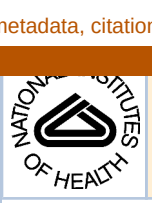

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Evaluating statistical methods used to estimate the number of postsynaptic receptors

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Abstract

Calcium levels in spines play a significant role in determining the sign and magnitude of synaptic plasticity [Yang et al., 1999, Malenka and Bear, 2001, Cummings et al., 1996]. The magnitude of calcium influx into spines is highly dependent on influx through N-methyl D-aspartate (NMDA) receptors [Sabatini et al., 2002], and therefore depends on the number of postsynaptic NMDA receptors in each spine. We have calculated previously how the number of postsynaptic NMDA receptors determines the mean and variance of calcium transients in the postsynaptic density [Yeung et al., 2004], and how this alters the shape of plasticity curves [Shouval and Kalantzis, 2005]. However, the number of postsynaptic NMDA receptors in the postsynaptic density is not well known. Anatomical methods for estimating the number of NMDA receptors [Takumi et al., 1999, Racca et al., 2000] produce estimates that are very different than those produced by physiological techniques [Nimchinsky et al., 2004]. The physiological techniques are based on the statistics of synaptic transmission and it is difficult to experimentally estimate their precision. In this paper we use stochastic simulations in order to test the validity of a physiological estimation technique based on failure analysis. We find that the method is likely to underestimate the number of postsynaptic NMDA receptors, explain the source of the error, and re-derive a more precise estimation technique. We also show that the original failure analysis as well as our improved formulas are not robust to small estimation errors in key parameters.

1 Introduction

A large contribution to the variability of calcium transients in spines might arise from the small number of postsynaptic NMDA receptors. Anatomical methods using electron microscopy (EM) and tagging of receptors so they can be identified, have produces estimates of 10-20 NMDA receptors [Takumi et al., 1999, Racca et al., 2000], whereas a physiological method produced the estimate of 1-3 receptors open at each presynaptic stimulus [Nimchinsky et al., 2004]. It is actually hard to directly compare these two methods because the anatomical techniques do not tell us what fraction of the receptors are not labelled, how many of the labelled receptors are functional, and what fraction of the functional receptors are open at each event. It would seem therefore that the more relevant number is given by the physiological techniques, if these techniques are indeed reliable.

One physiological method for estimating the number of postsynaptic NMDA receptors, which is called failure analysis, is based on the fraction of transmission failures [Nimchinsky et al.,

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2004]. Transmission failures occur due to two different reasons: first because of a presynaptic neurotransmitter release failure, and second because of a postsynaptic failure to open NMDA receptors. The more postsynaptic receptors there are in the spine the less likely is the occurrence of a postsynaptic failure given a release of neurotransmitter. Estimating the number of postsynaptic failures can tell us about the number or receptors.

In order to separate between pre and postsynaptic failures, Nimchinsky et al. (2004) suggested to use 3-(CR)-2-Carboxypiperazin-4-yl-propyl-1-phosphonic-acid (D-CPP), an NMDA channel blocker. The use of D-CPP will increase the number of postsynaptic failures without effecting presynaptic failures. Therefore, a comparison of the fraction of failures without D-CPP (f) and the fraction of failures with D-CPP (f') can help in estimating the number of postsynaptic NMDA receptors (M). The paper by Nimchisky et. al. provides a formula for extracting the number of NMDA receptors from the fraction of failures with and without D-CPP (see equation 1) below. We call this method differential failure analysis (DFA) because it uses the different number of failures under the two conditions in order to estimate the number of receptors.

In experimental systems there is no direct way to test the precision of this method. Therefore, in this paper we carry out stochastic simulations of synaptic transmission and receptor blocking by D-CPP. In this simulated system we have direct knowledge of the number of NMDA receptors and of NMDA receptors that open in each condition. Therefore we can use this system to test the precision of the failure analysis method. We find that it produced good estimates only for certain conditions. We next analyze this stochastic system, identify possible approximations that lead to the errors in the estimate. By taking into account the fluctuations in the number of receptors blocked by D-CPP, we develop a stochastic method of failure analysis (SFA). We compare the precision of the estimates obtained using both methods, show that SFA is more precise than DFA, show that DFA is a limiting case of SFA when fluctuations are small and therefore these two methods are in agreement in the case of a large number of receptors. However, estimation using SFA requires knowledge of an additional parameter. Additionally, we demonstrate that both methods are non-robust to small errors in the estimated parameters.

2 Material and Methods

We simulated transmission through stochastic NMDA receptors using Markov models of NMDA receptors with parameters obtained from fitting experimental data [Benveniste and Mayer, 1991, Sigworth, 1980, Destexhe et al., 1994].

The complete model of the NMDA receptor has in total eight states (Fig. 1), including states that represent its binding to the neurotransmitter glutamate or the D-CPP (Fig. 2a).

We used the following kinetic rates: $k_1 = 10(mM \cdot msec)^{-1}$, $k_{-1} = 4.7 \cdot 10^{-3} msec^{-1}$, $k_2 = 5(mM \cdot msec)^{-1}$, $k_{-2} = 9.4 \cdot 10^{-3} msec^{-1}$, $k_3 = 46.5 \cdot 10^{-3} msec^{-1}$, $k_{-3} = 91.6 \cdot 10^{-3} msec^{-1}$, $k_d = 8.4 \cdot 10^{-3} msec^{-1}$, $k_{-d} = 1.8 \cdot 10^{-3} msec^{-1}$. For the binding of DCPP we implemented three additional states with the following kinetic rates : $k_4 = 7.2 \cdot 10^{-3}(mM \cdot msec)^{-1}$, $k_{-4} = 1.1 \cdot 10^{-3} msec^{-1}$, $k_5 = 3.6 \cdot 10^{-3}(mM \cdot msec)^{-1}$, $k_{-5} = 2.2 \cdot 10^{-3} msec^{-1}$, $k_6 = 7.5(mM \cdot msec)^{-1}$, $k_{-6} = 9.4 \cdot 10^{-3} msec^{-1}$, $k_7 = 1.1 \cdot 10^{-3}(mM \cdot msec)^{-1}$, $k_{-7} = 3.6 \cdot 10^{-3} msec^{-1}$.

