gad *et al.* 1998), snake motor terminals (Teng *et al.* 1999; Teng & Wilkinson, 2000), rat hippocampal neurons (Takei *et al.* 1996) and goldfish bipolar cells (Paillart *et al.* 2003). These large invaginations occur away from the site of fusion and their formation is not thought to directly involve clathrin (Fig. 1*D*). The infoldings may be either pinched off from the surface, or may remain connected to the plasma membrane (Takei *et al.* 1996; Teng & Wilkinson, 2000). The current view is that the cisternae are not formed by the coalescence of internalised clathrin-coated vesicles as originally proposed (Heuser & Reese, 1973). Instead, synaptic vesicles are thought to be formed by CME from the infolded membrane, because clathrin-coated buds have been demonstrated on the cisternae in lamprey synapses and snake motor terminals (Shupliakov *et al.* 1997; Teng & Wilkinson, 2000).

Of these three basic mechanisms of retrieval, by far the best understood is CME. The molecules involved in CME are enriched at the synapse (Maycox *et al.* 1992; Ball *et al.* 1995; Ringstad *et al.* 1999; Roos & Kelly, 1999; Teng *et al.* 1999; Teng & Wilkinson, 2000) and clathrin-coated vesicles are observed at a number of synaptic terminals following stimulation (Heuser, 1989; Takei *et al.* 1996; Shupliakov *et al.* 1997). Rapid progress has been made in understanding how CME operates at the molecular level, although most of this information is derived from studies of endocytosis of transmembrane proteins in non-neuronal cells (Marsh & McMahon, 1999; Brodsky *et al.* 2001). The molecular mechanisms of CME are summarised in Fig. 2. The hetero-tetrameric adaptor complex AP-2 binds to transmembrane proteins at the surface and recruits clathrin triskelions that assemble in a lattice over the membrane surface. Formation of the lattice deforms the membrane to form a coated pit. Subsequent scission of the vesicle from the surface requires the large GTPase, dynamin. Within the cytoplasm, the clathrin coat is removed and the naked vesicle can then fuse with other endosomal structures. CME at the synapse is generally assumed to be similar to that in non-neuronal cells, although neuron-specific proteins may substitute for some of the key players shown in Fig. 2. An example is AP180, which can bind to clathrin, phosphoinositides in membranes (Ford *et al.* 2001) and possibly synaptobrevin, the v-SNARE in the vesicle membrane (Nonet *et al.* 1999). These properties suggest that AP180 may act together with AP-2 in the early stages of clathrin-coated vesicle formation.

Important questions regarding the role of CME remain. Clathrin-coated vesicles are not a consistent feature of stimulated synapses (Ceccarelli *et al.* 1973; Paillart *et al.* 2003) and where they do occur, it is often unclear whether they have budded directly from the surface membrane (Heuser & Reese, 1973), or from large invaginations still connected to the surface, or from endosomes (Takei *et al.* 1996). Another important issue is speed. The rate of receptor internalization by CME in non-neuronal cells is of the order of minutes, while many synapses possess a fast mode of endocytosis occurring on a time scale of 1 s or less.

The physiology of endocytosis at the synapse

For many years, our understanding of endocytosis at the synapse was limited by a lack of direct information about this process in living neurons. More recently, real-time measurements of endocytosis have been made at a number of synapses using electrophysiology and imaging methods.

Fast and slow modes of endocytosis operate in parallel at a number of synapses, as summarized in Table 1 and described in more detail below.

