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What is rate-limiting during sustained synaptic activity: vesicle supply or the availability of release sites

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For some types of synapses the availability of release-ready vesicles is a limiting factor during ongoing activity. Synaptic strength in this case is determined both by the recruitment of such vesicles and the probability of their release during an action potential. Here it is argued that not the availability of vesicles is the limiting factor for recruitment, but rather the availability of specific sites to which vesicles can dock.

Keywords: calyx of Held, endocytosis, exocytosis, neurotransmitter release, synapse

Many synapses in the central nervous system transmit tonically at high rates, particularly those mediating signals from sensory inputs (Rancz et al., 2007; Kopp-Scheinpflug et al., 2008; Lorteije et al., 2009). For instance, fibers of the auditory nerve fire up to 100 Hz, even in complete silence (Taberner and Liberman, 2005; Hermann et al., 2007) and some auditory synapses are capable of reliably transmitting at such high rates (Wu and Kelly, 1993; Taschenberger and von Gersdorff, 2000). A number of studies have addressed the question on how nerve terminals manage to maintain high release rates, given that there are a limited number of vesicles in a synaptic bouton. Vesicles have to be recycled and loaded with neurotransmitter before being ready for reuse. Almost exclusively vesicles and the vesicle cycle have been at the center of attention in previous studies; see (Fernandez-Alfonso and Ryan, 2004; Südhof, 2004; Rizzoli and Betz, 2005) for review. The issue has fuelled debates about short-cuts in the cycle, such as kiss-and-run exo/endocytosis (Stevens and Williams, 2000; Zhang et al., 2009) and the role of reserve pools (Kuromi and Kidokoro, 1998; Murthy and Stevens, 1999; Ikeda and Bekkers, 2009). There is, however, yet another possible bottleneck in the cycle, which is the availability of receptive release sites, to which vesicles can dock. Although a fixed number of release sites has been a firm concept of synaptic research since Katz (1969), representing the basis for all of binomical statistical analysis (Clements and Silver, 2000; Scheuss and Neher, 2001), little attention has been paid so far to the possibility that intact release sites may be the rate-limiting resource during high sustained demand; but see Dittman and Regehr (1996), Kawasaki et al. (2000), Pan and Zucker (2009). Here, I will assume that a release site (= vesicle docking site) can exist in three different states: (i) empty and accessible for a vesicle (ii) occupied, ready for its vesicle to exocytose (iii) empty and refractory (not accessible for a vesicle shortly after a fusion event); see also **Figure 1**. I will argue that, indeed, the demand on availability of release sites may be much more stringent than that on vesicle supply. Also, I will discuss some recent results, which invoke endocytic molecules in controlling a rapid step in the cycle, probably mediating clearance of release sites from vesicular components, with which they interacted before and during exocytosis.

EVIDENCE FOR A FIXED NUMBER OF RELEASE-SITES

The statistics of quantal release have provided early evidence for a limited number of release sites (see Clements and Silver, 2000 for review). Basically, this notion results from the finding that fluctuations in the number of quanta released per pre-synaptic stimulus become very small at very high release probability (e.g. under high calcium concentration). This is exactly what one expects for a fixed number N of release- (or vesicle docking-sites) and a release probability p_r near one. Most stimuli will then release nearly N vesicles with little trial to trial variability. Physiologists, therefore, have been quite confident, that a given synaptic connection is equipped with a well-defined number of release-sites, which are used repetitively upon stimulation. Quite a number of studies went even further demonstrating that the number N derived from release statistics agrees well with the morphologically determined number of active zones (AZ) at a given synaptic junction (Korn et al., 1981) or with the number of boutons (Silver et al., 2003). Since there can be several vesicles (≈3–6) morphologically docked at an AZ, it was postulated that there must be a so-called "single vesicle release constraint" in the sense that once one vesicle has been released at a given AZ, no further release events are possible at this AZ within some refractory period (Stevens and Wang, 1995). However, more recently several exceptions to that rule have been found (Wadiche and Jahr, 2001; Oertner et al., 2002). Also, it has been shown that postsynaptic receptor saturation may bias the statistical estimate of N, such that it artifactually seems to agree with the number of active zones (Meyer et al., 2001). At the calyx of Held nerve terminal (on which most of the quantitative arguments, to be

