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Estimation of Quantal Parameters With Multiple-Probability Fluctuation Analysis

Chiara Saviane and R. Angus Silver

Department of Physiology, University College London, Gower Street, London WC1E 6BT, UK

Summary

The functional properties of central synapses are difficult to study because they can be modulated either presynaptically or postsynaptically, each connection has multiple contacts and release at each contact is stochastic. Moreover, studying central synapses with electrophysiology is complicated by the fact that synapses are often remote from the recording site and signals are often difficult to resolve above the noise. This together with the fact that central synapses often have few release sites and have nonuniform quantal parameters makes classical quantal analysis methods difficult to apply. Here, we discuss an alternative approach, multiple-probability fluctuation analysis (MPFA), which can be used to estimate nonuniform quantal parameters from fits of the relationship between the variance and mean amplitude of postsynaptic responses, recorded at different release probabilities. We illustrate the experimental protocols and the analysis procedure that should be followed to perform MPFA and interpret the estimated parameters.

Keywords

synapse; transmitter release; quantal analysis; fluctuation analysis; MPFA; synaptic plasticity; CV analysis

1 Introduction

According to the quantal hypothesis of transmitter release (1, 2), neurotransmitter is packaged in discrete quantities (*quanta*) that are released in an all-or-none fashion. As release occurs at specific sites, synaptic efficacy can be described using three quantal parameters (2, 3): the number of functional release sites (N), the probability of release (P_R), and the amplitude of the response to a single *quantum* (Q). Changes in one or more of these parameters account for modifications in synaptic strength. Assuming that each site acts independently, binomial and Poisson statistics can be used to model the stochastic behavior of synaptic transmission, estimate quantal parameters, and identify the site of changes in synaptic efficacy. However, classical quantal analysis methods, based on the analysis of amplitude histograms or failure rate, can be difficult to apply at central synapses because of the low signal-to-noise ratio due to the small quantal size and because of the non-uniformity in P_R and Q (4). Therefore, new approaches inspired by stationary and non-stationary noise analysis of ion channels (5, 6) have been developed. These include stationary analysis of

mean and variance at different release probabilities (7, 8), the analysis of mean, variance and covariance (9, 10) during repetitive stimulation, and the analysis of higher cumulants (11) during prolonged release from voltage-clamped presynaptic terminals. Multiple-probability fluctuation analysis (MPFA) (8) (also known as variance-mean analysis [7]) is a method that can be applied to determine uniform or non-uniform quantal parameters when the signal-to-noise ratio is low. MPFA involves fitting the relationship between the variance and mean of the synaptic responses measured under different release probability conditions (8, 12, 13) with a binomial or multinomial model.

1.1 Statistical Models of Quantal Transmission

According to a binomial model of neurotransmitter release, the mean peak amplitude of the synaptic response I and the associated variance σ^2 can be expressed as a function of the number of release sites, the release probability and the quantal size:

$$I = NP_R Q, \quad (1)$$

$$\sigma^2 = NQ^2 P_R (1 - P_R), \quad (2)$$

so that a parabolic relationship holds between them (Fig. 1A):

$$\sigma^2 = IQ - \frac{I^2}{N}. \quad (3)$$

As several studies have shown that the assumptions required for applying a simple binomial model often do not hold for central synapses, a multinomial model has therefore been developed (8, 14) (Fig. 1B) in order to take into account quantal variability both at the single-site level (intrasite variability) and across sites (intersite variability). Intrasite variability arises from fluctuations in the size of quantal events from trial to trial and from asynchrony in their latency (13) and can be quantified in terms of the associated coefficients of variation (CV_{QS} and CV_{QL} , respectively). Intersite variability arises from differences in the mean quantal size from site to site, and the CV of this component is defined as CV_{QI} . Both intrasite and intersite variability can be quite easily implemented in a multinomial model, where the mean response and the variance are given by:

$$I = NP_R \bar{Q}_p, \quad (4)$$

$$\sigma^2 = N\bar{Q}_p^2 P_R (1 - P_R) (1 + CV_{QI}^2) + N\bar{Q}_p^2 P_R CV_{QI}^2 \quad (5)$$

and $\bar{Q}_p$ represents the mean quantal size at the time of the peak of the mean synaptic response and is therefore affected by asynchronous release. CV_{QI} includes all the variability observed at the single-site level, being defined by:

$$CV_{QI}^2 = CV_{QL}^2 + CV_{QS}^2. \quad (6)$$

Of course, the use of constant coefficients of variation assumes that the release latency and the quantal variability are independent of P_R .