Giant synapses

The capacitance technique is a particularly direct method of monitoring membrane retrieval because the electrical capacitance of the surface membrane is directly proportional to its area, and changes can be measured on a time scale of tens of milliseconds (Neher & Marty, 1982). This method can be applied to large synaptic terminals, such as the ribbon synapse of bipolar cells from the goldfish retina. Using this preparation, von Gersdorff & Matthews (1994) were the first to directly demonstrate the speed of vesicle retrieval. Following a brief stimulus, all the excess membrane was retrieved with a time constant of ~1 s. Neves & Lagnado (1999) then found that longer stimulation of this terminal (>100 ms) was followed by membrane retrieval occurring in two phases: fast endocytosis was followed by a slow mode, with a time constant of 10 s or more. An increase in stimulus duration increased the proportion of membrane retrieved by slow endocytosis and decreased the proportion retrieved by fast endocytosis, without altering the rate constant of either process. Fast and slow modes of endocytosis could also be distinguished by their dependence on Ca^{2+}. Limiting the spread of Ca^{2+} by introducing calcium chelators caused a proportion of the excess membrane to be retrieved by slow endocytosis, even after a very brief stimulus (Neves *et al.* 2001; Fig. 3*A*). It therefore seems that the Ca^{2+} microdomain triggering fast exocytosis also selected vesicles for fast endocytosis. The idea that fast and slow endocytosis in bipolar cell terminals are mechanistically distinct is supported by the observation that the slow mode of retrieval is selectively inhibited by raising the hydrostatic pressure inside the terminal (Heidelberger *et al.* 2002). Fast and slow modes of endocytosis have also been observed at the ribbon synapse of inner hair cells from the cochlea of the mouse, where fast endocytosis is also triggered by Ca^{2+} (Moser & Beutner, 2000; Beutner *et al.* 2001).

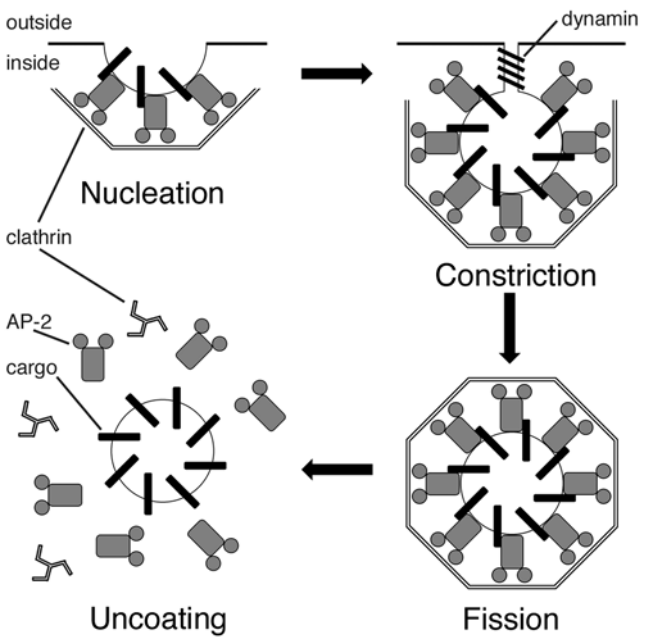

Figure 2. Molecular mechanisms of clathrin-mediated endocytosis

Schematic diagram to show the major steps of CME in non-neuronal cells. CME comprises four major steps: nucleation, constriction, fission and uncoating. Numerous other proteins such as amphiphysin, epsin and endophilin (Brodsky *et al.* 2001) have been omitted for clarity, and only the core molecular players are shown here. The transmembrane cargo protein has been proposed to be synaptotagmin in the case of synaptic vesicle retrieval.

Might the fast mode of endocytosis in bipolar cells occur by kiss-and-run? The possible existence of kiss-and-run has been tested using total internal reflection fluorescence microscopy (TIRFM), which allows fluorescent probes to be imaged within ~100 nm of the plasma membrane. The resulting improvement in signal-to-noise allows the visualization of individual vesicles labelled with the membrane dye FM1-43 and the diffusion of FM1-43 into the surface membrane following exocytosis. Zenisek *et al.* (2000, 2002) found that vesicle fusion in response to a stimulus lasting 500 ms was followed by complete loss of FM1-43, indicating free exchange of lipid. It therefore seems unlikely that kiss-and-run is an important mechanism of vesicle retrieval in bipolar cells, at least in response to this relatively strong stimulus. The proviso may be important, because capacitance measurements show that a 500 ms stimulus is followed by both fast and slow modes of endocytosis (Neves & Lagnado, 1999).