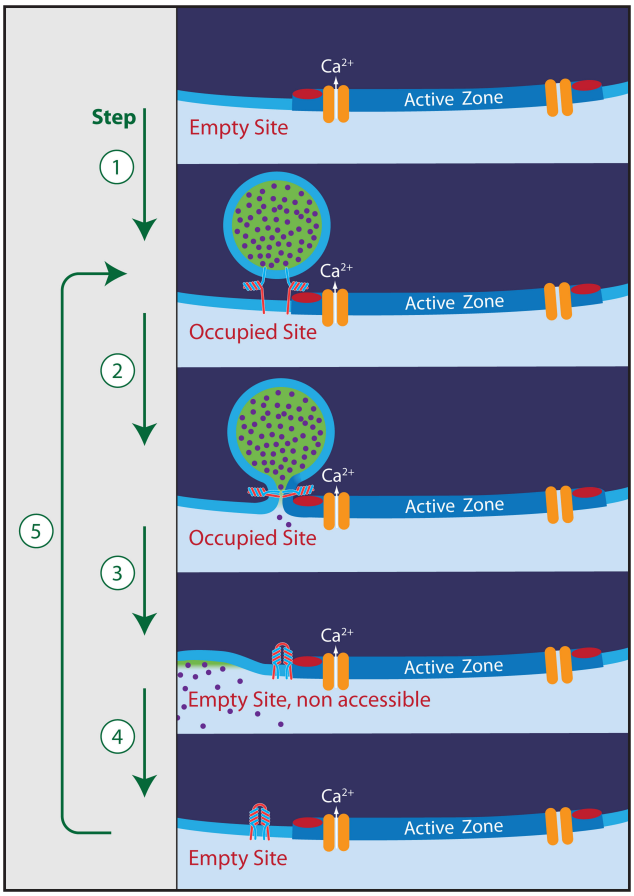

FIGURE 1 | The release cycle. Five steps can be identified: (1) Docking of a vesicle to an empty release site (red bar). Mean residence time of a vesicle is at least 40 s in the absence of stimulation. (2) Ca^{2+}-triggered exocytosis. (3) Removal of the vesicle by full collapse fusion or by kiss-and-run. Some vesicular components may still be bound to the release site, leaving it non-accessible for the docking of a new vesicle. (4) Clearance of the release site by dissociation of remaining interaction partners (must happen within 200 ms during high-frequency stimulation). (5) Docking of a new vesicle (equivalent to step 1). The symbol for the entity blocking the release site in the non-accessible state was chosen to resemble a *cis*-SNARE complex. Such complexes, in association with Rim, Munc-13, synaptotagmin, and possibly Ca-channels may, indeed, be the postulated blockers. However, this assignment and the relationship of such a complex to endocytosis is clearly a matter of speculation in the framework of this electrophysiology-based perspective.

discussed below, are based) a three-dimensional reconstruction of electron micrographs has identified about 500 active zones (Satzler et al., 2002). At least 1500 vesicles can be released at this synapse within a few milliseconds, without evidence for a single vesicle release constraint (Sakaba and Neher, 2001). For the following discussion I will, therefore, assume that there are three equivalent and independent release sites per active zone.