The release of Glutamate was simulated as a binary process, with a certain probability of release, usually set here to 0.5. For the estimation of the failure probability the concentration of the Glutamate was simulated by a step function of amplitude $1mM$ and duration 0.1 msec. The duration of Glutamate used in these simulations is shorter than that measured indirectly in cultures [Clements et al., 1992, Clements, 1996, Diamond and Jahr, 1997]. However, using those parameters would result in almost no postsynaptic failures of release, in contrast to

experimental results that indicate that in slices NMDA receptor responses are not saturated by a single release of glutamate [Mainen et al., 1999, Nimchinsky et al., 2004]. We calibrated the Glutamate dynamics on the experimental results of Mainen et al. (1999) who estimated that at most 56% of NMDA receptors are bound by a single synaptic release event.

We used a simple stochastic algorithm with a fixed time step dt = 0.01 msec (see Appendix), implemented in Matlab (The MathWorks, Natick, MA). Comparing our results with a smaller time step we found that 0.01 ms was sufficient to capture accurately the variability of our system.

The fraction of bound NMDA receptors at the steady state was estimated numerically from the model of the NMDA receptors of Fig. 1. Fig. 2b shows two examples of the transition of the NMDA receptors to the open state as well as their average. The probability that the receptor occupy the open state at time t is smaller when we apply the D-CPP, as was expected.

3 Results

3.1 Failure analysis applied to simulations of synaptic transmission

Using a realistic biophysical model for the NMDA receptors we tested the DFA method for estimating the number of open NMDA receptors during synaptic release of Glutamate. We carried out stochastic simulations for a small number of postsynaptic NMDA receptors, by implementing a stochastic Markov model for the NMDA receptors as shown in Fig. 1. Synaptic transmission parameters were chosen to produce results that are consistent with experimental results (methods). Simulation methods are discussed in the methods section and appendix B.

When we simulated the binding of D-CPP with the receptors we integrated the system for 4 sec before applying the Glutamate signal so the system would reach a steady state with the antagonist before the simulated glutamate release.

In figure 3a we see a histogram of the number of NMDA receptors that opened over 2000 simulations in the absence of D-CPP. The total number of NMDA receptors in that example was 6. In Fig. 3b we display the same type of histogram in the presence of a low level of D-CPP, which blocks on average 36% of NMDA receptors, and in Fig. 3c a higher level of DCPP is used which blocks on average 60% of receptors. In the presence of D-CPP a greater number of transmission failure occur (n = 0); due do to more postsynaptic failures.

The DFA method [Nimchinsky et al., 2004] is based on the following formula:

$$\frac{1-f}{1-e^{-n}}=\frac{1-f'}{1-e^{-nr}} \tag{1}$$

which is used to estimate the average number of NMDA receptors, n, that open for each presynaptic stimulation. The variables f and f' are the fraction of transmission failures with and without D-CPP respectively and r is the relative fraction of NMDA receptors not blocked by D-CPP.

From the simulations we count the fraction of total failures with different concentrations of D-CPP. Using the DFA method (eq. 1) we estimated the average number of NMDA receptors which open at least once and we compare these results with the results from our simulations. Applying DFA to the number of failures in our simulations we found that n = 0.2615 when the level of D-CPP is low and n = 0.3197 when it is high. However because we know n from our simulations we found that the average is $< n >$= 0.5226. Therefore the use of failures produces

underestimates for both low and high DCPP concentrations of 55% and 44% of the true value respectively. It is feasible that DFA may provides correct estimates for a different set of parameters, however as we show in our following analysis, the DFA makes errors in a more systematic way. As we shall explain, the main reason for those errors, is the fact that it ignores the fluctuations of the number of bound receptors with the D-CPP.

In the following section we will derive a formula for estimating the number of receptors using failure analysis, estimate its precision and explain the origin of the errors in the DFA method and its resulting estimates.

3.2 Analytical estimates of the validity and precision of failure analysis

Failures of synaptic transmission arise either from failure of neurotransmitter release or from a failure to open any of the postsynaptic receptors given that there was a release. In order to distinguish the two sources of failure, failures are monitored with and without D-CPP, and agent that blocks the NMDA receptors. The application of D-CPP should not alter the failure of release but will change the failures of opening any of the postsynaptic NMDA receptors. The key difference between the following derivation and the original DFA is that we take into account that blocking NMDA receptors by D-CPP is itself a stochastic process and as a consequence the number of NMDA receptors available at each synaptic event can vary. Because we take into account the stochastic fluctuations in the number of blocked receptors we call the formulas resulting from this derivation SFA. In the simulation of the previous section the two stochastic processes, receptor opening and blocking by D-CPP, are coactive. Throughout the analysis herein we assume that blocking of NMDA receptors by D-CPP occurs at a much slower time scale than synaptic transmission. We also assume that stimulation occurs at a frequency significantly lower than the time scale of D-CPP dynamics, so that each stimulus can be considered as an independent draw from the distribution of NMDA receptors not blocked by D-CPP.

The probability of failure without D-CPP is:

$$f=(1-P_r)+P_r(1-P_o)^M=1-P_r(1-e^{-\mu M})=1-P_r(1-K(M))$$

where f is the fraction of failures, P_r the probability of release, P_o is the opening probability of a single receptor, given that there was release, $\mu = -log(1-P_o)$ and $K(M) = e^{-\mu M}$. For $P_o \ll 1$ $\mu \approx P_o$ and $K(M) = \exp(-n)$ where n is the number of NMDA-R open on average since $n = M \cdot P_o$.

For the case blocked by D-CPP there is a distribution of the number of NMDA receptor unbound to D-CPP (M') described by the distribution function $P(M')$.

Therefore the average number of failures in the blocked case has the form:

$$f'=(1-P_r)+P_r\sum_{M'=0}^{M}P(M')(1-P_o)^{M'}=1-P_r\left(1-\sum_{M'=0}^{M}P(M')e^{-\mu M'}\right)=1-P_r(1-K'(M))$$

where f' is fraction of failures in the presence of D-CPP, and

$$K'(M)=\sum_{M'=0}^{M}P(M')\exp(-\mu M').$$

Therefore:

$$\frac{1-f}{1-K(M)}=\frac{1-f'}{1-K'(M)} \tag{2}$$

This equation is a correction of equation 1. If $P(M')=\delta(M'-rM)$ then this equation is equivalent to equation 1 with the conventions that $n=M\cdot\mu\approx M\cdot P_o$ and where r, the average fraction of NMDA-R unblocked by D-CPP, is estimated by the ratio of currents with and without D-CPP ($r=I'/I$), as suggested by Nimchinsky (2004).