The capacitance technique has also been applied successfully to the Calyx of Held, a large synaptic terminal in the auditory brainstem. Sun *et al.* (2002) measured capacitance in the presynaptic terminal while recording postsynaptic currents. By averaging the capacitance signal following hundreds of thousands of spontaneous miniature events, they measured the rate at which a single vesicle was retrieved. The time constant was surprisingly fast – just 56 ms at room temperature. The time constant of membrane retrieval increased to 115 ms at stimulation rates of 2 Hz, up to tens of seconds at higher frequencies. These findings were interpreted as indicating that a fast mechanism of endocytosis predominates during low frequency stimulation, but quickly saturates at higher frequencies, leaving vesicles to be retrieved by a slower mechanism (Sun *et al.* 2002). Buffering internal Ca^{2+} to different levels did not alter the rate of retrieval.

Capacitance recordings made in the cell-attached configuration have lower noise, allowing the fusion of individual vesicles to be detected (Neher & Marty, 1982; Albillos *et al.* 1997; Klyachko & Jackson, 2002). In chromaffin cells, the upward capacitance step caused by

the fusion of a single granule is sometimes followed by a rapid downward step of equal size, indicating that exocytosis of individual granules can be rapidly reversed. However, only about 5 % of granules run away after the kiss (Ales *et al.* 1999), indicating that the great majority of exocytotic events in chromaffin cells involve full fusion. Until recently, it was unclear how far the behaviour of granules 200–300 nm in diameter could be related to synaptic vesicles about 30–40 nm in diameter. The first study to resolve retrieval of individual small vesicles was carried out by Klyachko & Jackson (2002) by making capacitance recordings from cell-attached patches on large terminals of posterior pituitary neurons, which contain microvesicles 50 nm in diameter that are similar to small vesicles found at synapses. About 5 % of the step increases in capacitance generated by fusion of a microvesicle were paired with down-steps of equal size occurring within 2 s (Fig. 3*B*). The connection between the microvesicle and surface (the fusion pore) was open for an average of 0.31 s. Curiously, the pore had a conductance of only 20 pS (~0.3 nm radius), which would only allow a small trickle of neurotransmitter. The other 95 % of fusion events involving microvesicles were not obviously paired with a downward capacitance step, indicating that kiss-and-run was not the usual mode of exocytosis (Klyachko & Jackson, 2002).

Hippocampal boutons

The small presynaptic boutons of cultured hippocampal neurons have been studied by fluorescence imaging using FM1-43 (Cochilla *et al.* 1999) or fluorescent proteins associated with vesicles (Ryan, 2001). In earlier studies, the approach was to stain membrane compartments formed by endocytosis by applying FM1-43 at various times following strong stimulation. These experiments indicated that retrieval of stained membrane was relatively slow, of

