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Merits and Limitations of Vesicle Pool Models in View of Heterogeneous Populations of Synaptic Vesicles

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The concept of a readily releasable pool (RRP) of synaptic vesicles has been used extensively for the analysis of neurotransmitter release. Traditionally the properties of vesicles in such a pool have been assumed to be homogeneous, and techniques have been developed to determine pool parameters, such as the size of the pool and the probability with which a vesicle is released during an action potential. Increasing evidence, however, indicates that vesicles may be quite heterogeneous with respect to their release probability. The question, therefore, arises: what do the estimates of pool parameters mean in view of such heterogeneity? Here, four methods for obtaining pool estimates are reviewed, together with their underlying assumptions. The consequences of violation of these assumptions are discussed, and how apparent pool sizes are influenced by stimulation strength is explored by simulations.

Introduction

Recent advances in the ultra-structural analysis of active zones have allowed a greatly improved quantitative understanding of the relationship between presynaptic Ca^{2+} influx and neurotransmitter release (Herman and Rosenmund, 2015). In particular, the spatial and functional coupling between voltage-gated Ca^{2+} channels (VGCCs) and release-ready vesicles at the active zone has been a matter of intense interest (Chen et al., 2015; Eggermann et al., 2012; Holderith et al., 2012; Indriati et al., 2013; Keller et al., 2015; Nakamura et al., 2015; Sheng et al., 2012). An important conclusion of these studies was that the size and shape of VGCC clusters at active zones are quite variable and that vesicles should be docked at the perimeter of such clusters, some distance away (Keller et al., 2015; Nakamura et al., 2015). Variations in VGCC density and corresponding heterogeneity in release probability were measured by Sheng et al. (2012), performing cell-attached patch-clamp measurements. Many types of synapses seem to have variable-size clusters of VGCCs; see Cano et al. (2013) for review. Furthermore, evidence from molecular perturbations (Chen et al., 2015; Schlüter et al., 2006; Yang et al., 2010), from caged Ca^{2+} measurements (Wölfel et al., 2007), from minimal stimulation (Dobrunz and Stevens, 1997), and regarding the effect of modulators of release points to intrinsic differences between readily releasable vesicles, dubbed as "primed" and "superprimed" or "sleepy" and "wakeful" (Cano et al., 2012). Correspondingly, it is expected that release probability of vesicles is not uniform, but depends on the distance from VGCC clusters, on the number of channels in a nearby cluster, and on the intrinsic state of the release apparatus. In contrast, commonly used experimental techniques to determine key parameters of neurotransmitter release, such as release probability and the size of the RRP (the number of vesicles), assume homogeneity of the pool or at most two or three discrete sub-pools (see below). Therefore the meaning of such estimates is not clear, in spite of the fact that discrete pool models have been quite successful in the description of electrophysiological data, both at synapses (Dutta Roy et al., 2014; Goda and Stevens, 1998) and at neuroendocrine cells (Stevens et al., 2011). Although Pan and Zucker (2009), analyzing neurotransmitter release at the crayfish neuromuscular junction, rightfully concluded that "it may well be that RRP is a fuzzy concept, extractable from data, but not rigorously corresponding to any physical vesicle pool," it nevertheless turns out (see "pros and cons" and model simulations below) that such estimates are quite meaningful for the interpretation of changes in release properties in response to a variety of experimental manipulations.

It should be pointed out that the "pool" considered here is a subset of vesicle pools discussed in the context of optical assays of neurotransmitter release. In the nomenclature of Alabi and Tsien (2012), it is exclusively the RRP, together with its subdivisions. Pools that supply vesicles to the RRP, such as the so-called "recycling pool," are assumed not to be depleted during the relatively short episodes of stimulation required for estimating the RRP.

The actual situation at a synapse may be characterized by an (at least) two-dimensional probability function, for a given vesicle to be released during a given stimulus, $p(r,\mu)$, where r is some measure of location or distance of the vesicle with respect to VGCC clusters and μ represents the intrinsic state of the vesicle. The distance variable is likely to be continuous, while the state variable may well be discrete, representing defined molecular states of the release apparatus. The synapse as a whole in this view is represented at any given time by an ensemble of vesicles with abundance $n(r,\mu)$, such that the measured response may be understood as the integral over the product $n(r,\mu) \cdot p(r,\mu)$.

The researcher, studying release, is thus confronted with the problem of inferring from the overall responses the underlying distributions. The answer of experimentalists—describing the system in terms of vesicle pool models—may be seen as either

disregarding heterogeneity altogether or else as subdividing this partially continuous space into discrete subsections, with each sub-pool given its size at a certain moment, its release probability, and its rates of vesicle recruitment (priming) and conversion to other sub-pools. An example for such discretization is the subdivision of releasable vesicle pools at the Calyx of Held. A total of about 3,000–5,000 vesicles, which can be released by a step depolarization within a few milliseconds (Sakaba and Neher, 2001b; Leão and von Gersdorff, 2009), can be subdivided into a fast pool and a slow one (Sakaba and Neher, 2001a), which differ in release rate during a step depolarization by about a factor of 10.

In a recent study on the nanoscale distribution of presynaptic Ca^{2+} channels (Chen et al., 2015), responses to 1 ms and 10 ms step depolarizations were analyzed, which, according to the discrete pool model, release only the fast or the sum of fast and slow pools, respectively. In the same study corresponding release time courses were simulated in a numerical model involving Ca^{2+} influx, diffusion, and buffering, as well as the Ca^{2+} dependence of release, assuming vesicles with homogeneous intrinsic properties. The question was asked: where would vesicles have to be placed with respect to VGCC clusters in order to reproduce the experimental findings? The resulting distribution (number of vesicles versus distance) showed a main peak with mean distances from the edge of VGCC clusters of 16 nm and a tail with vesicle distances ranging from 30 to 100 nm. This result was obtained for calyces of older animals (P16–19), while larger distances had to be postulated for data from younger animals (P9–11), in agreement with previous conclusions based on discrete pool models (Fedchyshyn and Wang, 2005; Wang et al., 2008). A comparison of the simulations with the analysis of the experimental data in terms of a slow pool and a fast pool suggests that the fast pool comprises those vesicles that contribute to the main peak of the distribution, whereas vesicles of the tail are lumped together as slow ones. The example shows that pool models may well be able to capture heterogeneities among vesicle properties and that the differences between slow and fast pools, as well as their developmental changes, can be explained on the basis of morphological variations alone without having to postulate intrinsic differences. This does not exclude that intrinsic differences contribute. The question remains whether this positive outcome can be generalized, and which methods are best for estimating pool parameters.

Approaches for Estimating Pool Parameters

As a first step (Part A: Ideal Homogeneity of Pools), techniques of estimating vesicle pool sizes will be discussed, which make the assumption that the RRP is homogeneous. All vesicles within the pool are assumed to have the same release probability. Alternatively, the case of their subdivision into not more than two sub-pools is discussed. Later (Part B: More Realistic Cases), complications will be discussed for cases in which these assumptions do not hold.

