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DOI
10.1016/j.jneumeth.2019.108526

Publication date
2020

Document Version
Final published version

Published in
Journal of Neuroscience Methods

Journal of Neuroscience Methods 331 (2020) 108526

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Variance analysis as a tool to predict the mechanism underlying synaptic plasticity

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ARTICLE INFO

Keywords:
Electrophysiology
Synapses
Plasticity
AMPA-receptor
Variance analysis

ABSTRACT

Background: The strength of synaptic transmission onto a neuron depends on the number of functional vesicle release sites (N), the probability of vesicle release (P_r), and the quantal size (Q). Statistical tools based on the quantal model of synaptic transmission can be used to acquire information on which of these parameters is the source of plasticity. However, quantal analysis depends on assumptions that may not be met at central synapses.
New method: We examined the merit of quantal analysis to extract the mechanisms underlying synaptic plasticity by applying binomial statistics on the variance in amplitude of postsynaptic currents evoked at Schaffer collateral-CA1 (Sc-CA1) synapses in mouse hippocampal slices. We extend this analysis by combining the conventional inverse square of the coefficient of variation ($1/CV^2$) with the variance-to-mean ratio (VMR).
Results: This method can be used to assess the relative, but not absolute, contribution of N, P_r and Q to synaptic plasticity. The changes in $1/CV^2$ and VMR values correctly reflect experimental modifications of N, P_r and Q at Sc-CA1 synapses.
Comparison with existing methods: While the $1/CV^2$ depends on N and P_r, but is independent of Q, the VMR is dependent on P_r and Q, but not on N. Combining both allows for a rapid assessment of the mechanism underlying synaptic plasticity without the need for additional electrophysiological experiments.
Conclusion: Combining the $1/CV^2$ with the VMR allows for a reliable prediction of the relative contribution of changes in N, P_r and Q to synaptic plasticity.

1. Introduction

The ability of synapses to change their strength underlies experience-dependent adaptation in behavior (Kessels and Malinow, 2009; Roelfsema and Holtmaat, 2018) and cognitive disorders are often a consequence of aberrant synaptic plasticity (Kauer and Malenka, 2007; Volk et al., 2015; Torres et al., 2017). However, the origin and mechanism underlying synaptic changes are difficult to assess for many cases in which synaptic plasticity occurs.

The efficiency of synaptic communication is largely determined by three parameters: the number of functional vesicle release sites (N), the presynaptic release probability (P_r), and the postsynaptic response to the release of a single vesicle of neurotransmitter, i.e. the quantal size (Q). Presynaptic vesicle release is a stochastic process; when an action potential arrives at a synapse, it does not reliably evoke the release of a vesicle of neurotransmitter from that terminal. In the central nervous system, P_r varies greatly between synapses, depending on the size of the readily releasable vesicle pool (Dobrunz and Stevens, 1997; Kaeser and Regehr, 2017) and the efficiency of the vesicle release machinery (de Jong and Verhage, 2009). P_r has been estimated to be on average 0.3 for CA1 hippocampal synapses receiving Schaffer collateral (Sc) input (Hessler et al., 1993; Rosenmund et al., 1993; Oertner et al., 2002). Sc-CA1 synapses therefore usually do not release more than one vesicle per synapse, indicating that N can generally be considered equal to the number of synapses (Stevens and Wang, 1995; Schikorski and Stevens, 1997). Q depends on both the amount of neurotransmitter stored in a single presynaptic vesicle (McAllister and Stevens, 2000; Goh et al., 2011) and postsynaptic strength, which is determined by the density, conductance and open-channel probability of postsynaptic receptors (Korn and Faber, 1991; Kerchner and Nicoll, 2008).

A change in synaptic strength must be caused by a modulation of one or more of these three defining parameters N, P_r and Q. However, current electrophysiological techniques cannot directly distinguish which of these individual parameters are altered. Del Castillo and Katz described a statistical approach to investigate the mechanisms underlying synaptic plasticity: quantal analysis. This analysis is based on the

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https://doi.org/10.1016/j.jneumeth.2019.108526
Received 16 September 2019; Received in revised form 31 October 2019; Accepted 18 November 2019
Available online 19 November 2019

quantal model of synaptic transmission: the biological principle that neurotransmitter is released from the presynaptic release site in a probabilistic, all-or-none manner in discrete 'quanta', which are equal to the amount of neurotransmitter packaged within a single presynaptic vesicle (del Castillo and Katz, 1954). The postsynaptic response is the summation of multiple quanta and therefore graded by quantal steps (Kerchner and Nicoll, 2008). Due to the probabilistic nature of presynaptic vesicle release, repeated stimulation of presynaptic axon bundles produces a fluctuating postsynaptic response roughly following a binomial distribution (Korn and Faber, 1991). Both the mean amplitude (μ) of this postsynaptic response and its variance (σ^2) are determined by synaptic parameters *N, P_r* and *Q,* and can be described with formulas using binomial statistical models:

$$\mu = NP_rQ \quad (1)$$

$$\sigma^2 = NP_r(1 - P_r)Q^2 \quad (2)$$

Information about the mechanisms underlying synaptic plasticity can be extracted from these equations by making simple indices of the mean amplitude and variance of the evoked postsynaptic response and comparing the indices before and after an alteration in synapse strength. These indices are only dependent on two synaptic parameters instead of three, and therefore have a higher information value. An example of such an index is the quantal measure of the inverse square of the coefficient of variation ($1/CV^2$), which depends on *N* and P_r, but is independent of *Q* (Malinow and Tsien, 1990):

$$\frac{1}{CV^2} = \frac{\mu^2}{\sigma^2} = \frac{NP_r}{1 - P_r} \quad (3)$$

This $1/CV^2$ has been widely used to assess whether a change in synapse strength is predominantly of presynaptic (i.e. a change in P_r and/or *N*) or postsynaptic (i.e. a change in *Q*) origin. A second, lesser-known example is the variance-to-mean ratio (VMR), which is dependent on P_r and *Q*, but not on *N* (Lupica et al., 1992):

$$VMR = \frac{\sigma^2}{\mu} = (1 - P_r)Q \quad (4)$$

VMR values may be used to examine whether altered synaptic transmission is caused by a change in the number of active vesicle release sites (*N*). The VMR has not been systematically validated yet, but if proven reliable, combining the use of both $1/CV^2$ and the VMR would be of added value over using $1/CV^2$ alone.