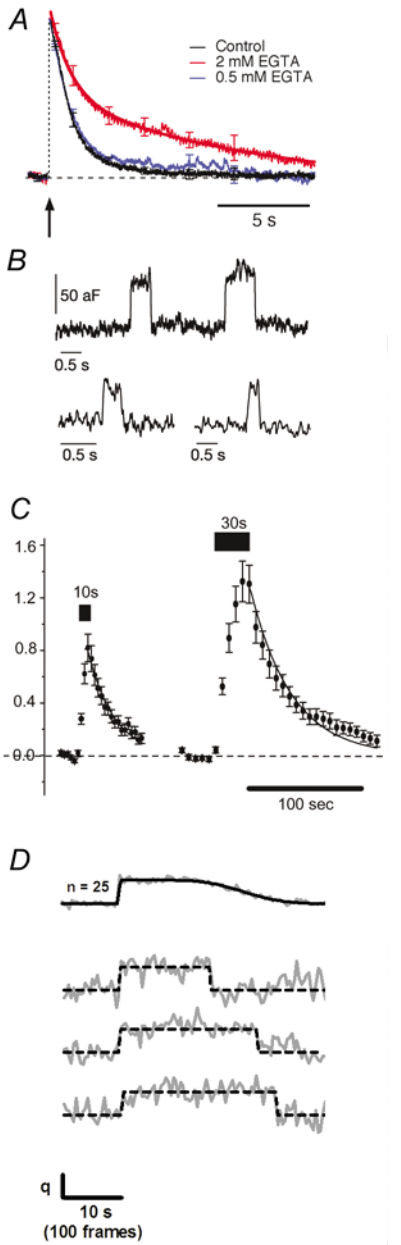

Figure 3. Physiological measurements of endocytosis

A, whole-cell capacitance recordings in goldfish retinal bipolar cells. A brief, 20 ms depolarisation (arrow) causes exocytosis and subsequent membrane retrieval by fast endocytosis. Introduction of EGTA to chelate intracellular calcium causes a proportion of membrane to be retrieved by slow endocytosis. *B*, low noise cell-attached capacitance recording in a pituitary nerve terminal reveals capacitance flickers that correspond to kiss-and-run of microvesicles. Note the similar size of the down-step following an up-step in the trace. *C*, average intensity of synapto-pHluorin fluorescence in hippocampal neurons. Transfected cells were imaged by confocal microscopy and stimulated at 10 Hz for 10 or 30 s. The decay of fluorescence at the end of the stimulus corresponds to reacidification of synaptic vesicles following endocytosis. *D*, exocytosis and endocytosis of single vesicles in hippocampal boutons. The top trace is an average of 25 events. The three individual traces below show stepwise changes in synapto-pHluorin fluorescence corresponding to exocytosis and endocytosis of individual vesicles. Figures reproduced from Neves *et al.* (2001), Klyachko & Jackson (2002), Sankaranarayanan & Ryan (2000) and Gandhi & Stevens (2003) with permission from the National Academy of Sciences and Nature Publishing Group.

the order of 30–60 s (Ryan *et al.* 1993; Ryan & Smith, 1995; Ryan *et al.* 1996), similar to estimates of the rate of CME at the NMJ (Miller & Heuser, 1984). Later studies used synapto-pHluorin, a pH-sensitive form of GFP fused to the intraluminal side of the vesicle protein synaptobrevin-2 (Miesenbock *et al.* 1998; Sankaranarayanan *et al.* 2000). This probe is quenched at the acidic pH inside a vesicle and fluoresces more brightly when a vesicle fuses. Because vesicles are thought to be re-acidified rapidly after retrieval, the rate of endocytosis can be estimated from the rate at which synapto-pHluorin is quenched again. Sankaranarayanan & Ryan (2000) found the average rate of endocytosis to be graded with the length of the stimulus period, with a time constant of 4 s after a brief stimulus and 90 s after a long one (Fig. 3*C*). Lowering the external $[Ca^{2+}]$ during stimulation slowed the rate of retrieval (Sankaranarayanan & Ryan, 2001).

The resolution of measurements using synapto-pHluorin has been dramatically improved by Gandhi & Stevens (2003), who measured the retrieval and reacidification of individual vesicles in response to single action potentials (Fig. 3*D*). Exocytosis of a single vesicle leads to a jump in fluorescence of fixed size (q) caused by unquenching of one vesicle's worth of synapto-pHluorin. The signal then abruptly steps down by an amount $-q$ on one of two time scales: fast endocytosis occurred within 400–860 ms and slow ('compensatory') endocytosis occurred within 8–21 s. The rapid events were identified as kiss-and-run involving a selective fusion pore because the retrieved vesicle could be loaded with the pH buffer Tris but not Hepes. Two other events triggered by an action potential were also identified; a step of q that did not decline, representing a 'stranded' vesicle, and a spontaneous step of $-q$, representing triggered retrieval and reacidification of a vesicle uncoupled from an exocytotic event. The frequency of 'stranded' vesicles and $-q$ events were similar, suggesting that 'stranded' vesicles were left on the surface until an action potential triggered their retrieval.

A notable feature of the results of Gandhi & Stevens (2003) is that stimulation with just one action potential could trigger full fusion of a vesicle followed by slow retrieval, which argues against the idea that full collapse of vesicles only occurs during strong stimulation. Intriguingly, the proportion of vesicles retrieved by each of the three modes of endocytosis differed according to the release probability of the bouton under study (Gandhi & Stevens, 2003). Synapses with a release probability of 0.2 used kiss-and-run in about 70 % of events, with the remainder of vesicles being 'stranded'. In contrast, synapses with a release probability of 0.42 only used kiss-and-run in about 25 % of events, with the remainder being 'compensatory'.