Part A: Ideal Homogeneity of Pools

Pool Size Estimates by Voltage-Clamp Depolarization, Ca^{2+} Uncaging, and the Application of Hypertonic Sucrose. The main aim of these methods is to obtain a ''snapshot'' of the size of the pool by applying a strong stimulus that empties the pool rapidly, before any recruitment and release of new vesicles can happen. Measuring release, either by the increase in membrane capacitance (Sun and Wu, 2001) or by integration of the postsynaptic current (PSC), then supplies a quantity that should be proportional to the pool size, i.e., the number of releasable vesicles that had been present at the time of the stimulation. Integration of postsynaptic response may require deconvolution and subsequent integration in case accumulation of neurotransmitter or ''spillover'' between neighboring synapses adds non-linear components to the signal (see Sakaba et al., 2002 for review). The stimulus may be a depolarization to the potential of maximum Ca^{2+} influx or flash-photolysis of caged Ca^{2+}, elevating intracellular Ca^{2+} concentration ($[Ca^{2+}]$) into the range of 20 μM or higher. On a much slower timescale, the stepwise application of hypertonic solution has been used extensively for the study of glutamate release in hippocampal cultures (Dutta Roy et al., 2014; Rosenmund and Stevens, 1996). Once the pool size has been determined, release probability during a PSC is calculated as the ratio of the recorded response divided by the pool size.

In order to determine the kinetics of release, stimuli have to be applied in a more defined manner. Using voltage-clamp depolarization at the Calyx of Held, Sakaba and Neher (2001a) showed that the cumulative release triggered by such stimuli could be fitted by two exponentials of about equal amplitude and with distinct time constants (fast and slow), which differed by about a factor of 10. This finding points to two vesicle populations of equal size with differing release rates (Schneggenburger et al., 2002). In cultured hippocampal neurons, Basu et al. (2007) applied sucrose in a stepwise manner. Varying the sucrose concentration and performing this experiment both in the presence and absence of the modulator phorbol ester, the authors observed release with varying time constants, which they interpreted in terms of the lowering of the energy barrier for release by hypertonicity and by phorbol ester.

In the case that the time course of release during application of the stimulus cannot be resolved, stimuli of varying lengths can be applied and the time course of release reconstructed by plotting the increments of the responses during the stimuli against the lengths of stimuli (Horrigan and Bookman, 1994).

A major problem with all pool-depleting methods is a possible non-linearity of the release assay. Given the requirement of strong stimulation for rapid pool depletion, postsynaptic receptors are likely to be saturated or to undergo desensitization. Both problems can be counteracted either by recording membrane capacitance (Sun and Wu, 2001) or, in the case of glutamatergic synapses, by pharmacologically antagonizing AMPA receptor desensitization and saturation (Elmslie and Yoshikami, 1985; Yamada and Tang, 1993). Also stimulus strength may be reduced, which, however, invokes the problem that pool depletion may not be complete, that it is slower, and that newly recruited vesicles may contribute to the response. The separation of such contributions from those of the pre-existing vesicles is a major issue for all kinds of weaker stimuli, in particular for the more physiological ones, and will be addressed in the next paragraph.

Pool Size Estimates Using Action Potential-Evoked Transmitter Release. Weaker stimulation, such as afferent fiber stimulation, is often preferred, since it is more physiological and applicable to most types of synapses. Although the

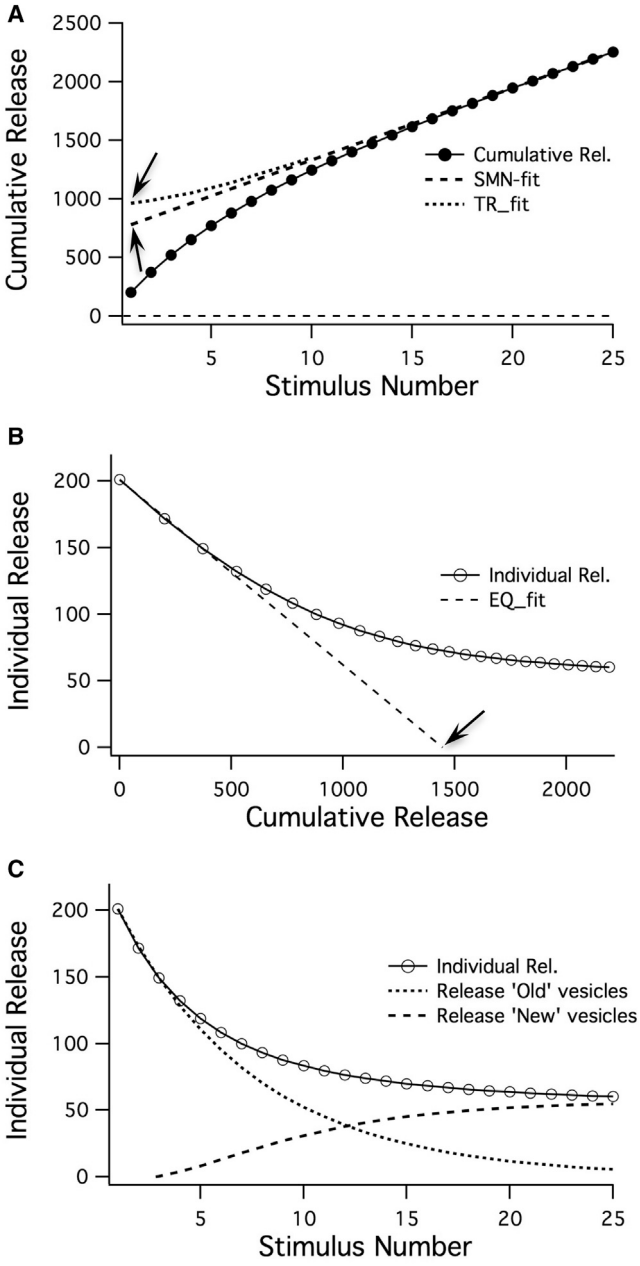

Figure 1. Schematic Diagrams of Plots for the Estimation of Vesicle Pool Sizes

(A) SMN plot and TR plot according to Schneggenburger et al. (1999) and Thanawala and Regehr (2013). In both plots the cumulative release during a high-frequency stimulus train is plotted against stimulus number (black solid line plus dots). A straight line is fitted to the late points of the cumulative trace (here through points 20–25; broken line). In the SMN plot, the y axis intercept of this line (lower arrow) is taken as the pool estimate, and the slope of the line is taken as a measure for the rate of vesicle recruitment. In the TR plot, a correction is applied to vesicle recruitment according to the argument that new vesicles can be recruited only after release sites have been vacated (upper arrow and dotted line, which starts with a shallower slope). In both plots release probability of the first EPSC is given by the ratio of its value and the pool estimate.

(B) EQ plot according to Elmqvist and Quastel (1965). Individual responses within a stimulus train are plotted against the cumulative release, which occurred before the given stimulus. A straight line is fitted to the early part of the curve, and the x axis intercept is taken as the pool estimate (arrow). Release probability is given by the negative slope of the line fit.