Because the binomial model underlying these quantal formulas depends on a number of assumptions that may not be met at central synapses, these formulas may not have the precision to accurately quantify absolute levels of *N, P_r* and *Q*. However, they should be able to detect changes in *N, P_r* and *Q*, as has been experimentally validated for the $1/CV^2$ in the CA1 region of the hippocampus (Manabe et al., 1993). Therefore, we set out to examine the merit of this extended variance analysis to predict the relative contribution of changes in these three parameters to synaptic plasticity triggered under experimental conditions at Sc-CA1 synapses. We conclude that calculating both $1/CV^2$ and VMR values allows for a rapid and reliable prediction of whether synaptic plasticity is caused by a change in *N*, P_r, and/or *Q*.

2. Materials and methods

2.1. Mice

Male and female C57BL/6 mice were used for this study. The mice were kept on a 12-h day-night cycle and the dams had ad libitum access to food and water. All experiments were conducted in line with the European guidelines for the care and use of laboratory animals (Council Directive 86/6009/EEC) and all experiments were approved by the experimental animal committee (DEC) of the Royal Netherlands Academy of Sciences (KNAW).

2.2. Electrophysiology

Organotypic hippocampal slices were prepared from P6-8 mice as described previously (Stoppini et al., 1991) and were used at 7–12 days in culture. Just before recording, a cut was made between CA3 and CA1 to prevent stimulus-induced bursting / recurrent activity. Two stimulating electrodes (two-contact Pt/Ir cluster electrodes; FHC), were placed between 100 and 300 μm down the apical dendrites, 200 μm apart laterally. Whole-cell recordings were made using 3–5 MΩ pipettes from borosilicate glass (Harvard Apparatus UK; $R_{access} < 20$ MΩ and $R_{input} > 10x\ R_{access}$) with an internal solution containing 115 mM $CsMeSO_3$, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 4 mM Na_2-ATP, 0.4 mM Na-GTP, 10 mM sodium phosphocreatine, and 0.6 mM EGTA, at pH 7.25. During recordings, the slices were perfused with artificial cerebrospinal fluid (ACSF) containing 118 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, and 1 mM NaH_2PO_4, gassed with 5 % CO_2/95 % O_2 at 29 °C, supplemented with 22 mM glucose, 4 mM $MgCl_2$, 4 mM $CaCl_2$ (or 1.5 mM for the low calcium condition of the P_r experiment), and 100 μM picrotoxin (Sigma). A wash-in of CNQX (0.6 μM; Hello Bio) was used to manipulate *Q* and 5 min later another recording was made. EPSCs were evoked with electrical stimulation (100 μs duration) at an inter-stimulus interval of 3 s. Stimulation intensity was adjusted to evoke postsynaptic currents of approximately half-maximal amplitude. When assessing a change in stimulation strength, the high intensity stimulation was set to evoke postsynaptic currents of about 75 % of the maximal amplitude and the low intensity stimulation was set at an intensity, which evoked a response with an amplitude that was approximately half of the amplitude of the response to high intensity stimulation. AMPAR-mediated EPSCs were measured following stimulation as the peak inward current at −60 mV. Data were acquired using a Multiclamp 700B amplifier (Molecular Devices). Signals were filtered at 3 kHz and sampled at 10 kHz. The recordings were analysed using pClamp 10 software (Molecular Devices).

2.3. Variance analysis

Values for the mean amplitude, VMR and $1/CV^2$ of synaptic transmission were obtained from the average and the variance in the responses of 30–50 sweeps. An important consideration in the use of the VMR and $1/CV^2$ is that the variance and mean should be calculated over an epoch where synaptic responses are stable, as instability will artificially increase the variance. Only recordings were used for which the average synaptic response of the last 5 sweeps was < 30 % different from the average synaptic response of the first 5 sweeps. Our recordings showed a good signal-to-noise ratio, with large evoked currents and a low, negligible, baseline variance. However, when calculating EPSC variance from recordings in which the difference between the baseline variance and signal amplitude is smaller, it might be useful to correct for baseline variance, as recommended by Faber and Korn (1991), by subtracting the variance due to background noise from the total variance. The experimenter was blind to the experimental conditions while analysing. When comparing VMR or $1/CV^2$ values for two periods, the same number of sweeps was used for each period.

2.4. Statistics

The Shapiro-Wilk normality test was used to test whether (the differences between) groups were normally distributed. To detect differences between two paired groups, the paired *t*-test or the Wilcoxon matched-pairs signed rank test (if the differences between the two groups were not normally distributed) was used. The one sample *t*-test was used to test whether a $\log_2$ fold change was significantly different from 0. For all tests, $p < 0.05$ was considered significant.