This approximation, which essentially ignores the fluctuations in the number of blocked receptors, produces an equation with the simple form:

$$\frac{1-f}{1-e^{-\mu M}}=\frac{1-f'}{1-e^{-\mu rM}} \tag{3}$$

which can also be rewritten as

$$\frac{1-f}{1-e^{-n}}=\frac{1-f'}{1-e^{-nr}}$$

As the number of NMDA receptors increases, it is reasonable to expect that the relative fluctuations in the number of blocked NMDA receptors will decrease thus making the approximation leading to the equation above more exact.

Uniform Case—Given these different formulas, it is not clear how similar the equations are to each other for non delta function $P(M')$. We will now make the simple assumption that $P(M)$ is a uniform distribution around the mean $M_0=rM$ such with a range $M_0-aM_0<M'<M_0+aM_0$, where the parameter $a\leq 1$ defines the range. Note that for a uniform distribution $a=\sqrt{3}\sigma$, where σ is the standard deviation of the distribution of M'. We need to take care that $a\cdot M_0<M$ and the $P(M)$ is normalized. We will now replace the sum $K'(M)=\sum_{M'=0}^{M}P(M')\exp(-\mu M')$. by the integral an d use $P(M')=1/(2aM_0)$ between M_0-aM_0 and M_0+aM_0. Using this:

$$\begin{aligned}K'(M)&=\frac{1}{2aM_0}\int_{M_0(1-a)}^{M_0(1+a)}e^{-\mu M'}dM'\\&=\exp(-\mu rM)\frac{1}{aM_0\mu}\sinh(aM_0\mu)\end{aligned}$$

Using $M_0=rM$, and $n\approx M\mu$, this produces the following correction to equation 1:

$$\frac{1-f}{1-\exp(-n)}=\frac{1-f'}{1-\exp(-nr)\sinh(\mathrm{nra})/\mathrm{nra}} \tag{4}$$

The solutions of this equation differ from those of equation 1. However, as a which determines the variance of $P(M')$ approaches zero this result converges to equation 1. Again this is reasonable because as $a \rightarrow 0$ the uniform distribution approaches a δ function.

To assess the difference between this equation and the uncorrected equation we compared their solutions for two several sets of parameters. We show two examples (Fig 4) in which have estimated n, for two different parameter sets and for values of a between 0 and 1.

We see that although the results are of the same order of magnitude, they diverge as a increases and can differ by as much as a factor of 2 for large a. For the parameters shown here, ignoring fluctuations results in underestimates in the number of receptors. Thus for a small number of NMDA receptors, such that it is reasonable that a is large a formula that ignores the fluctuations in the fraction of receptors blocked would yield results that have the correct order of magnitude but can still significantly under estimate the number of receptors.

Binomial Case—The assumption of a uniform distribution of unbound NMDA receptors in the previous section provides enables us to understand intuitively how fluctuations in the number of unbound receptors affect the estimates. However, the shape of this distribution is arbitrary, and its width is not related explicitly to the number of NMDA receptors. We can improve this assumption and employ a more complex binomial expression for the distribution of unbound receptors, which is justified if we can assume a separation of time scales.

The time scales of the D-CPP binding dynamics are slower than the glutamate binding dynamics, therefore it is approximately possible to separate these time scales. We can therefore assume that during each synaptic transmission event each NMDA receptor is either bound or unbound to D-CPP (This approximation would have been better for receptors with faster dynamics than NMDA receptors). Under this assumption each NMDA receptor has the probability p_{cpp} to be bound to D-CPP, we assume that the receptors are independent and the stimulation frequency is low enough so that these probabilities are independent across consecutive trials. We can then assume that the probability of having M' receptors unbound to D-CPP given a total of M receptors is binomially distributed such that:

$$P(M')=(1-p_{\mathrm{cpp}})^{M'}p_{\mathrm{cpp}}^{M-M'}\begin{pmatrix} M \\ M' \end{pmatrix}$$

Therefore:

$$K'(M)=\sum_{M'=0}^{M}P(M')e^{-\mu M'}=\langle e^{-\mu M'}\rangle_{P(M')}$$

Therefore $K'(M)$ is the characteristic function of the binomial distribution. Therefore

$$K'(M)=[(1-p_{\mathrm{cpp}})e^{-\mu}+p_{\mathrm{cpp}}]^{M}=[r\cdot e^{-\mu}+(1-r)]^{M}.$$

We see however that K' is a function of M, μ and p_{cpp}. It is no longer simply a function of n, and therefore has no unique solution for n but instead many different solutions in which each allowed choice of M will result in a different μ. Lets assume we know $p_{cpp} = 1 - r$.

If we use the relation $\mu = -log(1 - P_o)$ we obtain that $K(M) = exp(-\mu) = 1 - P_o$ and that: $K'(M) = 1 - (1 - rP_o)^M$. Therefore we obtain that in the binomial case equation 2 takes the form:

$$\frac{1-f}{1-(1-P_o)^M} = \frac{1-f'}{1-(1-rP_o)^M} \tag{5}$$

How sensitive are the estimates of n to the choice of M? We have estimated the value of n for every integer choice of M between 1 and 30 given the binomial assumption, for which SFA should produce precise estimates. These results are compared with the estimate of n given from equation 1. Results comparing estimates from the two approaches are displayed in figure 5. For some values of M it is not possible to find a P_o such that equation 5 has a solution. This is obvious for the case $M = 1$ for which the solution becomes independent of P_o, but can also occur for values $M > 1$, because this would require values of P_o outside the physically possible range. In such cases we do not display a result for n and these cases are marked by + symbols on the x axis.

The results in figure 5 demonstrate that the DFA method is not precise. As in the uniform case the estimates converge and the variability in the number of blocked receptors is reduced. In the uniform case this occurs when the variability parameter $a \rightarrow 0$ and in the binomial case this occurs when the true number of receptors is large.