Another approach to investigating endocytosis in hippocampal boutons has been to analyse the kinetics with which FM1-43 and its analogues are released from vesicles during stimulation of exocytosis (Klingauf *et al.* 1998; Pyle *et al.* 2000; Stevens & Williams, 2000). FM2-10 is more hydrophilic than FM1-43 and therefore partitions out of membrane more rapidly. Klingauf *et al.* (1998) found that destaining of boutons loaded with FM1-43 tended to occur more slowly than destaining of FM2-10, and suggested that FM1-43 was not always completely discharged from a vesicle during the time it was in contact with the external medium. By comparison with measurements of the rate at which FM1-43 partitions out of membrane, they estimated that a proportion of vesicles were retrieved by a mechanism with a time constant of ~1 s, and the remainder with a time constant of ~30 s. Aravanis *et al.* (2003) improved the resolution of this approach to the level of single vesicles, and found incomplete discharge of FM1-43 to be a common event in response to single action potentials. Some synaptic vesicles could fuse repeatedly with the plasma membrane, only losing a small proportion of FM1-43 each time.

The neuromuscular junction

A number of studies indicate that there are two distinct vesicle populations at the NMJ of a number of species. Richards *et al.* (2000) used FM1-43 and FM2-10 to distinguish these populations at the frog NMJ. They found that FM1-43 applied for 10 min *after* a 1 min tetanus and then washed away became trapped in large cisternae within the terminal, while FM2-10 applied in this way did not (Fig. 1*D*). These cisternae originated from deep invaginations of the surface membrane, from which FM1-43 was washed out more slowly than its more hydrophilic analogue FM2-10. This slow route of endocytosis operated long after the stimulus and regenerated vesicles slowly. However, *during* stimulation, a rapid endocytotic mechanism retrieved FM1-43 and FM2-10 with similar efficiency. This fast route of endocytosis selectively refilled the 20 % of vesicles in the terminal comprising the readily releasable pool, while the slow route refills the reserve pool. Rapid and selective recycling of vesicles into the readily releasable pool is qualitatively similar to the role of fast endocytosis in hippocampal boutons (Pyle *et al.* 2000). Recent data indicate that fast recycling of the readily releasable pool is sufficient to maintain transmitter output during relatively weak (2–5 Hz) stimulation, suggesting that the reserve pool need not be mobilised under these conditions (Richards *et al.* 2003). It remains to be seen whether the fast mode of retrieval at the frog NMJ is also kiss-and-run. Teng & Wilkinson (2003) have suggested that at the snake NMJ, clathrin-mediated endocytosis accounts for the 'delayed' route of endocytosis, which is sensitive to cooling to below 7 °C; while bulk retrieval or macropinocytosis may account for the pathway that is insensitive to this manipulation.

Electron microscopy and FM1-43 imaging experiments indicate that the *Drosophila* NMJ also contains two pools

of vesicles, as do photoreceptor terminals in this species. Vesicles available for immediate release are thought to be replenished by fast endocytosis close to the active zone (the cycling pool), while the reserve pool is refilled by a slower pathway away from the active zone (Koenig & Ikeda, 1999; Kuromi & Kidokoro, 1999, 2002). There is good evidence that the replenishment of the reserve pool is by CME (Koenig & Ikeda, 1996, 1999). The advantages of *Drosophila* for studying synaptic function rest with the genetic manipulations that this organism allows (Richmond & Broadie, 2002) and such experiments are discussed below.