(C) RBT plot according to Ruiz et al. (2011). The sum of an exponentially decaying curve (dotted line) and a suitably chosen function, rising from zero to the steady-state EPSC value (broken line), is fitted to the measured EPSC values (circles). The integral of the exponential is taken as the estimate for pool size.

emptying of the vesicle pools is spread out over a longer time period, desensitization and saturation may still be a problem (Taschenberger et al., 2005). However, two more problems arise with weaker stimulation, even when using the highest frequencies of stimulation that a given type of synapse can transmit reliably. These are:

(1) The stimulus may be too weak to deplete the whole pool in the presence of ongoing recruitment of new vesicles.
(2) Depletion may take long enough that an appreciable component of release stems from newly recruited vesicles.

In the following, these two problems will be discussed in terms of two classes of approaches, which differ in the way they separate the contributions of newly recruited vesicles from those of the pre-existing RRP. Two slightly different variants are compared in each case:

Estimating the Contribution of Newly Recruited Vesicles by Back-Extrapolation. The basis of this method is the assumption that late in a stimulus train the synapse is in a steady state in which vesicle release is balanced by recruitment of new vesicles (assumption 1). Thus, the quantal content of late release multiplied by the frequency of stimulation is equal to the rate at which vesicles are recruited. This rate is represented by the slope of a plot of cumulative EPSCs versus stimulus number (see Figure 1A).

One of the two variants (Schneggenburger et al., 1999), referred to as "SMN plot" in the following, makes the further assumption (assumption 2) that the rate of recruitment is constant throughout the train. This leads to a simple graphical procedure to determine pool size, namely back-extrapolation of a linear fit to the late section of the plot (Figure 1A). The y-intercept of this line fit is then taken as the pool estimate because it is viewed as the cumulative amount of release minus the release by newly recruited vesicles. It should be emphasized, however, that this estimate is not identical to the RRP, which had been release-ready prior to stimulation. Rather it reports a quantity very close to the decrement of the pool during stimulation (see Box 1 for a proof). In order to estimate the entire RRP, one needs to add those vesicles that prevail at steady state due to a balance of vesicle release and recruitment. This can be achieved within the framework of a simple pool model, provided that the release probability is constant throughout the train or else that the ratio of release probabilities at beginning and end of the stimulus train is known (see Box 1). Partial depletion of pools in a dynamic exchange has been demonstrated by Pan and Zucker (2009).

The second variant replaces assumption 2 (constant rate of vesicle recruitment) by the assumptions of a classical pool model with a restricted number of release sites and a fixed rate-constant of vesicle recruitment. The difference is that the actual rate of vesicle recruitment in terms of vesicles recruited per

Box 1. Theory of Cumulative Plots

CUMULATIVE PLOTS REPORT THE DECREMENT IN POOL SIZE, NOT THE ENTIRE POOL

For generality, a reaction scheme is considered in which vesicles can sequentially move between pools P_1 and P_m:

$$\xrightarrow{r_{0,i}} P_{1,i} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} P_{2,i} \leftrightarrow \cdots P_{m,i} \xrightarrow{release\left(=P_{m,i}\cdot p_i\right)} . \qquad \text{(Equation 1)}$$

The synapse is assumed to be stimulated repetitively and pool $P_{m,i}$ to release vesicles at stimulus i with release probability p_i $(i=1\ldots n)$. The ensemble of pools is supplied with new vesicles during the interval following stimulus i with the amount $r_{o,i}$. (Note: we use the symbol r and not k because we consider rates of recruitment for this step, not rate constants).
If the filling state of the whole ensemble before stimulation is π_0,

$$\pi_0 = P_{1,0} + P_{2,0} + \cdots P_{m,0}, \qquad \text{(Equation 2)}$$

and n stimuli are applied, then π_n, the filling state immediately before the last stimulus of the train, is given by

$$\pi_n = \pi_0 + \sum_{i=1\cdots n-1} r_{0,i} - \sum_{i=1\cdots n-1} P_{m,i} * p_i. \qquad \text{(Equation 3)}$$

The product $P_{m,I} \cdot p_i$ is the measured current y_i (neglecting desensitization), such that

$$\sum_{i=1\cdots n} y_i = \pi_0 - \pi_n + y_n + \sum_{i=1\cdots n-1} r_{0,i}. \qquad \text{(Equation 4)}$$

We see here already that the cumulative release Σy_i, is equal to the combined decrement of the pools considered, plus y_n, plus a term that represents recruitment. The back-extrapolation is an attempt to remove the recruitment term. It is correct if two conditions are fulfilled: (1) $r_{0,i}$ is constant throughout the train $(= r_o)$, and (2) the train is long enough that all pools have reached a steady state. Then the pool contents are constant, and release is balanced by recruitment $(r_{0,i} = y_i = r_0)$. The late section of a cumulative plot (Σy_i, versus stimulus number) is then well approximated by a straight line, and the increments between late points are equal to r_o. Back-extrapolation to $I = 0$ then subtracts $n - 1$ times this value, such that the result P_{back} is

$$P_{back} = \sum_{i=1\cdots n} y_i - (n-1)\cdot r_0 = \pi_0 - \pi_n + y_n. \qquad \text{(Equation 5)}$$

The theory presented here actually applies to any configuration of sequential and parallel pool states. In that case $r_{0,i}$, the rates of recruitment, must be replaced by the sum of recruitment rates to the ensemble of pool states from the "outside" (i.e., from pools that are not part of the explicitly considered ones). Again, as long as all recruitment and release rates are constant late in a stimulus train, the cumulative release after subtraction of recruitment is not the sum of all pools considered, but the sum of its decrements plus y_n. In the limit of a graded distribution of release probabilities (infinite number of pools), it is the difference between the initial distribution of vesicle numbers (n (r, μ); see Introduction) and the steady-state one.

CORRECTION FOR INCOMPLETE POOL DEPLETION

For most purposes the decrement in pool content $\pi_0 - \pi_n$ is not the quantity of interest. Rather, one would like to know the full number of vesicles available before onset of stimulation. For the special case of a single homogeneous pool, a simple correction can be applied to obtain an estimate for the latter: considering that both y_0 and y_n are products of a pool size and a release probability, we can write

$$y_0 = \pi_0 \cdot p_0 \text{ and } y_n = \pi_n \cdot p_n \qquad \text{(Equation 6)}$$

and divide the two equations between each other to yield

$$\pi_n/\pi_0 = y_n/y_0 \cdot p_0/p_n. \qquad \text{(Equation 7)}$$

Together with Equation 5 we obtain

$$\pi_0 = P_{back} + \pi_n - y_n = (P_{back} - y_n)/(1 - y_n/y_0 \cdot p_0/p_n). \qquad \text{(Equation 8)}$$

The ratio y_n/y_0 is readily measured, whereas p_0/p_n may need additional assumptions (see text for a discussion). The term y_n in the numerator of Equation 8 may be neglected, which is equivalent to the assumption that there is no recruitment during the first interstimulus interval. Thus, this simplification would partially accommodate the criticism raised against the SMN assumption of constant recruitment.
The required correction Equation 8 may be quite large. For instance, 10–20 Hz stimulation induces short-term depression at the Calyx of Held down to 40% of control ($y_n/y_0 = 0.4$) (Müller et al., 2010). At the same time, these authors found that release probability

may decrease by a factor of 2 during such stimulation (p_0/p_n = 2). Inserting these numbers into Equation 8 results in a correction factor for P_{back} of 5. This indicates that an SMN plot needs to be interpreted with extreme caution, unless EPSCs depress during trains to substantially less than 50%.