Another piece of evidence for a fixed number of release sites results from kinetic studies. If there were no limiting factors for vesicle docking, the number of docked and primed vesicles at steady-state, in the absence of stimulation, should be the result of a dynamic equilibrium between a docking/priming reaction and its reverse. "Undocking/unpriming" of vesicles has, indeed, been observed in TIRF studies on retinal bipolar cells (Zenisek et al., 2000) and its functional consequences have been studied in neuroendocrine cells (Dinkelacker et al., 2000). Also, the priming rate in adrenal chromaffin cells is strongly dependent on the intracellular Ca^{2+} concentration (Ca^{2+}). Large changes in the number of release-ready secretory granules when changing (Ca^{2+}) were observed in agreement with the expectation of a dynamic equilibrium (Voets, 2000). In contrast, no such increases in the size of the readily releasable pool of vesicles (RRP) could be detected in the calyx of Held terminal, when increasing (Ca^{2+}) (Sakaba and Neher, 2001; Hori and Takahashi, 2009) although the priming rate of vesicles in this preparation is highly dependent on (Ca^{2+}) (Hosoi et al., 2009). It is therefore concluded that during periods of rest the RRP fills up to a level, which is dictated by the number N of available sites. Given that no changes could be documented and assuming a change >20% might have been recognized, one may conclude that under normal resting conditions at least 80% of the sites are occupied. Together with the experimental finding that the basal priming rate (at zero Ca^{2+}) is about 0.1 pools/s, a simple model of reversible priming would predict that the "unpriming rate" is smaller than 0.025, or else that the mean residence time of a vesicle at a given site is at least about 40 s in the absence of stimulation. This view is in line with estimates for vesicle undocking times (2 min) in hippocampal neurons, using styryl dyes (Murthy and Stevens, 1999).

SOME NUMBERS FROM THE CALYX OF HELD

The calyx of Held (Held, 1893) is a large glutamatergic nerve terminal in the auditory pathway. The synapse, located in the medial nucleus of the trapezoid body, is amenable to simultaneous dual voltage clamp of the pre- and postsynaptic compartments. This allows one to perform a detailed biophysical analysis of synaptic transmission (Forsythe, 1994; Borst et al., 1995). Also a 3-D morphometric EM-reconstruction is available (Satzler et al., 2002) such that many aspects of this synapse are quantitatively well defined (Meinrenken et al., 2003; Neher and Sakaba, 2008). Fibers emanating from the globular bushy cells in the ventral cochlear nucleus, which form the calyx, fire at rates between 1 and 200 Hz in complete silence and can reach frequencies of 500 Hz during tone bursts (Kopp-Scheinpflug et al., 2008; Lorteije et al., 2009). In acute slices, using 100-Hz stimulation, the synapse of juvenile animals (postnatal day 12–14) has been shown to support quantal contents of about 40–50 vesicles per action potential for periods up to half a second (Taschenberger et al., 2005). The number of active zones is about 500–700 and the number of release sites – or else the RRP-size after periods of rest – is 1500–1800 (see Neher and Sakaba, 2008 for review). Thus, one can conclude that during the first second of 100-Hz stimulation each release site is used about three times per second (100 Hz × 45 quanta/stim divided by the RRP). A very similar number for site usage can also be calculated from the quantal content of steady-state responses under 500-Hz stimulation of mature calyces at 37°C (see **Table 1**). Remarkably, the same quantal content was found for recordings in 2 mM external (Ca^{2+}) and in 1.2 mM external (Ca^{2+}) (Lorteije et al., 2009), although release probability under the latter condition is quite low. The total number of vesicles in a calyx terminal was estimated between 77000 (Satzler et al., 2002) and 188 000 (de Lange et al., 2003). Thus, each vesicle is used every 13 or 30 s depending on which estimate is used and provided that vesicle pools intermix during such strong stimulation.

Table 1 | Kinetic and morphometric parameters[a] for the calyx of Held nerve terminal.