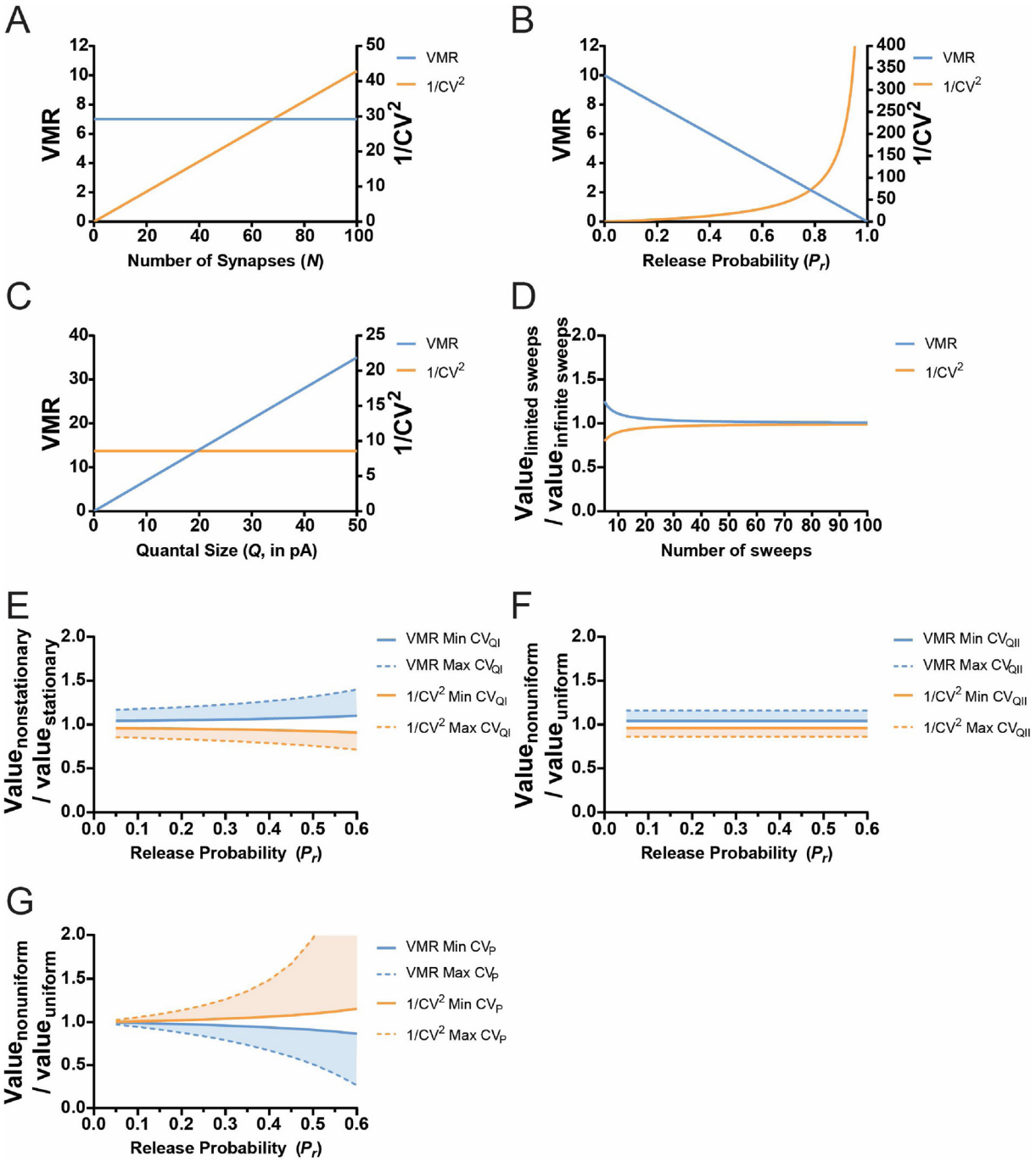

Fig. 1. Theoretical model of how VMR and $1/CV^2$ values are affected by changes in N, P_r, Q, sample size and variability in P_r and Q.
(A–C) Theoretical models of VMR (blue line; left axis) and $1/CV^2$ (orange line; right axis) values for increasing N (A), P_r (B), and Q (C), using the following values for the two constant parameters in each graph: $N = 20$, $P_r = 0.3$ and $Q = 10$. (D) The bias in the value of the VMR (blue) and $1/CV^2$ (orange) caused by a limited sample size, depicted as the ratio of the value with a limited number of sampled sweeps over the value considering an infinite sample size, plotted against the number of sweeps sampled. (E–G) Theoretical models of the bias in VMR (blue) and $1/CV^2$ (orange) values caused by intrasite variability in Q (E), intersite variability in Q (F) and intersite variability in P_r (G), depicted as the ratio of the value with variability over the value without variability, plotted against the release probability. The solid line represents the bias caused by the minimal amount of variability ($CV_{QI} = 0.2$; $CV_{QII} = 0.2$; $CV_P = 0.3$), the dashed line represents the bias caused by the maximal amount of variability ($CV_{QI} = 0.4$; $CV_{QII} = 0.4$; $CV_P = 0.7$) as reported in literature. A ratio of 1 indicates no bias.