Similarities and differences in synaptic vesicle endocytosis

The pattern that emerges from real-time measurements is that many synapses possess both fast and slow modes of endocytosis, with the fast mode predominating after weak stimulation, and the slow mode becoming more important after stronger stimulation (Table 1). The yawning gap in our understanding comes in attempting to relate these kinetically distinct modes of retrieval with the three basic modes of endocytosis described above. For instance, the kinetics of endocytosis in hippocampal boutons and bipolar cells is strikingly similar. In bipolar cells, increased stimulation causes a shift from fast endocytosis ($\tau = 1$ s) to slow endocytosis ($\tau > 10$ s) without altering the rate of either process (Neves & Lagnado, 1999; Neves *et al.* 2001). Fast and slow modes of endocytosis are also observed when retrieval of individual vesicles is imaged in hippocampal boutons, and these also have time constants of 0.4–0.8 and 10 s (Gandhi & Stevens, 2003). These similarities beg an obvious question – do fast and slow endocytosis in hippocampal boutons and bipolar cell terminals occur by the same mechanisms? While experiments in hippocampal boutons provide strong evidence that fast retrieval after a single action potential is by kiss-and-run (Aravanis *et al.* 2003; Gandhi & Stevens, 2003), TIRFM experiments in bipolar cells have failed to detect such a mechanism after stimuli lasting 500 ms (Zenisek *et al.* 2002). It therefore seems that these two terminals may possess different mechanisms of fast endocytosis, although before completely ruling out the existence of kiss-and-run in bipolar cells it would be good to test the effects of briefer stimuli that trigger exocytosis followed exclusively by fast endocytosis (Neves & Lagnado, 1999). The mechanism of slow endocytosis is just as elusive. Does this occur by a clathrin-dependent mechanism? And how rapidly might mechanisms of bulk retrieval operate?

It may be that synapses from different parts of the nervous system employ different mechanisms of endocytosis, depending on how the synapse operates. Strong stimuli applied to hippocampal boutons only cause the cycling of a subpopulation of vesicles (Harata *et al.* 2001*a*, *b*), and long depolarisations in high potassium solution cause little change to the ultrastructure (Sara *et al.* 2002). In contrast, strong stimulation of the frog NMJ and of bipolar cells causes release of many more vesicles than are originally docked to the membrane, as well as build-up of internal cisternae (Heuser & Reese, 1973; Lagnado *et al.* 1996; Paillart *et al.* 2003; Holt *et al.* 2003). These differences may indicate that hippocampal boutons rely more on a local and fast form of recycling compared to other synapses (Pyle *et al.* 2000). Gandhi & Stevens (2003) even found important heterogeneities within one type of synapse: kiss-and-run exocytosis was less prevalent at hippocampal boutons with high release probability compared with boutons with low release probability. It will be interesting to test whether stronger stimulation of boutons with low release probability causes a switch to the slow mode of endocytosis.

The gap between physiology and molecules

Although dynamin and clathrin have established roles in endocytosis at the synapse, we are still a long way from understanding the physiology of vesicle recycling in molecular terms.

A universal role for dynamin?

Dynamin is a GTPase that is involved in membrane scission and is thought to play an essential role in a wide range of endocytotic events, both at the synapse and in non-neuronal cells (Urrutia *et al.* 1997; Brodsky *et al.* 2001). The essential role for dynamin in retrieval of synaptic vesicles has been studied extensively using the *Drosophila* orthologue, *shibire*. A temperature-sensitive mutant of *shibire* exhibits paralysis at temperatures > 29 °C. Exocytosis at the NMJ is intact, but endocytosis is inhibited at > 29 °C and a total depletion of vesicles is seen following stimulation at 33 °C (Poodry & Edgar, 1979; Koenig *et al.* 1983).

Evidence from *Drosophila* indicates that dynamin may be essential for both fast and slow modes of endocytosis at the synapse. CME away from the active zone is blocked in *Drosophila* with mutations in the gene for *endophilin,* a protein that binds dynamin, resulting in enlarged boutons and fewer synaptic vesicles per bouton (Guichet *et al.* 2002; Verstreken *et al.* 2002). In the face of this, evoked release is still maintained at 15–20 % of wild-type levels, although FM1-43 is no longer taken up (Verstreken *et al.* 2002). These results indicate that while the majority of retrieval requires CME, a fast kiss-and-run mechanism of endocytosis continues to operate after CME is blocked. Interestingly, all endocytosis was blocked in double *shibire/endophilin* mutants, indicating that all forms of endocytosis at the *Drosophila* NMJ involve the GTPase activity of dynamin (Verstreken *et al.* 2002).