3.3 Comparison of the two methods for different NMDA receptor models

The previous analysis is based on the assumption that the opening of the NMDA receptors follows a binomial distribution. In order to verify the validity of the SFA method and compare the two estimation methods we simulate synaptic transmission using both a binomial model, for which our method should be exact as well as a more realistic Markov model for the NMDA receptors, that is based on experimental data (Fig. 1).

The SFA method (eq. 5) has two unknowns, the probability of opening P_o and the total number of NMDA receptors M. In order to test the validity of the estimation method we must assume that we know one of these. For the binomial receptor model we know exactly (in the simulations) the probability of opening given a release. In the following simulations we assumed a probability of release $P_r = 0.5$ and probability of opening $P_o = 0.15$. The probability of binding with D-CPP was either 0.65 (Fig. 6a) or 0.85 (Fig. 6b) and the number of the receptors M, was varied from 2 up to 20.

In Fig. 6 we compare the two methods for two different levels of D-CPP. These estimates are based on simulated data from 300,000 stochastic trials. The x axis is the number of the receptors in our simulations and the Y axis is ratio of the estimated number of receptors over the true number, which was a parameter of the simulations. In both cases (low and high D-CPP) the SFA estimation method (stars) is significantly more accurate than the DFA method. The DFA method results in large estimation errors for moderate blocking levels, however both methods converge as the number of the NMDA receptors increases.

These results are not surprising because our analysis was based on the binomial assumption. However, it is not clear how well this generalizes to more complex models of NMDA receptors. Therefore we compared the precision of the two methods for the more complex data driven model of NMDA receptors (Fig. 1). In order to apply the previous methodology for more complex models for the NMDA receptors, we need to calculate the probability of opening, or equivalently the probability that the NMDA receptor will not visit the open state even once. Solving the differential equations for the Markov diagram of figure 1, we find the probability

that the receptor occupy the open state at time t. For our purpose we shall follow a similar approach. We have made the assumption that during the release of Glutamate the receptor has occupied the C2 state and has not visited the open state. Since the kinetic rates for the transition from C1 to C2 is a function of the concentration of Glutamate we can treat the C1 state as a trap after the release of Glutamate. We have estimated the probability that the receptor will go to the closed state (or C1) without visiting even once the open state, given that at time t=0 it was at the state C2.

For this analysis we assume a small discrete time steps Δt. After n time steps the probability that the receptor return to state C2 without visiting the open state is given from by the following equation:

$$P_{C2}(n)=P_{C2}(n-1)\cdot(1-(k_{-2}+k_3+k_d)\cdot\Delta t)+P_D(n-1)\cdot k_{-d}\Delta t$$
$$P_D(n)=P_D(n-1)\cdot(1-k_{-d}\Delta t)+P_{C2}(n-1)\cdot k_d\cdot\Delta t$$

In the equations above, the first term on the right hand side represents the probability that there is no transition in the time interval Δt. The second term is the probability that the receptor during the time interval Δt will visit any neighbor state except the C1 or O.

Rearranging these equations and taking the limit $\Delta t \to 0$ we obtain:

$$\frac{d\overline{P}}{dt}=\begin{pmatrix} -(k_{-2}+k_3+k_d) & k_{-d} \\ k_d & -k_{-d} \end{pmatrix}\cdot\overline{P}$$

where

$$\overline{P}=\begin{pmatrix} P_{C2}(t) \\ P_D(t) \end{pmatrix}$$

The general solution of the above system two equations has the form (see Appendix) :

$$P_{C2}(t)=A_1\cdot e^{b_1 t}+A_2\cdot e^{b_2\cdot t} \tag{6}$$

For the kinetic rates that we have used in our simulations we find that $A_1 = 0.9963$, $A_2 = 0.0038$, $b_1 = -64.5\cdot 10^{-3}$, $b_2 = -1.6\cdot 10^{-3}$. Since we are interested in the probability of failure we have to multiply the above expression with the product $k_{-2}\cdot\Delta t$ and then integrate from $t = 0$ to $t = \infty$. Finally we find $P_{failure} = 0.1676$. So the probability that the receptor open at least once is $P_{success} = 1 - P_{failure} = 0.8326$

It is worth bearing in mind that an alternative way to estimate the probability of opening is the usage of stochastic simulations. Specifically for the kinetic rates of the NMDA receptor that we have used in our simulations and for duration of release 1 msec and amplitude 1 mM the probability of occupying the C2 state immediately after the release of Glutamate is 99.2%. The remaining 0.8% refers to the fraction of receptors that occupy the state C1. Note that the parameters of the glutamate dynamics do not effect transitions to or from the bound state and are therefore separable from the rest of the calculation. Using equation 6 we found that the

probability of opening is 0.8259. From our simulations we found that the probability of opening is 0.8182 (5000 trials, dt=0.0025 ms) and 0.8106 (5000 trials, dt=0.01 ms). These differ from the analytical estimates by less than 1% and 2% respectively.

In order to compare the two methods we used stochastic simulations. For the five states Markov model we calculated the probability of opening P_o. Since the kinetic rates are constant, we have multiplied the probability of success $P_{success}$ with the probability that the receptor occupy the state C2 immediately after the end of the Glutamate release. For a release duration of 0.1 msec that probability is 0.176. We make the assumption that the receptor has not visited the open state during the release, an assumption that is reasonable in our simulations (release duration: 0.1 msec).

Like in the experiments we estimated numerically the average number of blocked receptors with D-CPP before the release of Glutamate. Finally from the stochastic simulations we measured the fraction of failures with and without D-CPP, f' and f respectively Solving equations 1 and 5 we obtain the estimated number of receptors from the two methods. Figure 7 shows results from 6,000 simulation trials. In figure 7a the concentration of D-CPP is low producing a fraction of unbound receptors $r = 0.34$ and in Figure 7b D-CPP is high, resulting in $r = 0.2548$. Results indicate that SFA produces better estimates of the number of receptors, however both methods converge as the number of the NMDA receptors increase. Both methods produce better results with a higher concentration of D-CPP.

4 Robustness of the two methods

In order to estimate the number of receptors we need to experimentally measure key parameters such as f, f' and r. However, it might be difficult to estimate these precisely with the limited number of trials that is possible in experimental systems. Therefore we study how robust are these two methods given errors in the experimentally determined parameters.