3. Results

3.1. The influence of invalid assumptions on predictions made by variance analysis

In electrophysiological recordings of evoked excitatory postsynaptic currents (EPSCs), N vesicle release sites are activated by an evoked action potential in presynaptic terminals, each releasing a single vesicle of transmitter with probability P_r, producing a postsynaptic response with amplitude Q. In the simplified case in which values of Q and P_r are the same for all N release sites and invariant over the time of the recording, VMR and $1/CV^2$ values are affected by changes in N, P_r, or Q as predicted by formulas *3* and *4*. For instance, if we assume a homogeneous group of synapses, a change in N is reflected by a proportional

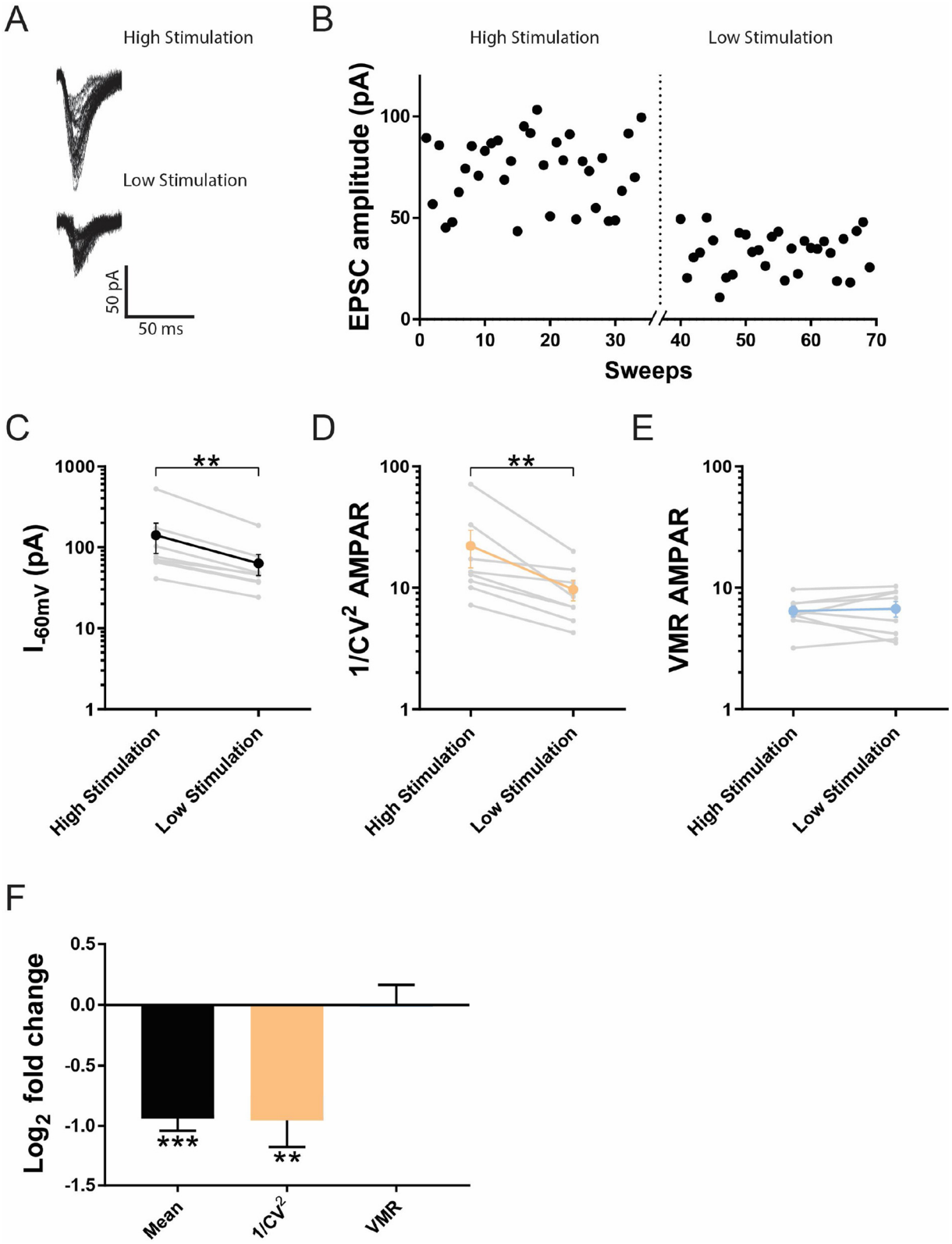

Fig. 2. $1/CV^2$ and VMR values predict a decrease in *N* upon lowering stimulation strength.
(A) Example traces of individual AMPAR-mediated EPSCs (30 sweeps) evoked by high and low intensity electrical stimulation in the same cell. (B) Example plot of an EPSC recording with high and low intensity electrical stimulation over the number of sweeps. (C) Line plot of the mean EPSC amplitudes evoked by high and low intensity electrical stimulation with individual recordings in grey and the average of all cells in black ($n = 8$). (D) Line plot of $1/CV^2$ values with high and low intensity electrical stimulation with individual recordings in grey and the average of all cells in orange ($n = 8$). (E) Line plot of VMR values with high and low intensity electrical stimulation with individual recordings in grey and the average of all cells in blue ($n = 8$). (F) Fold change (Log_2-normalized) in the mean amplitude (black), $1/CV^2$ (orange) and VMR (blue) of AMPAR-mediated EPSCs after lowering stimulation strength. Error bars indicate SEM, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

change in $1/CV^2$ without a change in VMR (Fig. 1A). An increase in P_r produces a superlinear increase in $1/CV^2$ and a decrease in VMR (Fig. 1B). A change in Q is reflected by an unchanged $1/CV^2$ and linear change in VMR (Fig. 1C). The relative changes in $1/CV^2$ and VMR may therefore be valuable to decipher the source of synaptic plasticity.

However, these formulas stem from a binomial model and are simplifications that rely on assumptions that are not necessarily met at synapses in reality. For instance, whereas quantal analysis assumes an infinite number of evoked EPSC responses, in experimental conditions the variance is influenced by the number of evoked responses sampled (n):

$$\sigma^2 = \frac{nNP_r(1 - P_r)Q^2}{n - 1} \quad (5)$$

The accuracy of VMR and $1/CV^2$ values thus increases with the number of evoked responses sampled and with an n of more than 20 traces, the bias is smaller than 5 % (Fig. 1D). When making a comparison between VMR or $1/CV^2$ values before and after manipulation, the same number of evoked responses should be used, so that the bias is equal and does not affect the comparison.