Substituting Eq. 4, the variance can be expressed in terms of I :

$$\sigma^2 = (\bar{Q}_p I - \frac{I^2}{N})(1 + CV_{QII}^2) + \bar{Q}_p I CV_{QI}^2 \quad (7)$$

According to the model of release used, fitting Eq. 3 or 7 to the relationship between the variance and the mean of the synaptic responses recorded in different release probability conditions provides estimates of the mean quantal size and N ; P_R can then be calculated from the mean response (Eq. 1 or 4).

Several groups have shown that the probability of release can also be non-uniform across release sites at central synapses (15, 16) (Fig. 1B). In this case, assuming that there are no correlations between release probability and quantal size across sites (8, 14), the relationship between the variance and the mean response (13) is given by:

$$\sigma^2 = [\bar{Q}_p I - \frac{I^2}{N}(1 + CV_P^2)](1 + CV_{QII}^2) + \bar{Q}_p I CV_{QI}^2 \quad (8)$$

where CV_P represents the CV of release probability across sites and changes with the mean release probability across sites at the time of the peak of the response ($\bar{P}_R$). CV_P and how it changes with $\bar{P}_R$ has been modeled (13) using families of beta functions $B(\alpha, \beta)$ so that:

$$CV_P = \sqrt{\frac{1 - \bar{P}_R}{\bar{P}_R + \alpha}} \quad (9)$$

and the variance-mean relationship depends on only one additional parameter α :

$$\sigma^2 = [\bar{Q}_p I - \frac{\bar{Q}_p I^2(1 + \alpha)}{I + N\bar{Q}_p \alpha}](1 + CV_{QII}^2) + \bar{Q}_p I CV_{QI}^2. \quad (10)$$

It follows that low α values indicate non-uniform release probability and the model reduces to the uniform P_R case for $\alpha \rightarrow \infty$.

Fitting Eq. 10 to the variance-mean relationship also gives an estimate of the non-uniformity in P_R . However, α is the least well-constrained parameter in Eq. 10 and its estimate should only be used as a rough indicator (13, 17). In practice, the variance-mean relationship is rather insensitive to α values larger than 2-3, which can give high CV_P at low $\bar{P}_R$, thus allowing the use of a uniform model (18).

MPFA can be combined with CV analysis of excitatory postsynaptic current (EPSC) amplitudes to track changes in quantal parameters during repetitive stimulation when P_R is uniform (19). After estimating quantal parameters for the first pulse in the train using MPFA, P_R can then be estimated for the subsequent synaptic responses in the train from the CV, assuming that N is constant:

$$CV = \frac{\sigma}{I} = \sqrt{\frac{(1 - P_R)(1 + CV_{QII}^2) + CV_{QI}^2}{NP_R}}; \quad (11)$$

$\bar{Q}_p$ can then be calculated from the mean response (Eq. 4).

1.2 Interpretation of Quantal Parameters

The interpretation of the results from MPFA is straightforward if quantal responses are independent and, therefore, sum linearly: $\bar{Q}_p$ represents the mean elementary postsynaptic response at the time of the peak, P_R is the release probability of a single *quantum* at the time of the peak (which is equivalent to the cumulative probability if the release function has a small tail [18]) and N corresponds to the number of release sites. N will also equal the number of synaptic contacts if a maximum of one *quantum* of transmitter is released at each site (uniquantal release). However, several studies have shown that at some synapses, multiple independent vesicles can be released at the same synaptic contact (20–22). If quantal responses sum linearly, N will indicate the number of functional release sites, but in this case, it will be larger than the number of synaptic contacts. Interpretation of quantal parameters becomes complicated when *quanta* do not sum linearly because of receptor saturation (23) which causes the quantal size to change with P_R . In the limiting case when the receptors are saturated by a single vesicle, $\bar{Q}_p$ would be independent of the number of vesicles released per site and N and P_R would represent the number synaptic contacts at which release occurred and the probability that release occurred at each contact.