ELMQUIST-QUASTEL PLOTS LINEARIZE A GEOMETRIC SERIES

In this type of plot, EPSC peak amplitudes y_i are plotted against the cumulative amplitudes up to y_{i-1}. For constant release probability, $p_i = p$, and no refilling pool size decreases during each stimulus by the relative amount of p, such that for a starting pool P_o the amplitudes y_n for the n-th EPSC are given by

$$y_n = P_0 \cdot (1-p)^{n-1} \cdot p. \quad \text{(Equation 9)}$$

This is a geometric series, for which

$$\sum_{i=1}^{n-1} y_i = P_0 p \frac{(1-p)^{(n-1)} - 1}{(1-p) - 1} = -P_0\left((1-p)^{(n-1)} - 1\right). \quad \text{(Equation 10)}$$

The quantity y_n is plotted against this sum, such that we can write

$$x_n = -P_0\left((1-p)^{(n-1)} - 1\right). \quad \text{(Equation 11)}$$

We solve this for $(1-p)^{(n-1)}$,

$$(1-p)^{(n-1)} = 1 - \frac{x_n}{P_0}, \quad \text{(Equation 12)}$$

and insert into Equation 9 for $i = n$

$$y_n = p \cdot (P_0 - x_n). \quad \text{(Equation 13)}$$

This is a straight line with x-intercept at P_o and a slope of $-p$. It should be noted that here an EPSC value is plotted against the sum of previous EPSCs, not including the given EPSC. If the given EPSC is included, then the slope s is $-p/(1-p)$ or else $p = -s/(1-s)$.

time is not assumed to be constant any longer (assumption 2, above) but is calculated as the product of a fixed rate-constant and the number of empty release sites. In other words: pool refilling is assumed to start only after some delay, when sites have been vacated (Hosoi et al., 2007; Thanawala and Regehr, 2013; Wang et al., 2013; Wesseling and Lo, 2002). Thanawala and Regehr (2013) simulated this type of model and concluded that the simple back-extrapolation (SMN plot) underestimates the real pool, because it assigns too much of the early release to newly recruited vesicles. Their simulation results suggest corresponding errors on the 10%–20% level (see Figure 1A). The postulate that vesicle recruitment starts slowly is well in line with standard kinetic modeling. However, experimental determination of recruitment rates at the Calyx of Held showed that recruitment starts as early as the second and third interstimulus interval and is relatively constant throughout 100 Hz trains (Lee et al., 2012). Thus, the error connected to assumption 2 may be quite small. In contrast, the correction for incomplete pool depletion may be large. In fact, the required correction may well increase the pool estimate several-fold, unless depression in the stimulus train is >60% (see Box 1). Wang et al. (2013) find a difference of a factor of ≈2 when estimating release probability on the basis of an SMN plot as compared to that reported by numerical fits with a classical pool depletion model. This difference may well result mainly from incomplete pool depletion.

Forward Extrapolation of Pool Depletion. This approach makes the assumption that early in the stimulus train almost all of release stems from vesicles in the pre-existing pool and the contribution of newly recruited vesicles is minor. A theoretical curve (see Figure 1B and below) is therefore fitted to the early release, and the contribution of newly recruited vesicles is taken as the difference between the measured late release and the extrapolation of the fit. We refer to these methods as methods based on "forward extrapolation," because the fit is based on early responses recorded during the stimulus train and the total release is determined by an extrapolation of that fit to the end of the train.

Two variants have been proposed for how to do the forward extrapolation. The first one was published in 1965 by Elmqvist and Quastel (1965). While studying depression of excitatory postsynaptic potentials (EPPs) in muscle, these authors explored the possibility that this form of short-term plasticity may be due to depletion of available resources. They argued that, given this hypothesis, it may be helpful to plot EPP size of a given stimulus against cumulative release prior to that stimulus (see Figure 1B). We will refer to this method as "EQ plot" in the following. Using such plots, the authors observed decays, which were well approximated by line fits, at least for the first few points. Later points showed more release than predicted by the line fits. This was interpreted to represent "mobilization" of

new vesicles during the train. Accordingly, the intercept of the line fit with the x axis (arrow in Figure 1B) was taken as an estimate for pool size. However, no formal treatment was presented to show under which conditions or assumptions the intercept agrees with the size of a pre-existing pool of vesicles.

In practice, a problem of the EQ plot is the choice of the fitting range. In particular, when synapses show a sequence of facilitation followed by depression, one cannot include the first two or three data points in the line fit. As a way out, the line is fitted to the region of steepest descent. This may be a narrow window, comprising three or four data points. For the case that such a fit describes the data well between points n_1 and n_2, the following two assumptions can lead to a quantitative justification of the intercept as a valid pool estimate:

(1) There is no release of newly recruited vesicles up to point n_2.
(2) Release probability of old vesicles is constant between point n_1 and the end of the train.

This implies that the only contribution from newly recruited vesicles is that given by the excess of the EPSCs beyond the line fit. A formal treatment of these statements is provided in Box 1. Key to this way of interpreting the method is the insight that, in the EQ plot, a geometric series is transformed into a straight line. EPSCs from a pool in which a constant fraction (release probability) of the remaining vesicles is liberated by each stimulus follow such a geometric series (the discrete form of an exponential). Therefore, a line fit starting from any point along the plot is exactly the prediction for release from a pool with constant release probability and no recruitment. The x axis intercept is then the cumulative release for an infinitely long stimulus train without recruitment, or else the total number of vesicles that had been present before stimulus onset. Release probability is given by the negative slope of the plot, as shown in Box 1.

The second variant of a method based on forward extrapolation was described by Ruiz et al. (2011) for the analysis of EPPs of mouse muscle (called RBT plot from here on). Here, EPPs were plotted against stimulus number and an explicit assumption was made about the contribution of newly recruited vesicles. This contribution was assumed to start at zero and to rise with some delay or in a sigmoid manner, asymptotically covering all late release. The whole time course of release was fitted by a sum of such a suitably chosen function and an exponentially decaying curve. The integral over the latter one was assumed to represent the contribution of pre-existing primed vesicles (see Figure 1C). Various shapes of the former contribution were explored (exponential rise, exponential after a delay, sigmoid rise), but as in the case of the QE method, best results were obtained when a delayed onset of newly recruited release was chosen. In particular, the authors could show that in this case the pool estimate agreed closely with estimates for the number of vesicles docked to the active zones of the neuromuscular junctions, as found by electron microscopy.

For both the RBT plot and the QE plot, no correction for residual pool is necessary due to the conceptual split of release contributions into a part that represents pre-existing vesicles (which by definition of a "releasable" pool decays to zero during prolonged stimulation) and its complement, representing newly recruited vesicles. In both variants recruitment of release of the latter is assumed to start late, and it is implied that release probability for the pre-existing component is constant. In fact, both methods are formally almost identical, if for the contribution of slow vesicles in the RBT plot an exponential is chosen with a delay that matches the endpoint of the fitting interval in the QE plot.