No. of active zones	500	Satzler et al. (2002)
Total no. of vesicles	77000–180000	Satzler et al. (2002), de Lange et al. (2003)
Recycling pool, no. of vesicles	20000–40000	de Lange et al. (2003), Yamashita et al. (2005)
No. of fast release sites	1500	Sakaba and Neher (2001)
No. of recycling vesicles per site	20	See above
No. of slowly releasing vesicles	1500	Sakaba and Neher (2001)
Residence time for vesicles at rest	>40 s	Estimated on the basis of a simple model for a docking/undocking equilibrium[b]
Quantal content at 100 Hz	40–50	Steady state no. of vesicles released per action potential[b]
Max. usage of specific release sites	3/s	Docking-release cycles at 100 Hz stimulation; p12–14; room temperature[b]
	3.5/s	At 500 Hz; adult; 37°C[c]
	1.5/s	At 60 Hz; adult gerbil; 37°C[d]
Max. time for reuse of a given vesicle	13–30 s	At 100-Hz stimulation, if all vesicles are recycling
	2.5–5 s	… If only the "recycling pool" is active
Max. rates of endocytosis	0.08–0.17/s	Vesicles endocytosed Per site per second, juvenile Room temperature[b]
	1.8/s	Posthearing; 37°C[e]

Parameters are given per release site, if not stated otherwise. [a]More parameters can be found in Meinrenken et al. [b]See main text. [c]Calculated from data of Lorteije et al. (2009). [d]Calculated from Hermann et al., (2007), assuming 1500 release sites. [e]Calculated from Renden and von Gersdorff (2007), on the basis of 80 aF/vesicle and three sites per active zone.

In case only the "recycling pool" participates, estimated to be 20000 vesicles (de Lange et al., 2003) to 40.000 vesicles (Yamashita et al., 2005), each of these vesicles would still be used only once every 2.5–5 s (see **Table 1** for a summary of parameters).

DEMANDS ON VESICLES AND RELEASE SITES

The comparison of these numbers regarding usage of release sites with those of vesicle usage shows that demands on sites are much more stringent than those on vesicles.

One may ask: Are these numbers comparable? After all, a vesicle has to be endocytosed after sorting of vesicular and plasma membrane components. It has to be uncoated and filled with transmitter before becoming available again for docking and priming. Compared to that, refurbishing a release site seems to be trivial. However, one must keep in mind that before exocytosis the vesicle is stably attached to the release site (**Figure 1**) with a residence time of 40 s or longer (see above). This interaction may occur between vesicular components (e.g. synaptobrevin, synaptotagmin, rab3) and those at the plasma membrane (SNARES, Munc13, Ca-channels, Rim). The mere process of exocytosis not necessarily weakens the interaction. However, the dissociation must happen in less than 200 ms once exocytosis has occurred (assuming that about half of the cycle time of release sites, 330 ms, is spent on docking/priming and the other half on this process). This involves a weakening of the interaction by a factor of 200 (= 40/0.200). The "catalytic mechanism", which brings about this weakening, or else the process, which mediates the rapid "run" (in kiss and run) still have to be found. In any case, the demand on the release-site cycle (see **Figure 1**) may not be trivial at all.