In order to test how the results of these methods depend on statistical estimation errors of the different parameters we examined how small perturbations in one variable of the equations 1 and 5 affect the results of the estimated number of receptors. In that way we test how sensitive are the estimates of M to slight changes of the parameters measured from experimental data. The set of parameters that we used are the same as in the Fig. 5a. We have chosen to vary two parameters (Fig. 8), the failures f' when D-CPP is applied and the fraction of unblocked NMDA receptors r. The y-axis in both plots is the ratio of the estimated number of receptors using the perturbed variable over the initial estimation. We can see that possible small errors in the evaluation of these parameters ($\sim$ 5%) from experimental data may result to large errors to the estimated number of receptors ($\sim$ 250%).

Such errors in estimating the experimentally determined parameters are likely to be larger for a smaller number of repeated runs. In order to evaluate how errors depend on the number of trials, we used the data from our simulations of binomial receptors for high level of D-CPP ($r = 0.15$). We divided the total number of trials (300, 000) into smaller data subsets of specific size. For each group of a given trial number we estimated the coefficient of variation of the estimated number of receptors M. In Fig. 9a the x-axis is the size of each sample subset where as the y-axis is the coefficient of variation for M. We see that even when the sample size is 2000 trials we still have large normalized variability in the estimated number of NMDA receptors, which tend to decrease as we keep increasing the sample size.

Using the same data subsets we examined if the DFA and SFA methods produce a biased estimate (Fig. 9b). For a small data sample both methods overestimate the number of the receptors. This can be explained from the results of Fig. 8a. In our simulations the probability

of opening P_o and the fraction of blocked receptors r is predefined. Consequently the only statistical errors arrive from our evaluation of the failures f and f' from the simulated data. The smaller is the data size the larger are the expected fluctuations of the estimates. Small errors in the evaluation of the parameter f′ result in large discrepancies for the estimated number of receptors and these errors are not symmetric (Fig. 9a). Therefore averaging over a range of $\delta f'$ results in biased estimations of the number NMDA receptors. These results demonstrate that both methods are highly sensitive to errors of the estimated values of f, f' and r and consequently the these estimates depend on the size of the experimental data.

5 Discussion

The number of NMDA receptors at a synaptic spine determines both the magnitude of calcium influx into synaptic spines as well as its variability. Physiological and anatomical methods for estimating this number yield very different results. However, these numbers cannot be simply compared because they actually measure different variables. Anatomical methods estimate the number of actual receptors at a spine whereas physiological methods measure the number of functional receptors at a spine. For most applications the physiological number is more interesting because it is more directly connected to the charge and calcium influx into the synaptic spine. In this study we set out to test the precision and sensitivity of one physiological method, DFA, which is based on measuring the fraction of transmission failures. The DFA method, is based on the usage of a NMDA receptor antagonist, D-CPP, which increases failure of postsynaptic opening without affecting release in order to differentiate between failures of release and opening.

Using simulations of synaptic transmission, we have shown that the DFA method [Nimchinsky et al., 2004] typically underestimates the number of functional NMDA receptors. We set the parameters of the NMDA receptors and the glutamate release on the basis of previous experimental studies [Benveniste and Mayer, 1991, Sigworth, 1980, Mainen et al., 1999, Clements et al., 1992, Nimchinsky et al., 2004], as explained in detail in the methods section. [Clements et al., 1992]. Therefore, it is likely that the errors reported based on this simulated test-bed would apply to the experimental systems as well. However, more generally, these errors independent of the parameter choice are sufficient to show that the method is not correct. Additionally, we carried out analysis to explain the origin of the errors of the DFA method. We carried out analysis and simulations to show the DFA method is not correct even for the binomial assumption of NMDA receptor, from which it is derived. We show that the DFA method implicitly ignores the fluctuations in the number of postsynaptic receptors blocked by D-CPP, and that this is one major contributing component to the errors generated by this method. In order to take these fluctuations into account we derive a new set of equations (SFA) which take into account the fluctuations in the number of NMDA receptors blocked by the antagonist. Using this method we obtain significantly better estimates for the number of NMDA receptors when the number of receptors is small. We show that SFA converges to DFA when the relative fluctuations of blocked receptors are small, or equivalently when the number of receptors is large. However, when there are many receptors both of these method often fail because it is hard to get any postsynaptic transmission failures.

The DFA method can be misleading, and is likely to produce erroneous results. However, it has the advantage that no additional parameters are necessary for obtaining an estimate of the number of receptors. The SFA method, although more precise does require an additional parameter, the opening probability given a release event. Although this parameter can be obtained from single channel types of experiments, as we have demonstrated here; errors in this estimate will adversely effect the precision of the method.

Finally we have shown that both DFA and SFA are not robust to errors in their experimentally measured parameters. Therefore, both of these methods are likely to produce estimates that are significantly different than the true number of functional receptors unless it is possible to very extensively stimulate each synapse in order to get very reliable statistical estimates. If such extensive statistics are available and if one can trust the external parameters used such as the probability of opening, then the SFA method is indeed better than the DFA method.

Appendix

A General form for the probability of failure

The probability of returning to the trap state C1 is given by the following equation:

$$\frac{d\overline{P}}{dt}=\begin{pmatrix} -(k_{-2}+k_3+k_d) & k_{-d} \\ k_d & -k_{-d} \end{pmatrix}\cdot\overline{P}$$

The general form of the eigenvalues and the eigenvectors of the above transition matrix is the following:

$$\lambda_1=\tfrac{1}{2}\cdot\left(-\alpha-\beta-\sqrt{\alpha^2-2\alpha\beta+5\beta^2}\right)$$
$$\lambda_2=\tfrac{1}{2}\cdot\left(-\alpha-\beta+\sqrt{\alpha^2-2\alpha\beta+5\beta^2}\right)$$
$$\overline{u_1}=\begin{pmatrix} -\frac{\alpha-\beta+\sqrt{\alpha^2-2\alpha\beta+5\beta^2}}{2\beta} \\ 1 \end{pmatrix}$$
$$\overline{u_2}=\begin{pmatrix} -\frac{\alpha-\beta-\sqrt{\alpha^2-2\alpha\beta+5\beta^2}}{2\beta} \\ 1 \end{pmatrix}$$

where $\alpha = (k_{-2} + k_3 + k_d)$ and $\beta = k_{-d}$. The solution has the following form:

$$\overline{P}=c_1e^{\lambda_1 t}\cdot\overline{u_1}+c_2e^{\lambda_2 t}\cdot\overline{u_2}$$

where the coefficients c_1 and c_2 can be found by the initial conditions. The probability of failure of opening is given by the following integral :

$$P_{\text{failure}}=\int_0^\infty k_{-2}\cdot c_1e^{\lambda_1 t}\cdot\overline{u_1}(1)+c_2e^{\lambda_2 t}\cdot\overline{u_2}(1)\,dx$$

The same methodology can be applied even for more complex Markov models of the NMDA receptor.