Summary of Assumptions and "Technical" Problems. Given the interpretation of methods, as outlined above, there are two major differences in the underlying assumptions. The first one relates to the contribution of vesicles that are recruited and released during the test stimulus. The SMN plot measures the rate of this contribution as the late slope in a cumulative plot and assumes that it is constant throughout. The TR plot measures it the same way, but interprets it as a rate-constant in a classical pool model. The EQ plot can be interpreted in the sense that the contribution of newly recruited vesicles is zero up to the last point included in the linear fit to that plot, and subsequently the difference between measured release and the linear fit is taken as the contribution of new vesicles. The RBT plot makes an explicit assumption about the contribution of new vesicles, as described above. Critical issues regarding these assumptions are the following: Both SMN- and TR plot results depend on the assumption that pools are at steady state in the late phase of stimulus trains. Trains may not be long enough to reach a truly steady state. Small increases in release probability or recovery from desensitization may lead to an apparent steady state, although pool contents are still declining. Moulder and Mennerick (2005) showed that such effects may influence pool estimates substantially. Late changes in release probability are also a major problem for both EQ and RBT plots, which assume constant release probability. Constancy of release probability is more likely to hold if late sections of the trains are considered, calling for a late placement of the fitting window in EQ plots. This, however, creates problems with the assumption of no recruitment before the end of the fitting window.

In both the EQ and the RBT plot, the resulting estimate is the size of the vesicle pool, which existed at the onset of stimulation. This is different for the case of the back-extrapolation methods. Both SMN and TR methods measure the pool decrement during stimulation, i.e., the difference between the pool size before stimulation onset and that during stimulation at steady state. This will result in an underestimate of the pool. Thus, when comparing results from EQ or RBT plots with those from SMN and TR plots, any differences not only reflect the different assumptions made, but also the degree of depletion at steady state, unless incomplete depletion is corrected for.

Contrary to the methods discussed here, which estimate the number of releasable vesicles, the analysis of current fluctuations, such as by multiple probability analysis (Silver, 2003), reports the number of release sites, N. This may not be equal to the number of release-ready vesicles, in case not all release sites are occupied at rest. Partial occupancy of about 70% of sites has been documented for inhibitory synapses (Trigo et al., 2012). This may lead to appreciable differences in estimates for both

Table 1. Comparison of Estimates for Release Probability and the Number of Vesicles/Sites for the Case of Partially Filled Pools

Method	Variance/Mean	SMN/TR	SMN/TR (Plus Correction)	EQ/RBT
No. of vesicles/sites	N	$N(p_{occ,o} - p_{occ,ss})$	$Np_{occ,o}$	$Np_{occ,o}$
Release probability	$p_{rel} \cdot p_{occ}$[a]	$(p_{occ,o} \cdot p_{rel}) / (p_{occ,o} - p_{occ,ss})$	p_{rel}	p_{rel}

It is assumed that there are N release sites, with probability of occupancy of sites $p_{occ,o}$ (before stimulation) and $p_{occ,ss}$ at steady state during stimulation. Release probability (p_{rel}) of a docked vesicle for simplicity is assumed to be constant. The column Variance/Mean provides values for multiple probability analysis (Silver, 2003), and the other columns report those for the various types of plots. The row Release Probability reports theoretical predictions from the ratio response over pool estimate.
[a]Multiple probability analysis requires measurements at a variety of stimulation strengths in order to determine N. Once this value is known, the product $p_{rel} \cdot p_{occ}$ can be determined for a given condition (Scheuss and Neher, 2001).

pool size and release probability, depending on the method used (see Table 1 for a summary).

Both EQ and RBT plots may overestimate pools, if substantial recruitment of new vesicles occurs in the early part of the stimulus train. This was pointed out by Thanawala and Regehr (2013), concluding that estimates from SMN and EQ plots are lower and upper boundaries for the true value of the RRP. However, when they compared results from TR plots with those from EQ plots, there was very close agreement, suggesting that the respective errors are small. EQ plots may also underestimate late contributions of RRP vesicles, if release probability decreases during the train, such that these vesicles release slower than predicted by the fit to the early data points and consequently are counted as newly recruited vesicles (see also below). Last but not least, as pointed out by Wesseling and Lo (2002) and Stevens and Williams (2007), pool estimates, which are based on peak amplitudes of EPSCs, underestimate the number of quanta released if these are not perfectly synchronized or if substantial asynchronous release occurs. The analysis of charge (current integral) may circumvent this problem, unless determination of the baseline for the integration poses a new problem.

A summary of problems and recommendations can be found in Table 2. But, as will become evident below, errors associated with such problems seem to be minor relative to the large variations in pool estimates with stimulus strength, which are observed if vesicle pools are not homogeneous.

Part B: More Realistic Cases

Depletable Pool and Graded Pool. The main conclusions drawn above are strictly valid only if the vesicle pools under study are homogeneous. The problems discussed so far can be viewed as "technical problems" connected to the way pool estimates are obtained. In reality, however, as outlined in the Introduction, the pool concept is an idealization for a distribution of vesicle properties, which may have one or several peaks (corresponding to one or several vesicle populations) or may be graded without distinct features. The question arises whether the pool concept is still useful in this case.

Inhomogeneous release probability violates the assumption about exponential decay of the release of RRP vesicles. If some of the vesicles have release probability higher than that of others, they will fuse earlier in a stimulus train, leaving behind vesicles with lower release probability. The fit in the EQ plot will report the higher release probability of fast vesicles and a good portion of the slower vesicles will be held to be newly recruited ones, leading to an underestimation of the RRP—partially compensating for the overestimate due to misassigned, newly recruited vesicles early in the train. Likewise, vesicles with much lower release probability will be released late in a train and may be mistaken in an SMN plot as newly recruited ones (see simulation below). Such vesicles are likely to reside at larger distances from channel clusters and may contribute even more, if the domain of elevated $[Ca^{2+}]$ spreads during stimulus trains, affecting more and more remote vesicles. In addition to such "pseudo-recruitment," synapses that can transmit in a sustained fashion must be equipped with "real recruitment," i.e., recycling of vesicles and refilling of release sites. Thus, increases in pool size estimates with increasing strength of stimulation may signal either a higher degree of depletion of a more or less homogeneous pool or else the release of vesicles with lower release probability, which release during strong stimulation, but would not do so within the duration of a weaker stimulus (see also Moulder and Mennerick [2005] for the case of sucrose stimulation).

Dependence of Apparent Pool Size on Stimulus Strength. At the Calyx of Held, differences in estimated pool size of almost a factor of two, depending on stimulation strength, were reported by Lou et al. (2008). To describe such changes, Wölfel et al. (2007) introduced the concept of "submaximal release."

Table 2. "Technical" Problems and Recommendations

Be aware of the assumptions and make sure they apply to your synapse.
Don't use back-extrapolation (SMN and TR plots) if the depression is less than 60%.
Use correction for residual pool (SMN and TR plots only), unless depression is more than 90%.
Aim at a compromise between speed of pool depletion and desensitization/saturation; use kynurenic acid to mitigate such problems.
Be aware that the apparent pool (or "accessible pool") varies with stimulation strength. Use stimulation somewhat stronger than "physiological" (e.g., 4 mM external $[Ca^{2+}]$).
When studying changes in pool size, aim at a constant release probability, maybe by adjusting $[Ca^{2+}]$.
When studying effects on release probability, interpret them with respect to changes observed by changing $[Ca^{2+}]$.
Be aware that estimates based on peak amplitudes of EPSCs underestimate the quantal content, unless evoked quanta are perfectly synchronized. Analyze charge (current integral), if possible.