TWO TYPES OF RELEASE EVENTS AT THE CALYX OF HELD

The discussion of neurotransmitter release at the calyx of Held so far dealt only with a fraction of its release capacity. Upon prolonged depolarization (under voltage clamp) an additional 1500 vesicle can be released slowly. These vesicles contribute only little to action-potential-induced release (Sakaba, 2006), but may play a role in asynchronous release during high-frequency stimulation. A striking property of this vesicle pool is that it recovers very rapidly during short rest periods, even faster than the rapidly releasing vesicles do at the highest possible (Ca^{2+}) (Sakaba and Neher, 2001). Unlike the rapidly releasing vesicles, the recovery of which is strongly retarded by blockers of calmodulin, their recovery is robust and not influenced by any of the modulators tested – except for depletion of ATP and interfering with the cytoskeleton (Sakaba and Neher, 2003b). A most interesting question is why this vesicle pool is faster in its recovery. This obviously is linked to a second question why these vesicles release more slowly than the other ones. Two answers have been provided to this latter question. Caged Ca^{2+} measurements (Wolfel et al., 2007) demonstrated that there is intrinsic heterogeneity with respect to the Ca^{2+} sensitivity of release-ready vesicles in the calyx of Held. This finding was proposed to be the basis for the kinetic heterogeneity in depolarization-induced release. However, another caged Ca^{2+} study (Wadel et al., 2007), which measured the Ca^{2+} sensitivity of vesicles remaining after depletion of the rapidly releasing vesicle pool by a short depolarization, found that the Ca^{2+} sensitivity of these vesicles (which should be slow ones) was only slightly reduced. This suggested that the main reason why slow vesicles do not respond so well to action potentials is their location relative to Ca^{2+} channels: If they were docked somewhat more remote from Ca^{2+} channels, they would not sense the localized Ca^{2+} microdomains, which build up and decay rapidly when Ca^{2+} channels open transiently during action potentials. Together with the concept of specific release sites at active zones, where a high density of Ca^{2+} channels prevails, this view also provides a reasonable explanation for the first question: Slowly releasing vesicles recover more rapidly, because they need not "find" a specific release site. Similar to the "newcomers", observed in TIRF microscopy (Zenisek et al., 2000), they can dock and develop their release apparatus anywhere near the active zone (and, maybe, elsewhere). Only vesicles, destined to be rapidly released from specific sites (near Ca^{2+} channels), need to either find such sites and/or wait until such sites become available.

SITE-CLEARANCE AND ENDOCYTOSIS

Several recent studies interfering with the function of "endocytic" proteins have reported inhibitory effects on vesicle exocytosis. Some of these effects develop too rapidly for being explained by a lack of recycled vesicles. The first study, pointing out this problem used the temperature-sensitive fly mutant "shibire" (Kawasaki et al., 2000). Shibire affects the protein dynamin, which facilitates the pinching off of vesicles (Mettlen et al., 2009). Kawasaki et al. observed that flies at non-permissive temperatures exhibited a fatigue phenotype within 20 ms. This is much shorter than the time required for endocytosed vesicles to become re-available for exocytosis, which is typically on the order of 10 s (Betz and Wu, 1995; Fernandez-Alfonso and Ryan, 2006). Therefore, Kawasaki et al. (2000) concluded that a block of endocytosis alone and the resulting lack of vesicles cannot be the cause for the paralysis. Rather, they postulated that clearance and re-priming of release sites is occluded by accumulation of endocytic intermediates at such sites. A number of other studies have interfered with various proteins, believed to be part of the endocytic machinery, and demonstrated very rapidly developing effects on exocytosis (Shupliakov et al., 1997; Chen et al., 2003; Ferguson et al., 2007). A very sensitive phenomenon in this respect is short-term synaptic depression (STD), which is observed in many types of glutamatergic synapses. STD is a reduction in synaptic responses, which typically sets in within the first 2–5 EPSCs, when stimulating at frequencies of 10–100 Hz. Part of this decrease is a reduction in the number of available vesicles. STD reaches a steady-state within some 10–20 stimuli, which reflects a balance between vesicle usage and recruitment of new vesicles. The typical phenotype of the relevant studies on endocytic proteins is an intact initial EPSC, but a deepening of STD and a slow-down of recovery from STD. Since such effects can also be observed early after blocking endocytosis (before the vesicle store is depleted) the limited availability of vesicles for docking and priming is unlikely to be the cause for this depression.