B Pseudo code for fixed time step stochastic algorithm

Initialization

T=total time of simulation

Dt=time step

Total number of NMDA receptors = N

state vector of NMDA = $N_{state}[1, N]$

Define states C,C1,C2,D,O

Define rates for transitions: K_{tr} = [RCC1 RC1C RC1C2 RC2C1 RC2D RDC2 RC2O ROC2]

Define probabilities for transition in time step dt $P_{tr} = K_{tr}*dt$

for i = 2:dt:T

$r_{tran} = rand(1, N)$

$N_{state}(find(N_{state} = C \,\&\, r_{tran} < P_{tr}(1, 1)) = C1$

$N_{state}(find(N_{state} = C1 \,\&\, r_{tran} < P_{tr}(1, 2)) = C$

$N_{state}(find(N_{state} = C1 \,\&\, r_{tran} \geq P_{tr}(1, 2) \,\&\, r_{tran} < P_{tr}(1, 2) + P_{tr}(1, 3)) = C2$

$N_{state}(find(N_{state} = C2 \,\&\, r_{tran} < P_{tr}(1, 4)) = C1$

$N_{state}(find(N_{state} = C2 \,\&\, r_{tran} \geq P_{tr}(1, 4) \,\&\, r_{tran} < P_{tr}(1, 4) + P_{tr}(1, 7)) = O$

$N_{state}(find(N_{state} = C2 \,\&\, r_{tran} \geq P_{tr}(1, 4) + P_{tr}(1, 7) \,\&\, r_{tran} < P_{tr}(1, 4) + P_{tr}(1, 7) + P_{tr}(1, 5)) = D$

$N_{state}(find(N_{state} = O \,\&\, r_{tran} < P_{tr}(1, 8)) = C2$

$N_{state}(find(N_{state} = D \,\&\, r_{tran} < P_{tr}(6)) = C2$

end

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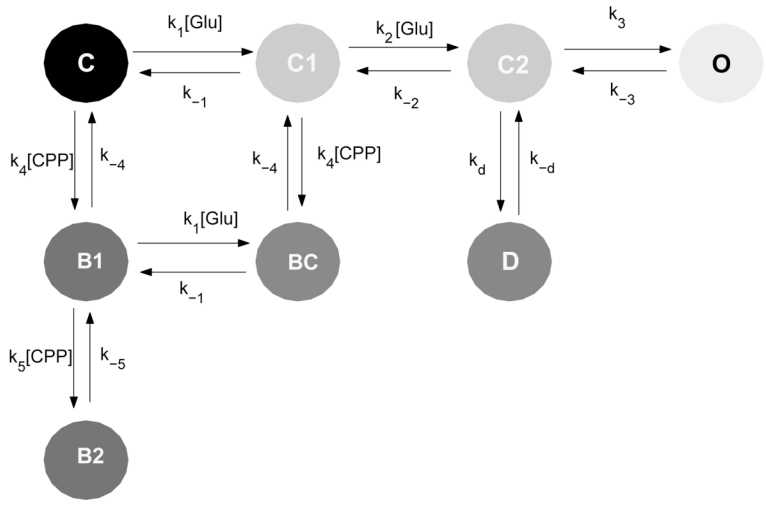

Fig. 1.

5-state Markov model for the NMDA receptor. Initially the receptors occupy the closed state C. The two binding sites of Glutamate or CPP are represented by the C1 and C2 states, whereas the open state is represented by the O, the desensitized by the D and the two binding sites for the antagonist by B1 and B2. After the release of Glutamate we may have transitions between the C2 and O or D states but not between C1 and C2.

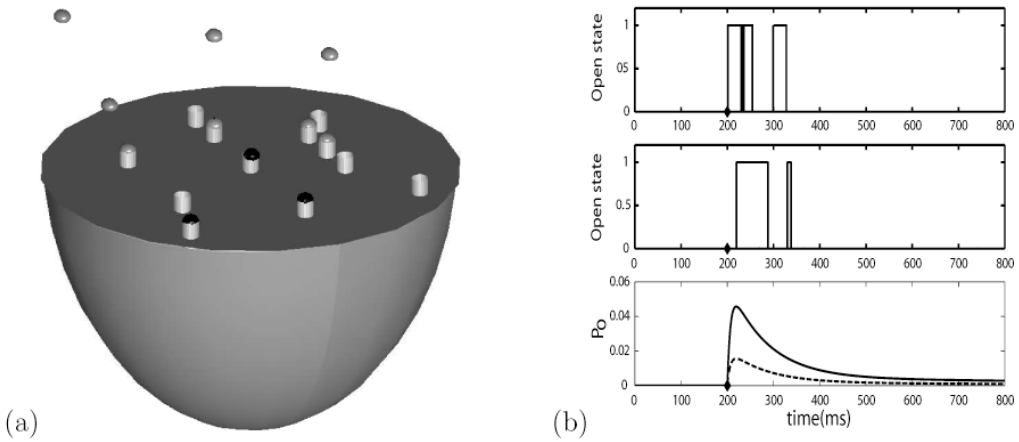

Fig. 2.

Stochastic synaptic transmission. (a) Postsynaptic spine: Both D-CPP (black spheres) and Glutamate (gray spheres) can bind with the NMDA receptors (gray cylinders) in a stochastic way. (b) Stochastic transmission through the NMDA receptors. The upper two panels show examples of stochastic transition to the open state of a simulated NMDA receptor. In the bottom panel the average probability of being in the open state, with (dashed line) and without (solid line) D-CPP is plotted. The lower probability of opening with D-CPP is due to the increased failures of opening since a fraction of NMDA receptors are blocked. The diamond point on the x axis denotes the time of release of Glutamate.