A primary concern with quantal analysis is that it assumes that P_r and Q are uniform across release sites and remain constant during an experiment (Korn and Faber, 1991; Oleskevich et al., 2000; Clements, 2003; Humeau et al., 2007). In reality, intra- and intersite variabilities in Q and non-uniformity in P_r contribute to the fluctuation in the amplitude of the synaptic response (Jack et al., 1981; Walmsley et al., 1988; Bekkers and Stevens, 1989; Biró et al., 2005). Upon inclusion of the coefficient of variation of P_r (CV_P), intrasite variability in Q (CV_{QI}) and intersite variability in Q (CV_{QII}), the variance is expressed as (Silver et al., 1998):

$$\sigma^2 = NP_rQ^2(1 - P_r(1 + CV_p{}^2))(1 + CV_{QII}{}^2) + NP_rQ^2CV_{QI}{}^2 \quad (6)$$

The physiological values of the CV_{QI} and CV_{QII} that are typically reported in literature lie between 0.2 and 0.4, and the CV_P can vary between 0.3 and 0.7 (Isaacson and Hille, 1997; Murthy et al., 1997; Silver et al., 1998; Clements and Silver, 2000; Biró et al., 2005; Nusser, 2006). These non-uniformities in Q and P_r cause a bias in VMR and $1/CV^2$ values (Fig. 1E–G), thereby severely limiting the use of variance analysis as a means to quantify absolute values of N, P_r and Q. A comparison of Q and P_r before and after the synaptic modulation can remain valid as long as the values of CV_P, CV_{QI}, and CV_{QII} are not different following a synaptic modulation, because the relative error in the estimates of Q and P_r remains unchanged. However, in the case that the variability in Q or P_r changes upon synaptic plasticity, for example when either strong synapses are selectively weakened or predominantly weak synapses are strengthened, this complicates the interpretation of the results. In addition, the effect of CV_{QI} and particularly of CV_P on $1/CV^2$ and VMR values are dependent on P_r (Fig. 1E, G). Particularly for synapses with a high P_r, when P_r changes, the magnitude of $1/CV^2$ and VMR changes will be an overestimation or underestimation of the magnitude of synaptic changes. Taking these imperfections of the model into account, we assessed how experimentally obtained Sc-CA1 synaptic currents fit the theoretical models.

3.2. Validation of variance analysis upon a change in N

To experimentally validate that $1/CV^2$ and VMR values are in practice modified by changes in N, P_r, or Q as predicted by formulas *3* and *4*, changes in the $1/CV^2$ and VMR were examined following interventions known to affect one of the three parameters. In organotypic hippocampal slices, electrical stimulation was applied to the Schaffer collateral inputs onto synapses of CA1 pyramidal neurons and the mean amplitude, VMR and $1/CV^2$ were obtained from the resultant EPSCs recorded in whole-cell configuration in pyramidal CA1 neurons before and after manipulation. Electric stimulation was performed with a low (0.3 Hz) sampling rate to exclude variance in N due to incomplete replenishment of release-ready synaptic vesicles, variance in P_r due to fluctuations in intracellular calcium, or variance in Q due to desensitization of postsynaptic glutamate receptors (Humeau et al., 2007; von Engelhardt et al., 2010).

We first aimed to verify that the $1/CV^2$ but not the VMR decreases when the number of active vesicle release sites (N) was lowered under experimental conditions. To change N, the strength of electrical stimulation was varied, which changes the number of recruited Sc-fibers (Manabe et al., 1993). Stimulation strength was lowered such that EPSC amplitudes were on average 47 % reduced ($p = 0.008$; Fig. 2A–C). Variance analysis revealed that while the $1/CV^2$ decreased to the same extent, by on average 44 % ($p = 0.008$; Fig. 2D), the VMR did not significantly change upon lowering stimulation strength (5 %; $p = 0.659$; Fig. 2E). An assumption within quantal analysis that is relevant for this experiment is that the applied electrical stimulation causes reliable axonal activation (Clements, 2003). If lowering stimulation strength reduces the chances of evoking an action potential in axons, this could add another source of variance, causing the value of P_r to be underestimated (Faber and Korn, 1991). Since the VMR, and thus P_r, remained unaltered upon changing stimulation strength, the change in stimulation strength did not seem to affect the reliability of evoking action potentials onto Sc-axons. Thus, in accordance with the model predictions, a decrease in N did not impact the VMR and proportionally lowered $1/CV^2$ (Fig. 2F).

3.3. Validation of variance analysis upon a change in P_r

We next investigated the consequences of selectively altering P_r on $1/CV^2$ and VMR values. An important assumption concerning vesicle release is that the release of quanta at any one of the release sites must be independent of whether or not release occurs at any other site (Clements, 2003). This assumption was shown to be appropriate for several different synaptic connections, including synapses in the hippocampus (Redman, 1990; Silver et al., 1998; Clements, 2003; Biró et al., 2005).

The presynaptic release machinery is directly dependent on the levels of calcium ions in the presynaptic bouton, and lowering the extracellular Ca^{2+} concentration decreases P_r (Dodge and Rahamimoff, 1967). Notably, changes in extracellular Ca^{2+} do not affect Q provided that the concentration of extracellular magnesium ions is kept high (e.g. 4 mM) (Hardingham et al., 2006). To selectively decrease P_r, the extracellular calcium concentration was lowered from 4 mM to 1.5 mM, while maintaining Mg^{2+} levels at 4 mM (Fig. 3A, B), which resulted in an on average 59 % decrease in EPSC amplitude ($p = 0.002$; Fig. 3C). This decrease in average EPSC amplitude was associated with a 70 % decrease in $1/CV^2$ ($p = 0.005$; Fig. 3D), whereas the VMR was increased by on average 50 % ($p = 0.039$; Fig. 3E). These experimental data fit the theoretical model predictions that a change in P_r is associated with a superlinear change in $1/CV^2$ and an inverse change in VMR (Fig. 3F).