A universal role for dynamin in endocytosis at the synapse has been called into question by Heidelberger (2001) who carried out capacitance experiments indicating that endocytosis at the ribbon synapse of bipolar cells was dependent on ATP but not GTP. The possibility that membrane scission at this ribbon synapse depends on an enzyme other than dynamin is intriguing.

Clathrin-mediated endocytosis

Evidence for a key role of CME at the synapse comes both from electron microscopy (Fig. 1*B*) and from experiments disrupting this mechanism in living neurons (Brodin *et al.* 2000; Richmond & Broadie, 2002). In the giant reticulospinal synapse of the lamprey, introduction of proteins and peptides that inhibit the interaction between amphiphysin and the proline-rich domain of dynamin cause an activity-dependent accumulation of clathrin-coated vesicles (Shupliakov *et al.* 1997). Similarly, peptides that block the interaction between clathrin heavy chain and AP180 or AP2 were found to inhibit clathrin-coated vesicle formation and reduce the number of synaptic vesicles at the squid giant synapse (Morgan *et al.* 1999, 2000). Peptides that inhibit the uncoating of clathrin-coated vesicles by auxilin also cause an increase in the number of clathrin-coated vesicles per active zone (Morgan *et al.* 2001). However, it seems likely that CME is not the only mechanism of endocytosis in the lamprey and squid giant synapse. Vesicles still remain after perturbation of CME at lamprey synapses (Shupliakov *et al.* 1997; Ringstad *et al.* 1999) and squid terminals have been reported to contain a clathrin-independent pathway involving the vesicle protein synaptophysin (Daly *et al.* 2000).

It is also unclear whether disruption of CME affects fast or slow modes of retrieval. An acute disruption of synaptic transmission would be expected if CME played an immediate role in vesicle retrieval, but it is also possible that fast endocytosis occurs by a mechanism that is independent of clathrin, with CME having a longer-term role, such as retrieval of vesicle proteins that leak into the surface membrane (Li & Murthy, 2001). Studies at squid and lamprey synapses have not assayed endocytosis directly; gross changes in the rate of retrieval were inferred from changes in postsynaptic responses measured electrophysiologically and changes in the ultrastructure revealed by electron microscopy. To determine the role of clathrin it will be necessary to disrupt CME at synapses in which endocytosis can be assayed in real-time using capacitance or fluorescence imaging techniques.

A key step in CME is the recognition by the AP-2 adaptor complex of a molecule in the surface membrane, usually a receptor (Marsh & McMahon, 1999; Brodsky *et al.* 2001). What molecule(s) could fulfil this role when vesicles are retrieved at the synapse? Interest has centred on synaptotagmin as a membrane receptor for AP-2 (Zhang *et al.* 1994), although it is not clear whether the high affinity of this interaction *in vitro* accurately reflects the situation *in vivo*. While synaptotagmin remains an attractive candidate to trigger CME (Jorgensen *et al.* 1995), other molecules resident on the synaptic vesicle membrane, such as neurotransmitter transporters or proton pumps, might also play this role.

Several observations suggest that actin may play a role in CME of synaptic vesicles, the strongest evidence coming from electron microscopy at the lamprey reticulospinal synapse (Slepnev & De Camilli, 2000; Shupliakov *et al.* 2002). Disruption of F-actin inhibits vesicle cycling at the *Drosophila* and snake NMJ (Kuromi & Kidokoro, 1998; Cole *et al.* 2000), although these results might be explained by inhibition of exocytosis rather than endocytosis. In contrast, capacitance measurements in bipolar cells and FM1-43 measurements in hippocampal neurons demonstrate that exocytosis, endocytosis and refilling of release sites at the active zone all continue at normal rates after the actin cytoskeleton has been disrupted (Job & Lagnado, 1998; Holt *et al.* 2003; Sankaranarayanan *et al.* 2003). A lack of actin-dependence does not necessarily rule out CME at these synapses, because clathrin-dependent internalization of membrane receptors is not always sensitive to disruption of the actin cytoskeleton (Fujimoto *et al.* 2000).