A recent study (Hosoi et al., 2009) observed this same phenotype for six different manipulations involving proteins, which either participate in endocytosis or else have to be sorted before endocytosis. These manipulations were

- infusion of a peptide which interferes with the binding of dynamin to amphiphysin
- external application of dynasore, which blocks the GTPase activity of dynamin
- infusion of a dynamin antibody
- infusion of a peptide disrupting the interaction between AP2 and amphiphysin
- infusion of a peptide from the N-terminal proline-rich domain of synaptobrevin
- infusion of the C2B domain of synaptotagmin 2

The authors concluded that "block of endocytosis has a limited retrograde action on exocytosis, delaying recruitment of release-ready vesicles and enhancing short-term depression". They noted that this effect develops rapidly, while endocytosis itself (as measured by a change in membrane capacitance) is a relatively slow process, taking tens of seconds until completion. It is, therefore, likely that the respective manipulations affect a step in between exo- and endocytosis, which is necessary for the restoration of a release-site. Translocation of synaptic components from sites of exocytosis to the "periactive zone" (Roos and Kelly, 1999) may be a candidate for this step, which may be perturbed by these manipulations or simply slowed by the accumulation of vesicular proteins. It should be stressed that this step, whatever it is molecularly, has important physiological roles, since it determines synaptic strength during second-long episodes of sustained activity (Hosoi et al., 2009) and was shown to be a target for modulation by second messengers, such as Ca^{2+} and cAMP (Sakaba and Neher, 2003a).

A second limitation on sustained release is set by endocytosis itself (**Figure 2**). Maximum rates of endocytosis after short, strong stimuli were found to be 10–20 fF/s (Sun et al., 2002; Yamashita et al., 2005; Hosoi et al., 2009). Assuming a value of 80 aF for the capacitance of a single vesicle (Sakaba, 2006) this corresponds to 125–250 vesicles per second or 0.08–0.17 vesicles per release site (or about 0.4 vesicles per active zone). This is about 25 times smaller than the maximum rates for reuse of sites and will lead to further slow depression, once the recycling pool of vesicles (20 000–40 000 vesicles, see above) is being used up. At 100-Hz stimulation this is expected to occur after about 6 s. The number of stimuli (600) is similar to that typically used for labeling the recycling pool in hippocampal nerve terminals (Murthy and Stevens, 1999). Also, the rates of endocytosis per release site are similar between the two types of synapses (Sankaranarayanan and Ryan, 2001) and they are of the same order of magnitude as rates of vesicle replenishment after long periods of intense stimulations (Wesseling and Lo, 2002). In this sense, the properties of the juvenile calyx of Held, discussed here, are very much in line with those at other glutamatergic synapses (see also a comparison of different synapses by Fernandez-Alfonso and Ryan (2006), but note that numbers are given per active zone, not per release site. Conversion: three sites per active zone). If such slow rates of endocytosis were the physiologically relevant ones, one would expect that the synapse undergoes deep depression after intense stimulation for more than a few seconds. The recycling pool of 20 000 vesicles would be expected to be refilled only after a few minutes. In contrast, Hermann et al. (2007), stimulating calyces of the gerbil at 60 Hz, found that after an initial fast phase of depression release remained constant for several seconds at a quantal content of approximately 50 quanta (assuming an mEPSC of 50 pA), which further decayed within a minute only by about 30% and remained constant thereafter. Thus, this synapse can sustain much higher rates of vesicle turnover than expected on the basis of measured membrane retrieval rates (1.5 quanta per sec and site, assuming 1500 sites). Several findings may explain the discrepancy: (i) Faster rates of membrane retrieval have been observed following excessive stimulation (Richards et al., 2000; Sun et al., 2002; de Lange et al., 2003). (ii) Endocytic capacity increases with age and temperature (Renden and von Gersdorff, 2007), see **Table 1**; (iii) species differences; (iv) endocytic capacity is partially lost during prolonged whole-cell recording (Smith and Neher, 1997; Renden and von Gersdorff, 2007); (v) vesicles of the reserve pool may be mobilized (Ikeda and Bekkers, 2009).