3.4. Validation of variance analysis upon a change in Q

In quantal analysis, it is generally assumed that quanta summate linearly (Reid and Clements, 1999; Clements and Silver, 2000; Humeau et al., 2007), such that the peak of an evoked EPSC represents the number of released quanta times the mean amplitude of the synaptic response evoked by the release of one quantum. Because vesicle release is not perfectly synchronous, the contribution of individual quanta to the peak amplitude of the evoked EPSC will be somewhat smaller than expected (Isaacson and Walmsley, 1995; Bellingham et al., 1998), leading to an underestimate of mean quantal size. Furthermore, dendritic filtering alters the amplitude and time course of synaptic responses that originate at a distance from the recording site (Bekkers and Stevens, 1990), making the contribution to the evoked EPSC of quanta released further from the recording site smaller. Although this means

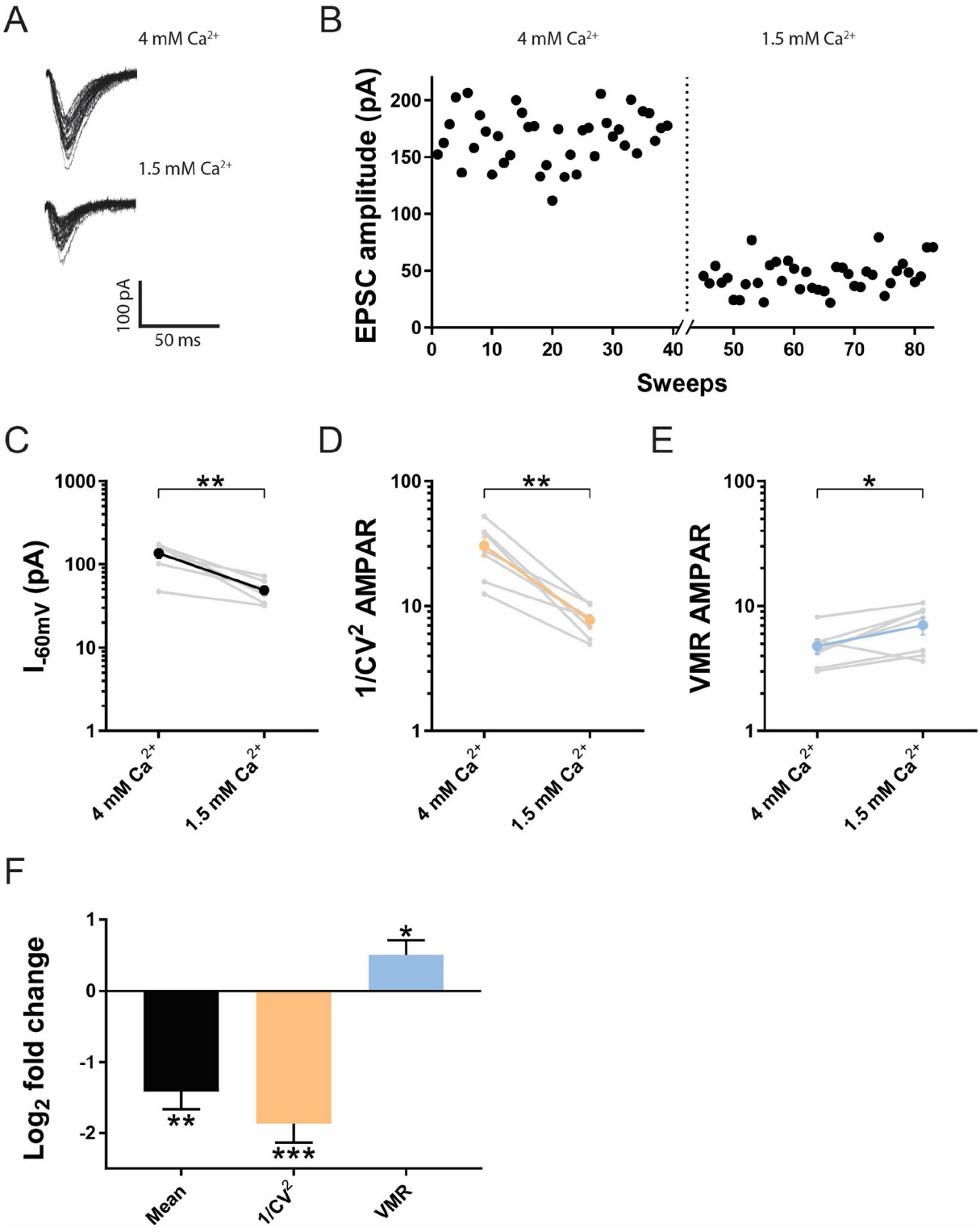

Fig. 3. $1/CV^2$ and VMR values predict a decrease in P_r upon lowering extracellular Ca^{2+}.
(A) Example traces of individual AMPAR-mediated EPSCs (30 sweeps) recorded from the same cell with 4 or 1.5 mM extracellular [Ca^{2+}]. (B) Example plot of an EPSC recording with 4 or 1.5 mM extracellular [Ca^{2+}] over the number of sweeps. (C) Line plot of the mean EPSC amplitudes at 4 and 1.5 mM [Ca^{2+}] with individual recordings in grey and the average of all cells in black ($n = 7$). (D) Line plot of $1/CV^2$ values at 4 and 1.5 mM [Ca^{2+}] with individual recordings in grey and the average of all cells in orange ($n = 7$). (E) Line plot of VMR values at 4 and 1.5 mM [Ca^{2+}] with individual recordings in grey and the average of all cells in blue ($n = 7$). (F) Fold change (Log_2-normalized) in the mean amplitude (black), $1/CV^2$ (orange) and VMR (blue) of AMPAR-mediated EPSCs after decreasing extracellular calcium concentration. Error bars indicate SEM, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