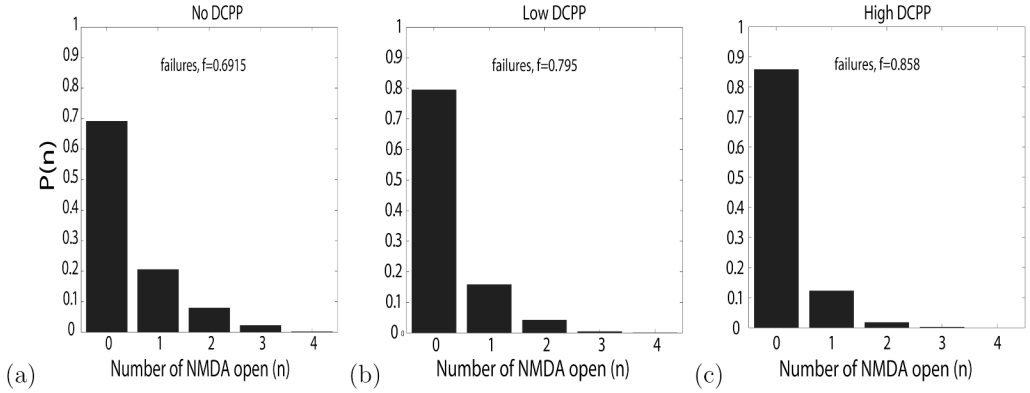

Fig. 3.

Histograms from stochastic simulations of the number of open NMDA receptors *n* for different levels of D-CPP. By applying the DFA formula 1 to the failures in our simulations we found that by using equation 1 we underestimate the average number of open NMDA receptors for both low and high D-CPP concentrations of 55% and 44% of the true value respectively.

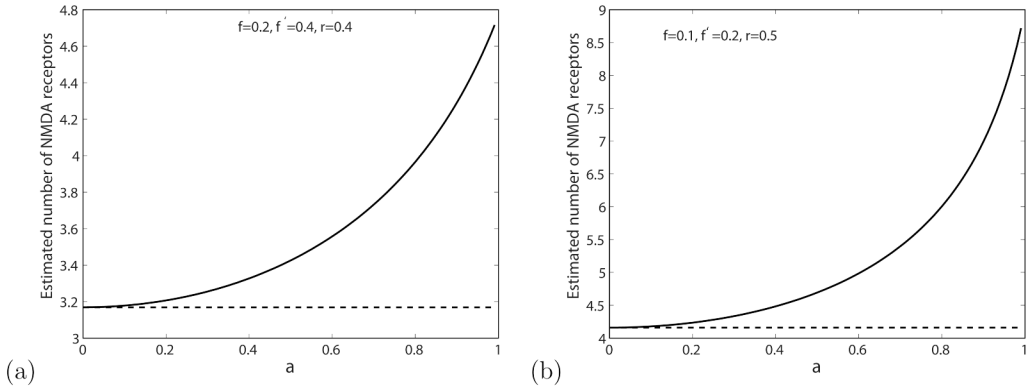

Fig. 4.

Difference between number of receptors calculated by the DFA (dashed line) formula and the corrected formula (equation 4) which assumes a uniform distribution (solid line) of blocked receptors. These are plotted as a function of *a* which characterized the width of the distribution.

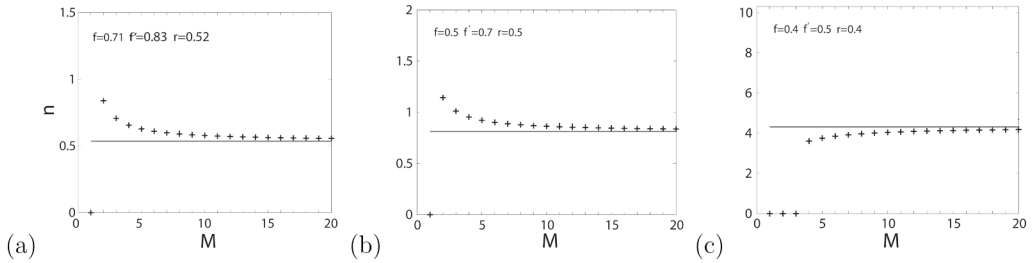

Fig. 5.

Estimates of the number of open NMDA receptors *n* depend on the number of total receptors *M*. Three examples comparing the estimate of *n* from the DFA method with the SFA method. The flat solid line is the estimate according to DFA, + symbols represent the estimate according to equation 5. The different plots are for different values of *f*, *f*′ and *r*. (a) *f* = 0.71, *f*′ = 0.83, *r* = 0.52, for *M* = 2 the estimate using the binomial distribution is 56% higher than the zero variance estimate. (b) *f* = 0.5, *f*′ = 0.7, *r* = 0.5, here the estimate at *M* = 2 differs by 41%. c. *f* = 0.4, *f*′ = 0.5, *r* = 0.4, here larger estimates for *n* are obtained and they are relatively independent of *M*. For large *M* or parameters that result in a larger estimate of *n* (*n* > 3) the zero variance result is a good approximation.

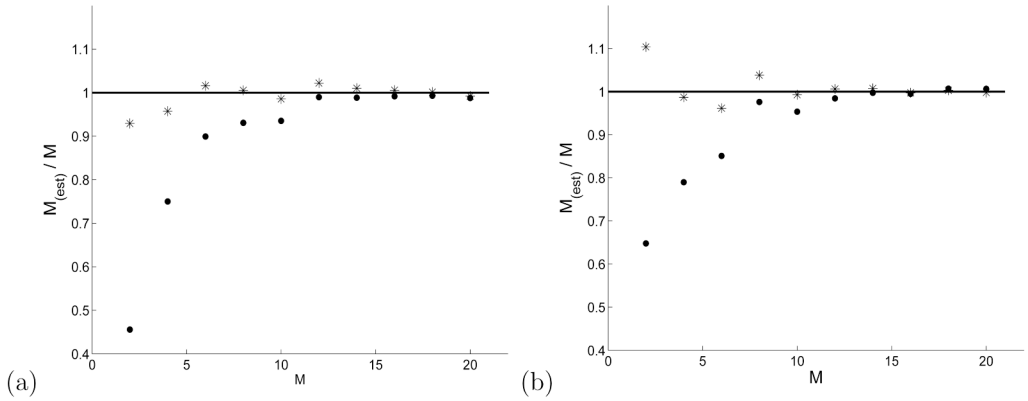

Fig. 6.