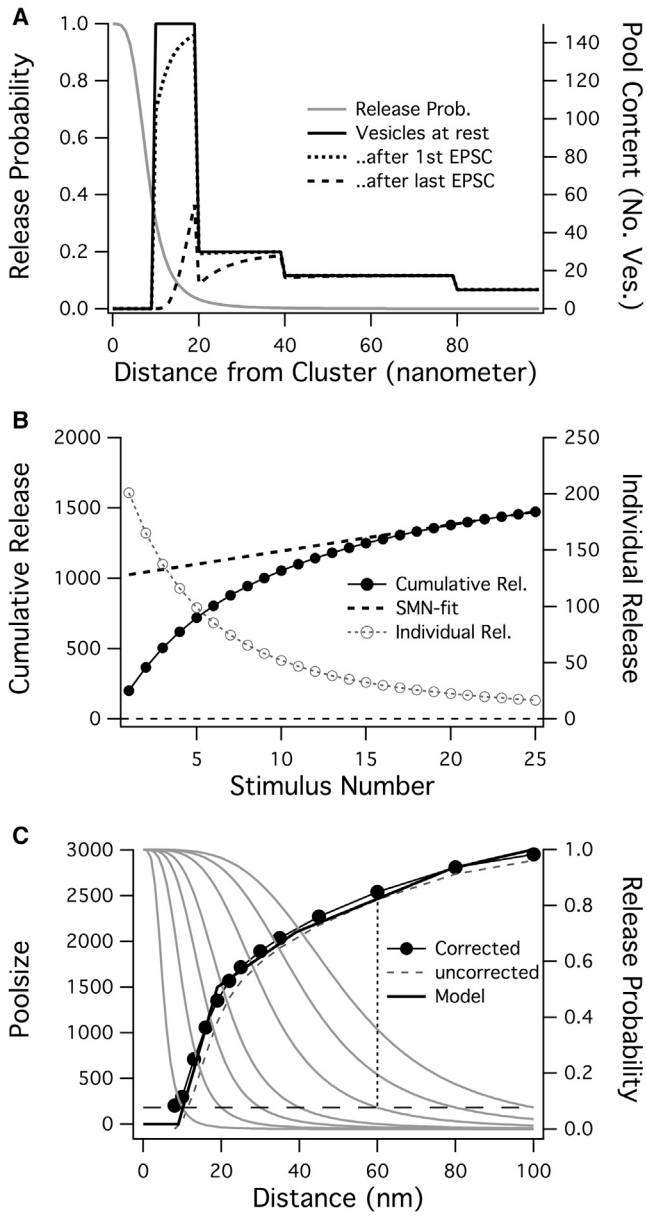

Figure 2. Simulation of Release from an Inhomogeneous Vesicle Pool

(A) Pool parameters as a function of distance between vesicles and a VGCC cluster: A distribution similar to that of Chen et al. (2015) was assumed (see Figure 3 for a graphical representation). The solid box-like trace shows vesicle numbers per nanometer before onset of stimulation (right ordinate). The thin smooth line is the release probability (left ordinate). It was calculated as $p_{rel} = 1/(1+(const \cdot r/I_{Ca})^{3.7})$, where r is the distance from a Ca^{2+} channel cluster and I_{Ca} is the Ca^{2+} current, which was assumed to be constant during a given stimulus train. The thin dotted curve shows the vesicle distribution after the first EPSC, and the broken line does so for the situation after the last stimulus. The first stimulus releases predominantly fast vesicles (at distances between 10 and 19 nm), while the whole stimulus train depletes most of the fast pool and also releases vesicles in the distance range 20–40 nm. Here the ratio $const/I_{Ca}$ was chosen such that 200 vesicles were released during the first stimulus. No recruitment was assumed. Marginal distance, r_0, for this run was 15.6 nm (see below).

(B) Release as a function of stimulus number (from the same simulation as in A). Gray circles are responses to individual stimuli (right ordinate), black dots (solid trace, left ordinate) are cumulative values, and the broken line is an SMN-fit to the last six points of the cumulative plot. The back-extrapolation yields an estimate for the pool size, which is 1,007 vesicles. The correction for residual pool (Equation 8, Box 1, assuming $p_0/p_n = 1$) is small, since depression of the responses is large (92%). This simulation does not assume any recruitment of vesicles. A very similar simulation including recruitment is given in Figure 1A (note different y scale).

(C) Pool estimates as a function of stimulus strength. Simulations were performed, as shown in (B), but including recruitment of vesicles to the fast pool at a rate of 3.3 pools/s. Values for $const/I_{Ca}$ (see above) were chosen such that release probability for a given run had the value of 0.0773 at a given "marginal" distance r_0. Release probability as a function of distance (right ordinate) is plotted for a few examples of r_0 (10, 20, 30, 40, 60, 80, 100 nm). For one case ($r_0 = 60$), the calculation of r_0 is illustrated as the intersection of the trace for release probability with the horizontal dashed line at a y value of 0.0773. Both corrected (large dots) and uncorrected (broken line) estimates for pool size are plotted against r_0. In addition, the cumulative number of vesicles up to a given distance is plotted (left ordinate) as a function of distance from the VGCC cluster. The uncorrected estimates for pool size seem to be almost identical to corrected ones at the resolution of the display. It should be pointed out, though, that relative differences between corrected and uncorrected values are 30%–50% in the steep region of the traces and that the assumed recruitment rate is relatively small.

More recently, Thanawala and Regehr (2013) addressed this problem in an extensive study, in which they varied Ca^{2+} influx by several methods. This led to changes in the estimates for release probability and pool sizes using both cumulative plots and EQ plots. To discuss the observed changes, they introduced the term "effective pool size" and concluded that the Ca^{2+} dependence of release is only partially caused by changes in release probability, an appreciable part being due to changes in effective pool size.

According to the analysis presented here (Box 1), part of the differences in SMN pool size estimates is due to incomplete pool depletion and the question arises whether the correction for incomplete pool depletion should be applied to the "effective pool." Although this question is a matter of interpretation, there is a good reason that it should be applied in the case of cumulative plots (SMN and TR plots) and also to the "submaximal release," since it would be required in such plots for obtaining the correct result, even if the underlying pool were an ideally homogeneous one (see also discussion below).

How do changes in apparent pool size with stimulus strength relate to vesicle properties, if the latter are not homogeneous? Intuitively one would expect that increasing stimulation strength will release more and more vesicles, even if these have below-average release probability. A simulation of such a scenario is shown in Figure 2. Here it was assumed that vesicles are distributed with respect to channel clusters in a way similar to what was postulated by Chen et al. (2015)—a "fast pool" of 1,500 vesicles at 10–19 nm and a "slow pool" of 1,500 vesicles at 20–100 nm (see Figure 3 for a graphical representation). It had been shown before that afferent fiber stimulation releases predominantly vesicles from the fast pool, with slow vesicles contributing very little (Sakaba, 2006). Thus, one would expect that pool estimates from standard plots report values close to those of the fast pool, if action potential-evoked release is studied. The simulation was performed in order to test this expectation.