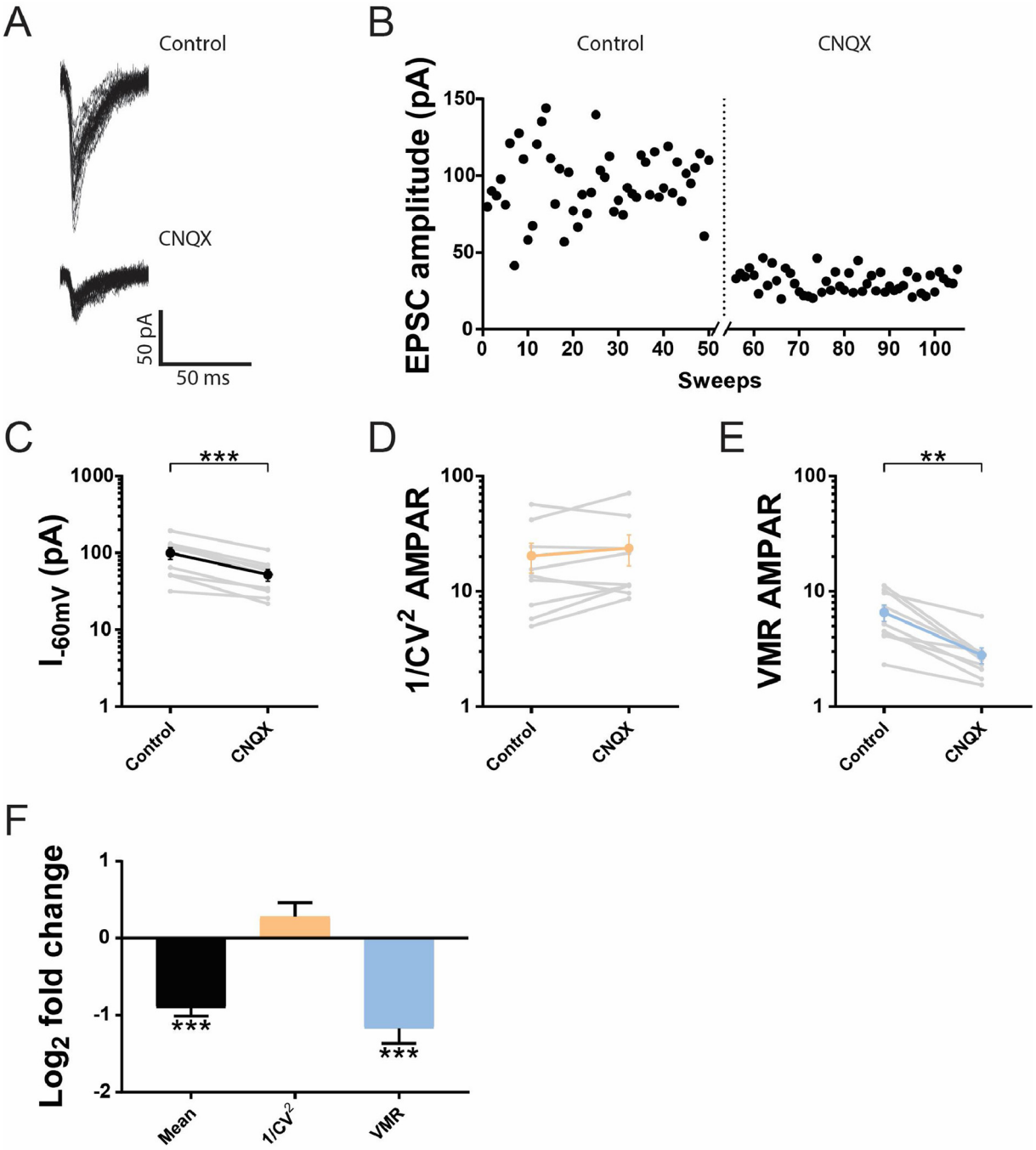

Fig. 4. $1/CV^2$ and VMR values predict a decrease in *Q* upon wash-in of AMPA-receptor antagonist CNQX.
(A) Example traces of individual AMPAR-mediated EPSCs (30 sweeps) recorded from the same cell before and after wash-in of 0.6 μM CNQX. (B) Example plot of an EPSC recording before and after wash-in of CNQX over the number of sweeps. (C) Line plot of the mean EPSC amplitudes in absence and presence of CNQX with individual recordings in grey and the average of all cells in black ($n = 9$). (D) Line plot of $1/CV^2$ values in absence and presence of CNQX with individual recordings in grey and the average of all cells in orange ($n = 9$). (E) Line plot of VMR values in absence and presence of CNQX with individual recordings in grey and the average of all cells depicted in blue ($n = 9$). (F) Fold change (Log_2-normalized) in the mean amplitude (black), $1/CV^2$ (orange) and VMR (blue) of AMPAR-mediated EPSCs after wash-in of CNQX. Error bars indicate SEM, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

that the exact value of *Q* cannot be precisely determined from the variance, this systematic measurement error does not invalidate the use of variance analysis as a tool to investigate the relative contribution of a change in *Q* to synaptic plasticity, because *Q* will be underestimated by the same factor before and after modulation (Reid and Clements, 1999).