Comparison of the precision of the DFA (solid line) and SFA (stars) methods, using a binomial NMDA receptor model. The Y axis of these two plots is M_{est}/M. where M_{est} is the number of receptors estimated by failure analysis and M is the true number of receptors. This therefore shows the average precision of the estimate with 1 being totally precise. Two different levels of D-CPP were used producing different levels of blocked NMDA receptors. (a) The fraction of unblocked receptors is $r = 0.35$. (b) The number of unblocked receptors is: $r = 0.15$. In both cases DFA underestimate the number of receptors if their number is small whereas SFA produces improved estimates. The two methods agree as the number of NMDA receptors increases.

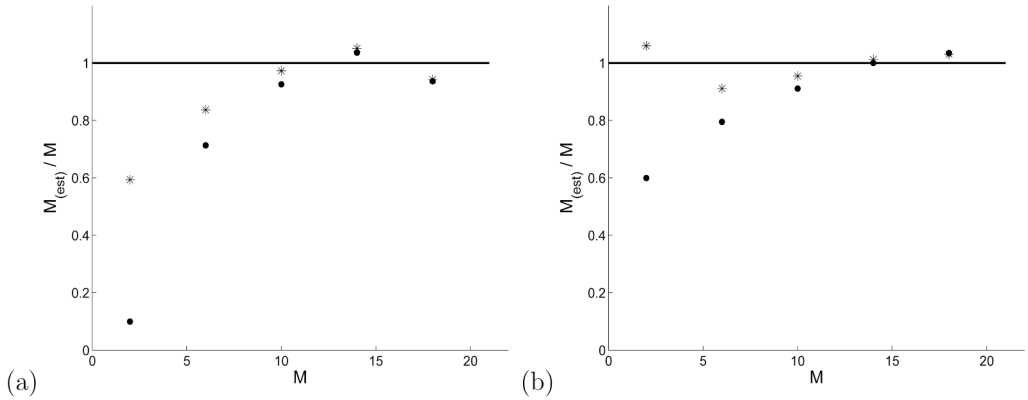

Fig. 7.

Comparison of the DFA and SFA estimation methods, using a Markov model for the NMDA receptors. The y-axis is the ratio of the estimated number of NMDA receptors over the one that was used in the simulations. Duration of Glutamate release is 0.1 ms and probability of release 0.5. The concentration of D-CPP is 0.22 mM for the left panel and 0.3 mM for the right panel.

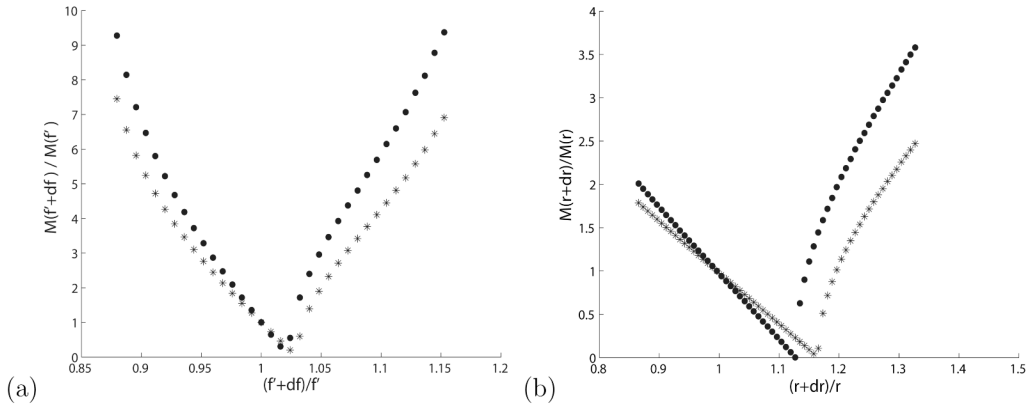

Fig. 8.

Sensitivity of DFA (dots) and SFA(stars) when small perturbations applied on one variable. (a) Small changes of the relative fraction of failures with D-CPP ($f' \rightarrow f' + df'$) result in large deviations of the estimated number of receptors *M*. (b) Small deviations in the relative fraction of unblocked receptors result in large errors in estimating *M*. We see that both methods are highly sensitive. For instance an error of 5% in the estimation of the failures f' results in an error (~ 250%) in the estimated number of NMDA receptors M.

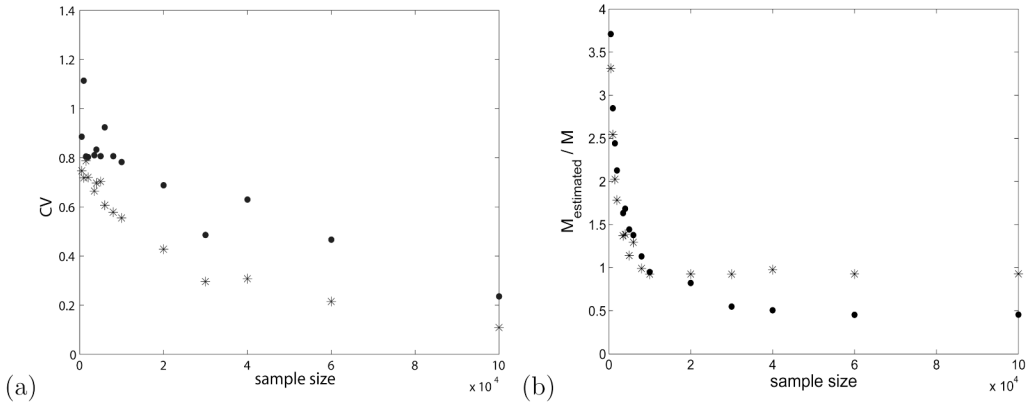

Fig. 9.

Dependence of the estimated number of receptors on the data set size. The x-axis shows the number of trials for each subset. (a) Normalized variability of *M* for both methods (DFA circles, SFA stars) for different sizes of data sets. For small number of trials the estimated number of NMDA receptors show high variability for each subset. (b) Biased estimates of M as a function of the sample size. For small size of data sets both methods overestimate M. Increasing the sample size the DFA method underestimate M where as the SFA method converge to the correct number.