It was assumed that local $[Ca^{2+}]$ drops with the inverse of distance from a channel cluster, that it is proportional to I_{Ca}, and that release probability during an action potential is given by a Hill function of local $[Ca^{2+}]$ with an exponent of 3.7

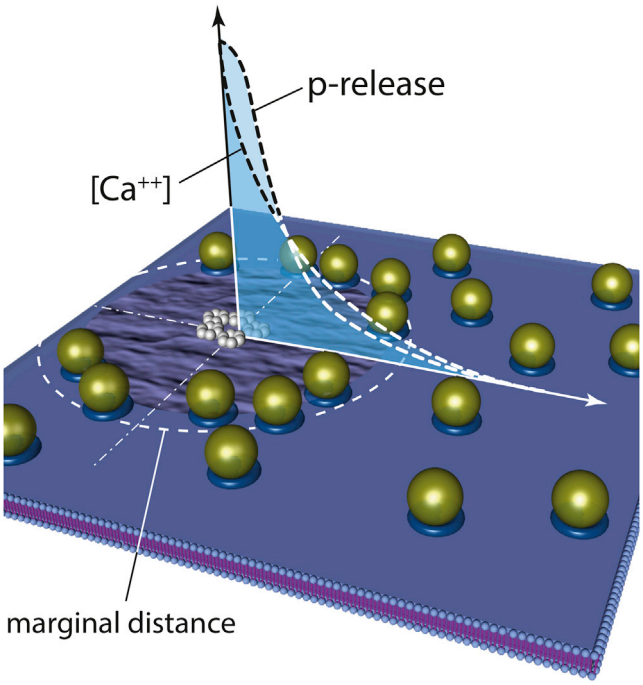

Figure 3. Graphical Representation of the Simulation
A cluster of five VGCCs is assumed to be surrounded by "fast" vesicles at a certain perimeter. Some more "slow" vesicles are spread at larger distances. Local calcium concentration during an action potential ($[Ca^{2+}]$) is assumed (for simplicity) to decay inversely with distance. Release probability (p-release) decays as a power function of the latter. The strength of stimulation, as drawn, is such that p-release reaches the marginal value (0.0773) at a radius (marginal distance) that includes most of the fast vesicles. According to the simulation (see text), these vesicles, but not slow ones, would show up in the pool estimate of an SMN plot. For stronger stimulation (larger $[Ca^{2+}]$ and p-release), the marginal distance would be larger, and more vesicles would be included in the pool estimate. The drawing shows more vesicles per VGCC clusters than are actually present at the Calyx of Held, for better visualization of the vesicle distribution.

(see Figure 2 legend for details). For Figures 2A and 2B, Ca^{2+} current was adjusted, such that 200 out of the 1,500 vesicles of the fast pool were released during the first action potential in a high-frequency stimulus train, as observed experimentally for standard conditions (Taschenberger et al., 2005). Figure 2A shows the release probability (thin, smooth curve) and the vesicle density describing this inhomogeneous pool before onset of stimulation (solid line), after the first stimulus (thin dotted line), and after the 25th stimulus (broken line), all as functions of distance from the VGCC cluster. No refilling of pools was assumed to take place for this first simulation. The figure shows that the first stimulus releases predominantly vesicles from the fast pool (at 10–19 nm distance). After the 25th stimulus, the fast pool is almost completely depleted, but the first few bins of the slow pool are also affected. Upon performing an SMN plot, it turns out that the late section of such a plot can quite well be fitted by a straight line (Figure 2B), suggesting a pool of 1,007 vesicles and an apparent recruitment rate of 1.86 pools/s, although no recruitment was included in this first run of the model. This result demonstrates that vesicles with lower release probability can show up in the cumulative plot as if they were newly recruited. The pool estimate is about two thirds of the fast pool. The correction for incomplete depletion, as suggested in Box 1, has little effect, since the steady-state value is very low in the absence of recruitment. Adding recruitment into the fast pool at a rate of 3.3 pools/s (assuming 100 Hz stimulation) did not lead to major changes in the SMN result (1,025 vesicles), if the correction for residual pool was applied. This shows that the back-extrapolation method successfully corrected for "real" recruitment. The EQ plot (not shown) with a line fit on the first three data points reported a pool of 1,158 vesicles without recruitment and 1,441 vesicles including recruitment. Thus, both methods in the absence of recruitment come up with estimates, which are smaller than the fast pool, since part of the fast pool is interpreted as recruitment. The EQ estimate (1,441 vesicles) is surprisingly precise in the presence of recruitment. The reason is a compensation of this underestimation by including newly recruited vesicles early in the train in the pool estimate.

The simulations shown so far were performed assuming relatively weak stimulation, and therefore vesicles at longer distances from VGCC clusters did not release during stimulus trains. It has been the general experience that stronger stimulation leads to more robust and larger pool estimates. Figure 2C therefore explores how pool size estimates change if stimulation strength is varied. Here, as in Figure 2A, release probability was calculated as a function of distance from a VGCC cluster, but now for various I_{Ca} values. SMN plots were performed and the resulting pool estimates plotted against the distance at which release probability for the given I_{Ca} value was 0.07732. This value is indicated as a horizontal dashed line in Figure 2C. The particular position (as marked for one example as a dotted vertical line) was chosen because the total probability for a vesicle at that location to be released up to the onset of the linear fit (the 20th stimulus) amounts to 0.8 ($= 1 - (1 - 0.07732)^{20}$). It was expected that most of the vesicles at distances shorter than this "marginal distance," which have release probability higher than that of the "marginal vesicles," will be released early in the train. More distant ones may not be released or else be mistaken as newly recruited vesicles. Therefore the pool estimate should be close to cumulative numbers of vesicles up to the marginal ones. This was, indeed, found. The closed symbols in Figure 2C are pool estimates plotted against the location of marginal vesicles (see legend for details). They closely agree with the cumulative vesicle density function of Figure 2A, as it was assumed for the modeling (continuous line "Model" in Figure 2C).

This simulation shows that the apparent pool varies strongly with stimulation strength and that it can in principle recover the essential features of a heterogeneous vesicle distribution. It should be pointed out, though, that the simulations as presented here span a more than 10-fold range in assumed Ca^{2+} current, which is hard to obtain experimentally. It has been shown that physiological stimulation in the Calyx of Held can release not much more than the fast pool (Sakaba, 2006), which in this simulation corresponds to the 1,500 vesicles up to 20 nm. This is confirmed in Figure 2C, where 1,530 vesicles are estimated for a marginal distance of 20 nm. Increasing Ca^{2+} current 1.5-fold, corresponding to an increase in the marginal distance from 20 to 30 nm, increases release, but only by 19%. In order to tap the remainder of the slow pool, much larger currents are required,

which cannot readily be obtained with action potential-like stimulation. Prolonged depolarization under voltage clamp or else Ca^{2+} uncaging, however, will be able to release these vesicles (Sun and Wu, 2001). Nevertheless, there is a plateau region covering the range of stimulus strength just above that typically used for electrophysiological experiments in which the pool estimate is close to the fast pool (see also discussion of ''pros'' and ''cons'' below). Decreasing stimulation strength, however, leads to dramatic reduction in the apparent pool size. Thus, the modeling suggests that within certain limits of stimulus strength, the pool estimates are relatively robust (see below for a comparison with experimental data). It should be stressed, though, that the mapping of the distance scale (x axis in Figures 2A and 2C) onto ''real'' distances may be quite complex, involving realistic modeling of Ca^{2+} spread in the nanodomain and the sensitivity of the release apparatus, as determined by caged Ca^{2+} experiments. Any intrinsic heterogeneity of vesicles will require even more complexity, involving the two-dimensional distribution $n(r,\mu)$, as discussed in the Introduction. Likewise, heterogeneity with respect to priming would need extra attention.