Whether multivesicular release occurs, or not, can be investigated by examining the fractional block of the synaptic response in the presence of a rapidly equilibrating competitive antagonist under low and high release probability conditions (18, 20, 22, 24) (Fig. 2). If the fractional block is reduced at high P_R , this suggests that the synaptic receptors are exposed to higher concentrations of glutamate at higher P_R and therefore that multiquantal release is present (see Note 1). In contrast, if the fractional block is

independent of P_R , this suggests that unquantal release predominates. However, it should be noted that this result relies on the assumption that the competitive antagonist can equilibrate during the rise of the EPSC.

Here, we describe the experimental protocols and analysis procedures that should be used to estimate and interpret quantal parameters using fluctuations analysis, explaining how to quantify intrasite and intersite variability for a multinomial model of release.

2 Materials

1. Extracellular solution (Artificial cerebrospinal fluid, ACSF) typically used for experiments in acute cerebellar or cortical slices (19, 24): 125 mM NaCl, 2.5 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 25 mM glucose, 2 mM CaCl₂, 1 mM MgCl₂ (pH 7.4 when bubbled with 95% O₂, 5% CO₂).
2. Different release probabilities conditions are imposed by changing the [Ca²⁺] and [Mg²⁺] in ACSF (18, 19, 24): for example, 1 and 5, 1.5 and 2, 2 and 1, 3 and 0.75, 5 and 0.5, 8 and 0 mM, respectively. The osmolarity is kept constant by varying [glucose] between 10 and 25 mM. A phosphate-free solution (with NaCl increased to 126.25 mM) is used in high [Ca²⁺] (8 mM) to avoid phosphate precipitation.
3. Specific combinations of drugs might need to be added to ACSF to isolate a particular synaptic component. These will depend on the type of receptors and on the preparation. In the case of glutamatergic connections, blockers of N-methyl-D-aspartate (NMDA) receptors should be used to prevent Ca²⁺-mediated plasticity processes (18, 19, 24). A rapidly equilibrating competitive antagonist is added to ACSF to test for multivesicular release (18, 24) and interpret the estimated parameters (for example, kynurenic acid or γ -D-glutamylglycine (γ -DGG) for alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors).
4. Intracellular solution for whole-cell recordings, voltage-clamp configuration (19) (see Note 2): 110 mM KMeSO₄, 40 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), 4 mM NaCl, 1 mM KCl, 1.78 mM CaCl₂, 5 mM ethylene glycol tetraacetic acid (EGTA), 4 mM MgATP, 0.3 mM MgGTP (pH 7.3; free [Ca²⁺] approximately 50 nM at physiological temperature).
5. Data acquisition and analysis can be performed using different programs, such as Axograph, PClamp (Molecular Devices, USA) or Igor PRO (Wavemetrics, USA), for which the acquisition and analysis packages NClamp and NeuroMatic are freely available on <http://silverlab.org/software-resources/>.

3 Methods

MPFA is best applied at single-synaptic connections since the number of stimulated fibers must be constant from trial to trial. This can be achieved by paired recordings (24) or, in some preparations, by extracellular stimulation (8). Quantal parameters are estimated by fitting the variance-mean relationship obtained by recording synaptic responses in different

$[Ca^{2+}]/[Mg^{2+}]$. However, the first step is to verify that it will be possible to interpret the estimated quantal parameters by testing for multiquantal release. Moreover, if a multinomial model of release is used, the different types of quantal variability have to be estimated.

In the following sections, we will describe the general procedures for data acquisition and analysis and then focus on the different types of experiments.

3.1 Data Acquisition

1. To avoid aliasing and obtain a reliable reconstruction of the signal, the sampling frequency should be set at least four to five times the highest frequency component of interest. For MPFA at the mossy fiber-granule cell (MF-GC) synapse in the cerebellum (18, 19), currents were filtered to 7.1 kHz, with a cascade of two Bessel filters with 10 kHz cutoff frequency, and digitized at 100 kHz.
2. If extracellular stimulation is used across different recording conditions, it is important to know how the stimulation threshold changes under the different conditions. Stimulation threshold should be checked in each condition and, if multiple fibers preparations are used, additional controls, such as measuring the afferent volley, should be carried out.
3. The fastest stimulation frequency that gives time-stable EPSCs in each condition (see Section 3.2.3) and the minimum time necessary for solution exchange should be established (for example, we have used 2, 0.5, 0.2 Hz at 1, 2, 8 mM $[Ca^{2+}]$, respectively, at the MF-GC synapse [18, 19]).