Unidentified molecules

We have very little understanding of the mechanisms of bulk retrieval at the synapse. The formation of large invaginations of the surface appears to be independent of clathrin because the infolded membranes are not coated (Fig. 1*D*). Although the molecules tubulating membrane at the synapse have not been identified, liposomes containing phosphoinositides can be tubulated *in vitro* by epsin and amphiphysin, two proteins that are enriched at the synapse (Takei *et al.* 1999; Ford *et al.* 2002). This tubulating activity occurs in the absence of clathrin or other factors, so one might speculate that retrieval of large sections of uncoated membrane might represent a 'stripped-down' mechanism that occurs when other elements of the clathrin-dependent pathway are depleted. It seems likely that dynamin is required for scission of these large compartments, because tubular invaginations remain connected to the plasma membrane in *shibire* mutants at the restrictive temperature (Koenig & Ikeda, 1996).

Bulk retrieval at the synapse may be related to macropinocytosis in non-neuronal cells, which is dependent on the actin cytoskeleton and the activity of phosphatidylinositol 3-kinase (Nichols & Lippincott-Schwartz, 2001). In terminals of goldfish bipolar cells, inhibitors of actin remodelling and phosphatidylinositol 3-kinase activity block bulk retrieval measured by uptake of large fluorescent dextrans (Holt *et al.* 2003). In contrast, inhibition of phosphatidylinositol 3-kinase at the frog NMJ blocks a clathrin-dependent step that occurs *after* bulk internalisation (Rizzoli & Betz, 2002). The role of phosphoinositides in regulating endocytosis at the synapse will certainly be an important area of future research.

An important factor regulating exocytosis at many synapses is the Ca^{2+} signal triggering exocytosis (Table 1). The evidence for a stimulatory effect of Ca^{2+} at some

synapses is strong enough to ask what is the calcium-sensitive molecule linked to fast endocytosis? Synaptotagmins are attractive candidates, given their role in exocytosis (Chapman, 2002), but there are several other calcium-binding proteins associated with vesicles and the presynaptic membrane (Marks & McMahon, 1998). The identification of the Ca^{2+}-binding molecule(s) involved in regulating endocytosis may become clearer when the fast and slow modes of endocytosis are defined more clearly at the molecular level.

Conclusions

Our understanding of endocytosis at the synapse is still at a relatively rudimentary stage. The evidence for neurotransmitter release by kiss-and-run has become much firmer in the last year (Klyachko & Jackson, 2002; Aravanis *et al.* 2003; Gandhi & Stevens, 2003), but we do not know what molecules are involved. What is the fusion pore made of? The role of bulk retrieval in recycling vesicles to a reserve pool has been characterized (Richards *et al.* 2000; Paillart *et al.* 2003), but we do not understand the mechanisms by which large membrane compartments are formed. The mechanism of clathrin-mediated endocytosis is understood in much greater detail through work on non-neuronal cells, but we do not have a clear understanding of the importance of this process in retrieval of synaptic vesicles. How rapidly does CME recover vesicles following fast exocytosis triggered by calcium?

We also have little understanding of the ways in which these various routes of retrieval interact. It is clear that the relative importance of fast and slow endocytosis depends on the strength of stimulation (Table 1), and it seems that after fusion, a vesicle can 'choose' between different routes of retrieval (Neves *et al.* 2001; Gandhi & Stevens, 2003). How is this choice made? Might different synapses employ different mechanisms of endocytosis according to their release properties, as suggested by Gandhi & Stevens (2003)?

How will we obtain answers to these basic questions? In our opinion, a major bottleneck in our understanding of endocytosis at the synapse is the gap between physiology and molecules. Bridging this gap will require the coupling of real-time methods for directly assaying endocytosis to preparations that allow manipulation of specific proteins. The search for answers to these questions will surely provide exciting times for the scientists that pursue them.

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