The simulation also implies that it is not straightforward to assay changes in pool size with certain manipulations (molecular or pharmacological) if these also influence release probability. Compensation of the latter by adjustment of Ca^{2+} concentration may be necessary to isolate the effect on pool size, or else apparent changes in pool size need to be probed at maximal stimulation strength (caged Ca^{2+}; voltage step depolarization, high sucrose). Likewise, changes in release probability, induced by some agent, may be distorted by changes in apparent pool size. Again, compensation of such effects by ''titration'' with $[Ca^{2+}]$ may be advisable, resulting in a relative potency of a given agent with respect to $[Ca^{2+}]$ changes.

Summary and Perspective

Ideally, the researcher would like to study the properties of a given release site: the release probability of a docked and primed vesicle, how it changes with stimulus strength, and how such properties are distributed among the population of vesicles, which are release-ready at a given time. The common analysis tools used to determine such parameters can only give cumulative numbers and averages over pools of vesicles, which may be quite heterogeneous. There are experimental manipulations to better define the pool or a sub-pool of interest, such as inclusion of EGTA in the intracellular medium, which acts by limiting the extent of Ca^{2+} microdomains. Also, there are experimental means to mitigate possible errors due to release of newly recruited vesicles, such as inclusion of calmodulin blockers (Sakaba and Neher, 2001a) or else latrunculin (Lee et al., 2012), which slow down recruitment of such vesicles. In any case, however, one should be aware of the assumptions made in a particular type of analysis, likely problems related to those (see Table 2), and to the fact that ''readily releasable pool'' means ''vesicles releasable by a given type of stimulus.''

With this in mind, we may consider the ''pros'' and ''cons'' of pool estimates with respect to three themes:

(1) ***Dependence on Stimulus Strength.*** The ''contra'' definitely centers around the notion (Pan and Zucker, 2009) that ''RRP is a fuzzy concept … not rigorously corresponding to any physical vesicle pool.'' In the Calyx of Held, this leads to the situation that pool estimates based on strong stimulation (by flash-photolysis of caged Ca^{2+} or else using step depolarization) are in the range of 4,000–6,000 vesicles (Sun and Wu, 2001; Leão and von Gersdorff, 2009), which is up to 4–6 times higher than estimates based on SMN plots using fiber stimulation (Schneggenburger et al., 2002). Part of this discrepancy may be due to the fact that the former values were obtained using membrane capacitance as an assay, which is not subject to desensitization. Another factor of two probably reflects the finding that fiber stimulation releases only the fast pool, whereas stronger stimulation releases all primed vesicles. Restricting the analysis to fiber stimulation, changes in synaptic strength with experimental manipulations may well be broken down into changes in release probability and those of pool size, if variations in stimulation strength are confined to a certain range. A recent study at the Calyx of Held (Thanawala and Regehr, 2013) demonstrated large decreases in effective pool size when lowering stimulation strength by various means, but it also showed that such changes are confined to the 10%–20% level if Ca^{2+} influx is increased or decreased by less than 50% around a reference value obtained with 2 mM external $[Ca^{2+}]$. Thus, given the steep dependence of release on Ca^{2+} influx, there is a relatively wide range of synaptic responses within which release can be manipulated without major changes in the pool estimate. The simulation of Figure 2C also shows that above a marginal distance of 20 nm, a 50% increase in I_{Ca} leads to about 20% increase. This is similar, but somewhat more than what was found experimentally at the Calyx, which may mean that the difference between slow and fast vesicles is indeed more pronounced in reality than assumed in the model. Variation of the model distribution with the aim of more accurately reproducing the experimental dependence of the RRP estimate upon stimulus strength may be an interesting exercise to learn more about the relative sensitivity of slow and fast vesicles.

(2) ***The Sucrose Pool.*** A major concern (con) of this method is the ignorance about the mechanism by which application of hypertonic solution causes release. However, it is quite likely that sucrose application releases all primed vesicles, not only those in close proximity to Ca^{2+} channels. In fact, Moulder and Mennerick, 2005 concluded that ''reluctant'' vesicles may contribute to the sucrose pool (but see Stevens and Williams, 2007). As a consequence, estimates of release probability calculated on the basis of sucrose pools are typically very low and not consistent with the rapid decay of EPSCs during high-frequency stimulus trains. Nevertheless, normalization of EPSCs measured in hippocampal cultures with respect to the sucrose pool of a given neuron has been very helpful in numerous studies employing molecular or pharmacological manipulations of neurotransmitter release. As a ''pro'' of the method, it should also be

stated that it is independent of [Ca^{2+}] changes and thus eliminates influences of a number of Ca^{2+}-dependent processes and that it normalizes with respect to cell size, number of synapses, etc. Also, constancy of the sucrose pool may well imply constancy of the pool relevant for evoked release, since the latter quite likely is a sub-pool of the former.

(3) ***The Debate on Vesicle Recruitment.*** A problem (con) of all methods discussed is the correction for newly recruited vesicles. As a ''pro'' it should be stated that it is clearly established that recruitment during stimulus trains, as measured by the slope of SMN or TR plot, is much higher than recruitment at rest or during recovery from short-term depression. However, it is a matter of debate how fast recruitment sets in after onset of stimulation. As discussed above, evidence has been provided that it may set in faster than compatible with simple pool models. On the other hand, RTB plots provided best agreement between pool estimates and the number of morphologically docked vesicles under the assumption of a delayed onset of vesicle recruitment. More detailed studies of these aspects may reveal features of vesicle docking and priming, which go beyond a simple one-step reaction.

Irrespective of the pros and cons of pool models, the simulation of Figure 2C suggests a likely scenario of why pools are heterogeneous and how this translates into varying pool estimates. Given the steep dependence of release probability upon distance from Ca^{2+} sources (see Figure 2C and more detailed modeling in Nakamura et al., 2015) and assuming that local [Ca^{2+}] is the major determinant of release probability, one may view the RRP as the sum of all those vesicles located within a certain radius from a Ca^{2+} source for which release probability is higher than about 0.08, the exact value of this probability depending on the number of stimuli preceding those EPSCs on which the back-extrapolation is based. As shown in Figure 2C, increases in stimulus strength, which enlarge this radius, may then be related to the increasing number of vesicles docked within this larger radius. Systematic experiments of this type, together with models of the spread of Ca^{2+} microdomains and the Ca^{2+} dependence of the release apparatus, may in the end provide an idea about the way how vesicles are distributed around clusters of VGCCs at the active zone.

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