3.2 Data analysis

1. Individual traces are baseline subtracted using a 0.5 to 1.0 ms window (Fig. 3A, W_B) just before the stimulus.
2. The stimulus artefact is removed by subtracting a double-exponential fit to the decay of the artefact current.
3. In each recording condition, a statistical test, such as the Spearman rank-order test, should be used to find the largest set of contiguous events that are time stable (25) (Fig. 2A). This can be done by measuring for each event either the peak amplitude or the mean amplitude of the points within a 0.1 ms window around the time of the peak of the mean current (see point 4 below). Both individual and mean currents can be digitally low-pass filtered using a Gaussian filter to improve the accuracy of the peak detection (for example, at the MF-GC synapse [26], we use a cutoff frequency of 1.9-4 kHz). However, all the amplitude measurements for MPFA should be made on the original traces.
4. The synaptic response amplitude and the background noise are measured for each event using two brief measurement windows of equal duration (for example, 0.1 ms; Fig. 3A, W_P and W_N , respectively). The former should be centered at the time at which the average waveform peaks and the latter should be 'reflected' before the stimulus at a time such that the center of the baseline window is equidistant between the centers of the two measurement

windows (see Note 3). For each event, the synaptic amplitude and pre-baseline amplitude are calculated from the mean of the points within the two windows.

5. Once measurements have been made from all the stable events in a certain condition, the overall mean, sample variance and the error in the sample variance (see Note 4) can be calculated from the amplitude measurements in W_p . The synaptic variance can then be corrected for the baseline variance by simply subtracting the variance of the pre-baseline window from the variance of W_p .

3.3 Discriminating Between Univesicular and Multivesicular Release

1. Synaptic responses should be recorded in control and in the presence of a rapidly equilibrating competitive antagonist under low and high release probability conditions (18, 20, 22, 24) (Fig. 2).
2. The mean peak amplitude in the different conditions is then measured to examine the fractional block (Fig. 2). If it is independent of release probability, MPFA can be performed and the interpretation of quantal parameters will be straightforward. Otherwise, MPFA should be performed in the presence of the antagonist (27) to estimate N and P_R . However, this can be complicated if the quantal size becomes very small because it is important to quantify the quantal variance in the presence of antagonist as it may increase as the receptor occupancy is reduced.

3.4 Quantifying Quantal Variability

1. Total quantal variability ($CV_{QT} = \sqrt{CV_{QI}^2 + CV_{QII}^2}$) can be determined from evoked stimulus-aligned successes under low probability conditions when the proportion of multiquantal responses is negligible (13, 18) (Fig. 3A). Ideally, a failure rate of approximately 80-90% and at least 20 successes should be used.
2. After baseline and artefact subtraction, successes and failures in the stable epoch are distinguished using an amplitude criterion set to three times the standard deviation of the noise. This can be done using 0.75 to 1 ms measuring windows, with W_p centered slightly later than the time of the peak of the mean response to capture most of the amplitude.
3. CV_{QL} is determined from the difference in variability of stimulus-aligned (Fig. 3A) and rise-time-aligned successes (Fig. 3B). Events are usually aligned on the 10 or 20% rise-time point (see Note 5).
4. CV_{QS} can be estimated from rise-time-aligned events recorded at a single site (see Note 6). If the estimation of CV_{QI} is not possible, the best strategy is to use $CV_{QI} = CV_{QII}$ which is likely to introduce little error (28). This value can be compared with the upper estimate of CV_{QI} that can be obtained from the variance remaining when release probability is maximal ($P_R \sim 1$; $\sigma^2 \approx N\bar{Q}_p^2 CV_{QI}^2$).

3.5 MPFA

1. Ideally 100 EPSCs per condition and six to eight different conditions should be used to construct the variance-mean relationship. However, this is feasible only at connections where a relatively high stimulation rate can be applied with no synaptic depression and reasonably long recordings can be performed. Otherwise, the number of traces and/or the number of probability conditions can be reduced. At least three different recording conditions are needed to test for the statistical significance of the fit for a model of release with uniform release probability and four for the non-uniform case. The minimum number of traces depends on P_R (see Note 7) and the type of analysis to be done. High $[Ca^{2+}]$ should be used last to avoid any plasticity processes.
2. The quantal parameters $\bar{Q}_p$ and N and their uncertainties are estimated by a weighted fit (17) of the variance-mean relationship to a binomial (Eq. 3) or multinomial (Eq. 7 or 10) model of release where each data point is weighted by the reciprocal of its variance (Fig. 4A). P_R is calculated at each $[Ca^{2+}]$ from the mean current and its uncertainty can be calculated by error propagation.
3. Fits should be accepted when the χ^2 value gives a $P > 0.05$. If an accurate estimate of N is to be made, it is important that the P_R at the highest $[Ca^{2+}]$ is greater than 0.60 (13).