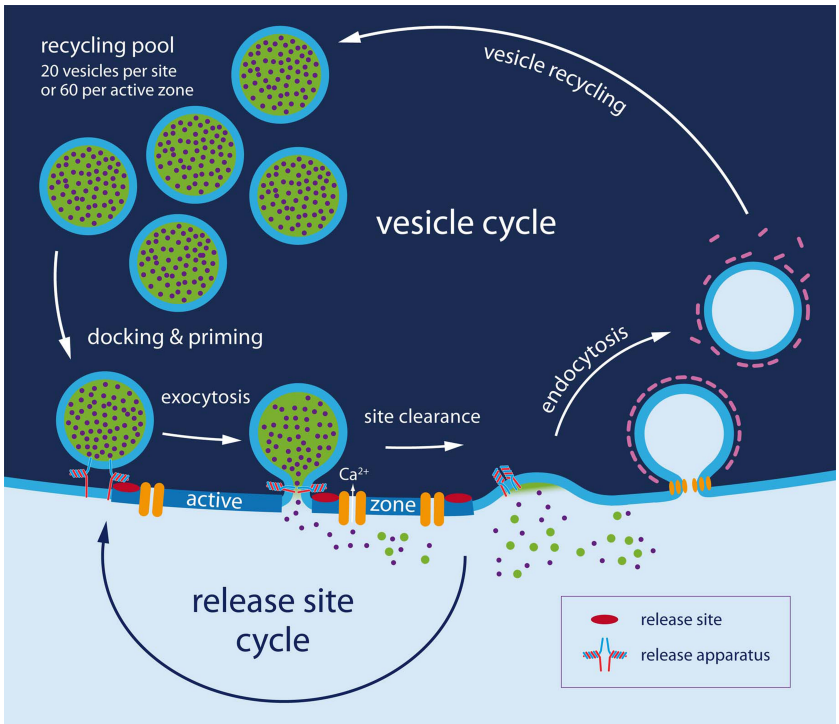

FIGURE 2 | The dual cycle. At least two entities need recycling: The release site (shown in detail in **Figure 1**) and vesicles. Regarding vesicles three additional steps must be considered. (1) Endocytosis, which takes several seconds in patch clamped calyces and hippocampal cultures. (2) Vesicle recycling: 10 s of seconds (3) Vesicle docking and priming. It is proposed (Neher and Sakaba, 2008; Hosoi et al., 2009) that vesicle docking and priming actually occurs very rapidly (within 100–200 ms) once a release site has been cleared and that site clearing is slow (200 ms to 10 s), being modulated by (Ca^{2+}) and other signaling pathways.

Taken together these effects may elevate membrane retrieval to a level close to that of maximum rates of sustained exocytosis. If so, reuse of vesicles, which happens on the seconds to minutes time scale, is not likely to be limiting in the normal operation of a synapse, given that there is a large reserve of vesicles and provided that there is the more stringent and activity-dependent limitation for rapid reuse of release sites discussed here.

CONCLUSION

Scarcity of release-ready vesicles during sustained activity has been an issue of synaptic research since the early studies by Katz et al. on the neuromuscular junction. It is well established that in many types of synapses the level of neurotransmitter release during ongoing activity reflects a balance of vesicle supply and vesicle consumption by exocytosis. Here, it is argued that recycling of vesicles may actually not be the rate-limiting step during physiological activity patterns. Rather the availability of specific sites, to which vesicles can dock, might be the major bottleneck. The main arguments are

- requirements on such sites are more stringent than those on vesicles
- strong interactions between vesicular components and the active zone must be broken after exocytosis
- refilling of the sites is perturbed by manipulations affecting endocytosis and this effect sets in faster than the time required for reuse of vesicles
- vesicle docking and priming can be very fast, in case vesicles do not target special sites on the active zone.

Unfortunately, all of these points are conjectures, based on indirect arguments or else on findings, which individually may have alternative interpretations. Together, however, they strongly suggest that the dynamics of release sites should be considered as a limiting and modulatory element in synaptic transmission.

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