To examine how a selective decrease in *Q* affects the variance analysis measures, *Q* was manipulated by partially blocking non-NMDA glutamate receptors with a non-saturating concentration of CNQX (0.6 μM). CNQX wash-in reduced the EPSC amplitudes by on average 45 % ($p = 0.0009$; Fig. 4A–C). This decrease in synaptic currents was not associated with a significant change in the value of $1/CV^2$ (29 %; $p = 0.390$; Fig. 4D), but was accompanied by an on average 52 % decrease in VMR ($p = 0.003$; Fig. 4E). Thus, upon a selective reduction in *Q*, the mean EPSC amplitude and VMR decreased to a similar extent, while the $1/CV^2$ did not significantly change (Fig. 4F), which is in line with the model prediction.

4. Discussion

We tested whether variance analysis can be applied to predict the source of synaptic plasticity at Sc-CA1 synapses in hippocampal slice cultures. First, we experimentally confirmed that selectively decreasing the number of synapses (i.e. vesicle release sites; N) lowered $1/CV^2$ to roughly the same extent as it depressed the mean EPSC amplitude, while having no effect on the VMR value. Secondly, we showed that decreasing the probability of vesicle release (P_r) decreased $1/CV^2$ superlinearly and increased the VMR, following the predictions of the model. Finally, we verified that reducing postsynaptic strength (Q) lowered the VMR value proportionally, but did not significantly affect $1/CV^2$. These experiments confirm that the $1/CV^2$ depends on N and P_r but is independent of Q, while the VMR is dependent on P_r and Q but not on N. Therefore, calculating both VMR and $1/CV^2$ values of evoked synaptic currents can be informative for deciphering the mechanisms underlying synaptic plasticity. Our experiments demonstrate that variance analysis may be used as a simple tool to predict which of the three individual components contributing to synapse strength are changed in a certain type of synaptic plasticity.

The use of variance analysis to predict the source of synaptic changes requires correct definitions for N, P_r and Q. For example, the notion that N reflects the number of functional synapses is only valid as long as synapses release only one vesicle of neurotransmitter upon successful activation, which has been confirmed for e.g. different types of CA1 synapses, mossy fiber-CA3 interneuron synapses and layer 4-layer 2/3 cortical synapses (Gulyás et al., 1993; Hanse and Gustafsson, 2001; Lawrence et al., 2003; Silver et al., 2003; Biró et al., 2005). Multivesicular release may occur when P_r is elevated (Oertner et al., 2002; Conti and Lisman, 2003; Christie and Jahr, 2006), in which case N represents the number of active release sites instead of the number of synapses. Furthermore, the notion that Q reflects postsynaptic strength only holds when the amount of neurotransmitter in a vesicle remains constant during the recording. Glutamate release, including at Sc-CA1 synapses, might not always lead to saturation of postsynaptic glutamate receptors (Mainen et al., 1999; McAllister and Stevens, 2000). This means that since Q is defined as the size of the postsynaptic response to the release of a single vesicle of neurotransmitter, its value is determined by both the postsynaptic strength and the amount of neurotransmitter per vesicle. There is evidence that the amount of neurotransmitter stored in synaptic vesicles can exhibit a certain, although small, degree of variation (Hanse and Gustafsson, 2001; Wu et al., 2007; Goh et al., 2011; Takamori, 2016). Therefore, a change in VMR may reflect either a change in neurotransmitter concentration per vesicle or a postsynaptic change in AMPA-receptor currents.

It is important to keep in mind that the quantal formulas are simplifications of reality and therefore do not allow the determination of the exact values of N, P_r and Q. We here consider and discuss several of the assumptions that govern the quantal formulas. Most of these assumptions do not influence the determination of the relative change in $1/CV^2$ and VMR, since they equally affect synaptic currents in the 'before' and 'after' condition. The main concern with the use of variance analysis is related to the fact that synapses are not uniform in their values for P_r and Q, causing extra variance in synaptic responses (Jack et al., 1981; Walmsley et al., 1988; Bekkers and Stevens, 1989; Biró et al., 2005). When this level of non-uniformity in P_r or Q changes among a group of synapses after manipulation, the magnitude of a change in $1/CV^2$ and VMR will be an over- or underestimation of the change in N, P_r, or Q. A similar over-/underestimation will be made when P_r changes after a synaptic manipulation, since the influence of non-uniformity and intrasite variability on $1/CV^2$ and VMR values is dependent on P_r. Particularly for synapses with a high P_r the influence of non-uniformity and intrasite variability on the variance in synaptic responses will be substantial.

Although other more complex models, which rely on fewer assumptions but often require more elaborate experiments, obviously might fit the data better, the proof-of-principle experiments have shown that the VMR is a valid tool to detect changes in synaptic parameters. Conveniently, since VMR values are independent of the number of stimulated axons, they can be directly compared as a relative measure of average synapse strength between neuronal preparations from different mice. Although quantal analysis has its shortcomings, it allows the determination of relative values of the synaptic parameters N, P_r and Q, at least in the CA1 region of the hippocampus, and thereby provides a reliable prediction of the synaptic changes underlying plasticity. In conclusion, our experiments validate this extended variance analysis as a relatively simple method to generate hypotheses on the mechanism behind a synaptic plasticity phenomenon, which can be used to efficiently guide further experiments that provide direct experimental confirmation.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

We thank Roberto Malinow, Taco Werkman and Natalie Cappaert for critically reading the manuscript. This work was supported by ZonMw – The Netherlands Organisation for Health Research and Development, grant nr. 733050106, dementia research and innovation programme 'Memorabel' as a part of the 'Deltaplan for Dementia'.

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