3.6 Quantal Determinants of Short-Term Plasticity

1. Trains of stimulation can be used to look at changes in quantal parameters during short-term plasticity (Fig. 4B-D). If long trains are used, stability should be checked on the first EPSC and on the steady-state level at the end of the trains. A lower repetition rate might be needed to avoid depression.
2. When performing CV analysis (using Eq. 11) the number of traces recorded should be maximised to reduce the sampling error in the CV estimate. We have recently used (19) a minimum of approximately 150 traces at the MF-GC synapse in 2 mM $[Ca^{2+}]$, but this depends on P_R in the condition under investigation.
3. It is important to identify the time of the peak of the current at each pulse in the train and measure the CV at the right time point. If the current does not go back to baseline within the interpulse interval, it is necessary to subtract the residual component of the preceding EPSC. Trains of different length can be alternated, and the fit to the preceding EPSC scaled and subtracted from each trace (19) (Fig. 4B).

4 Notes

1. The linear summation of *quanta* can also be tested by comparing the estimate of N , obtained from the quantal content when release is maximal (for example, 8 mM $[Ca^{2+}]$ when $NP_R \sim N$), in the presence and absence of 1 mM kynurenic acid (18). This can be obtained by dividing the mean currents by their respective quantal sizes measured from the σ^2/I in low probability conditions (13) (for example, 1.25 mM $[Ca^{2+}]$). If the estimate of N is unchanged in the presence of kynurenic acid, no new release sites are revealed by lowering receptor occupancy, suggesting that *quanta* summate linearly over the entire range of P_R .

2. Synaptic responses should be measured in voltage-clamp configuration to alleviate driving force issues and avoid dependence on voltage-gated channels. However, if synapses are far from the soma and the quality of the voltage-clamp is poor, postsynaptic potentials should be recorded and a correction for the deviation in driving force should be applied (24, 29).

3. This arrangement ensures that the peak measurement and pre-baseline measurements pick up similar frequency components of the background noise.

4. The expression used to estimate the error on the sample variance depends on the number of release sites (17). A general expression for all likely conditions is given by:

$$\text{var}(s^2) = \frac{n}{(n-2)(n-3)} \left[\frac{3(3-2n)(n-1)^2 - n(n-2)(n-3)^2}{(n^2-2n+3)(n-1)^2} m_2^2 + m_4 \right] \quad (12)$$

that, for large N , simplifies to:

$$\text{var}(s^2) = \frac{2n}{(n-1)(n-2)(n-3)} \left[\frac{n^2(n-2)(n-3) - 3(3-2n)(n-1)}{(n^2-2n+3)(n-1)} m_2^2 - m_4 \right], \quad (13)$$

where n is the number of traces acquired and m_2 and m_4 are sample central moments about the mean and can be calculated as $m_r = \frac{1}{n} \sum_{i=1}^n (X_i - \bar{X})^r$.

In case of large sample size, neglecting terms to the order n^{-1} , m_2 can be used to estimate the variance, whereas proper estimators of its variance are given by:

$$\text{var}(m_2) = \frac{m_4 - m_2^2}{n}, \quad (14)$$

which simplifies for large N to

$$\text{var}(m_2) = \frac{2m_2^2}{n}. \quad (15)$$

As the reliability of individual estimates is higher for the simplified expression, the use of this estimator could be more appropriate if it is known *a priori* that a large number of release sites are present. Moreover, maximizing the number of samples improves the estimators' reliability and is therefore anyway desirable.

5. To minimize the effects of sampling error, CV_{QL} can also be measured by simulating stochastic quantal release at a release site using the mean measured quantal waveform and the mean latency distribution measured under low probability conditions (18). For each

connection, the mean quantal response is obtained by averaging the rise-time-aligned successes and by fitting it with an expression (30) modified from **ref.** 31 to minimize errors due to noise:

$$EPSC(t) = A_1 \left(1 - \exp\left(-\frac{t-t_0}{\tau_{rise}}\right) \right)^n \left(A_2 \exp\left(-\frac{t-t_0}{\tau_{decay1}}\right) + (1 - A_2) \exp\left(-\frac{t-t_0}{\tau_{decay2}}\right) \right) \quad (16)$$

The mean cumulative latency distribution was obtained after normalising and aligning on the 50% point the cumulative latency distributions obtained for each cell from the 20% rise-time point of the successes.

6. To test for the single site connections, the measured mean amplitudes of successes at the different $[Ca^{2+}]$ are compared with the predicted amplitude (A_s) assuming one or two release sites (18, 25). According to a binomial model of release, this can be calculated for a given N , knowing the quantal size and the failure rate f :

$$A_s = \frac{I}{1-f} = \frac{NQ P_R}{1-f} = \frac{NQ(1-f^{\frac{1}{N}})}{1-f} \quad (17)$$

7. For MPFA, the number of traces recorded in each condition could be as low as 30-50. However, the distribution of variances obtained at extreme P_R from few synaptic currents at synapses with few release sites can become skewed, and the use of a weighted least squares optimization routine under these conditions might lead to biased results (17).

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Fig. 1.

Binomial and multinomial models of synaptic transmission. **(A)** Monte Carlo simulations of synaptic currents (15 traces superimposed) and mean current (black trace, average from 200 traces) for a synapse with $N = 5$, $Q = -20$ pA at three different release probabilities and associated variance-mean relationship (bottom; Eq. 3). The black dots represent the theoretical data points associated with the tested release probabilities (Eqs. 1 and 2), whereas the empty squares are values from sets of 200 simulated synaptic currents. **(B)** Same as in panel **A** for synapses with intrasite and intersite quantal variability ($CV_{QS} = CV_{QH} = 0.3$ and $CV_{QL} = 0.2$). The current traces and the black line and markers in the variance-mean plot refer to a synapse with uniform P_R (Eqs. 4-7), whereas the grey line and markers in the graph refer to a non-uniform release probability case ($\alpha = 1$; Eq. 10). The dashed line shows the variance contributed by intrasite variability. The mean quantal amplitude across sites was set to -20 pA that gave $\bar{Q}_p = -17.2$ pA in the presence of asynchronous release. (Modified from ref. 17 with permission from Elsevier.)

Fig. 2.

Use of a rapidly equilibrating competitive antagonist to test for multivesicular release. **(A)** EPSC amplitudes in 2 and 8 mM (dashed bar) $[Ca^{2+}]$, in the absence or presence of 1 mM kynurenic acid (Kyn; thin grey bar). The thick grey bars indicate stable epochs as assessed with the Spearman rank-order test. **(B)** Mean EPSCs from the four stable epochs in panel **A** (artefact blanked). **(C)** Fractional block of the EPSC by 1 mM Kyn in 2 and 8 mM $[Ca^{2+}]$ across cells. (Modified from Fig. 4, ref. 18 with permission from The Society for Neuroscience.)

Fig. 3.

Measuring quantal variability in low probability conditions. 500 synaptic currents were simulated for a multinomial model of release with uniform $P_R = 0.02$. 15 successes (bottom) are shown stimulus aligned (A) and rise-time aligned (B). The black lines represent the mean (bottom) and variance (top) obtained from all successes. Open bar (W_B) in A indicates an appropriate window for baseline-correcting individual traces. Vertical lines, marked W_N and W_P , indicate windows for measuring mean and variance of the baseline noise and peak of the EPSC, respectively. Because of the asynchrony in quantal release, the mean of the stimulus-aligned successes is smaller and thus the variability larger.

Fig. 4.

Combining MPFA and CV analysis to determine quantal parameters during trains. **(A)** Variance-mean plot obtained from EPSCs recorded in different release probability conditions at a MF-GC connection. Errors are calculated according to Eq. 12. The line shows the weighted fit according to a multinomial model (Eq. 7). **(B)** Mean EPSCs for single pulses, 4-pulse and 5-pulse 100 Hz trains in 2 mM $[Ca^{2+}]$. Double-exponential fit of the mean single pulse or 4-pulse train were scaled and subtracted from each trace for analysing the second or fifth EPSC, respectively. **(C and D)** P_R and $\bar{Q}_p$ during 5-pulse 100 Hz trains for 9 connections. (Modified from ref. 19.)