Facilitation at the Lobster Neuromuscular Junction: A Stimulus-Dependent Mobilization Model

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Worden, Mary Kate, Maria Bykhouvskia, and John T. Hackett. Facilitation at the lobster neuromuscular junction: a stimulus-dependent mobilization model. J. Neurophysiol, 78: 417-428, 1997. Frequency facilitation is a process whereby neurosecretion increases as a function of stimulation frequency during repetitive synaptic activity. To examine the physiological basis underlying facilitation, we have estimated the frequency dependence of the synaptic parameters n (number of units capable of responding to a nerve impulse) and P (average probability of responding) at the lobster neuromuscular junction. Both n and P increase as a function of frequency, suggesting that the efficiency of quantal docking and quantal fusion is regulated by repetitive synaptic activity. In experiments in which facilitation is strong and quantal content does not saturate over the frequency range tested, the value of P saturates at low frequencies of stimulation, and increases in quantal content at higher frequencies of stimulation are due to an increase in n. Therefore the value of P does not limit facilitation. We propose that transmitter release is limited by the rates of quantal mobilization and demobilization, and that each excitatory stimulus causes additional mobilization of quanta to dock at the presynaptic release sites. In such a model the binomial parameter n will correspond to the number of quanta docked at the release sites and available for release. We have developed and solved kinetic equations that describe how the number of docked quanta changes as a function of time and of stimulation frequency. The stimulus-dependent mobilization model of facilitation predicts that the reciprocal value of the quantal content depends linearly on the reciprocal product of the stimulation frequency and the probability of release. Fits of the experimental data confirm the accuracy of this prediction, showing that the model proposed here quantitatively describes frequency facilitation. The model predicts that high rates of quantal demobilization will produce strong frequency facilitation.

INTRODUCTION

Facilitation of synaptic transmission is a process whereby the efficacy of neurosecretion increases as a function of repetitive stimulation. Frequency facilitation is a form of short-term plasticity that is induced during brief trains of repetitive stimuli and lasts only a few hundred milliseconds after the cessation of stimulation (Magleby 1979; Zucker 1974, 1977). Even though short-term or frequency facilitation rapidly decays, its activation may be important for the initiation of other forms of synaptic plasticity induced by longer stimulation protocols, such as augmentation and potentiation (Kamiya and Zucker 1994) and long-term facilitation (Sherman and Atwood 1971).

At both invertebrate and vertebrate neuromuscular junctions, the process of facilitation is entirely presynaptic in origin (del Castillo and Katz 1954b; Dudel and Kuffler 1961) and is due to an increase in quantal content (m). del Castillo and Katz (1954a) first suggested that m is the product of the number of units capable of responding to a nerve impulse (n) and the average probability of responding (P). Johnson and Wernig (1971) observed that transmitter release at crustacean neuromuscular synapses follows binomial statistics, measured values for n and P, and suggested that the binomial parameter n may represent the number of releasable quanta, with each quantum having a probability P of fusing with the presynaptic membrane and releasing neurotransmitter into the synaptic cleft. The fact that synaptic output could be described by binomial (rather than Poissonian) statistics suggested that some factor limits the number of releasable quanta. Zucker (1973, 1977) proposed that the limiting factor might be the finite number of release sites in the presynaptic terminal, each of which may release a single quantum per impulse, and that the number of release sites corresponds to the synaptic parameter n. These conclusions were based on a quantal analysis of paired pulse and frequency facilitation in the crayfish in which the probability of quantal release P increased directly with stimulation frequency, whereas the synaptic parameter n did not change (Zucker 1973). However, the amount of facilitation was sufficiently small that estimates of n became unreliable because of large SE estimations. In studies in which higher stimulation frequencies were employed, Wernig (1972) and Wojtowicz et al. (1994) observed increases in the values of both n and P; however, there were few experimental observations and no statistical evaluation of the significance of changes in the quantal parameter values.

Because of the discrepancies in the literature, it is important to reinvestigate whether under steady-state conditions the presynaptic quantal parameters n and P both vary as a function of stimulation frequency. If the parameter n increases during facilitation, it becomes necessary to reevaluate the physical interpretation of n. One possibility is that within the nerve terminal there are a number of release sites, each with a specific value of P, and that some of these sites are inactive (have values of P approaching 0) at low stimulation frequencies. The value of n obtained from statistical analysis will therefore represent only the subset of release sites defined as “active,” with values of P sufficiently larger than zero. Under conditions of repetitive stimulation,
the value of $P$ may increase at some release sites, including previously inactive sites, thereby increasing the value of $n$ as previously “silent” synapses are recruited (Wojtowicz et al. 1991). More recently, Wojtowicz et al. (1994) have further refined this model to suggest that $n$ represents the number of complex synapses (those with $\geq 2$ dense bodies). According to this model, complex synapses are preferentially active at low frequencies of stimulation, and the recruitment of silent synapses into the active pool at high frequencies of stimulation occurs as the probability of release at simple synapses (those with 0 or 1 dense body) increases and the simple synapses undergo morphological conversion into complex synapses.

The assumption underlying each of these models of facilitation is that the number of quanta released is limited by the number of active release sites. However, an alternate possibility is that the synaptic output is limited not by the number of release sites but by the rates of quantal mobilization and demobilization (Elmqvist and Quastel 1965; Maeno and Edwards 1969). According to this alternate model, the value of $n$ may increase with facilitation as more quanta become mobilized to the release sites and thereby become “releasable,” defined as being properly positioned at the release sites (Docked) and able to undergo fusion. In this case, the value of $n$ is not related to the number of release sites, but will be an approximation of the number of docked quanta under the recording pipette.

In this paper, we consider how these different models of quantal release may apply to frequency facilitation at the lobster neuromuscular junction. The distinction between the models of facilitation described above is important because if $n$ represents the number of docked quanta (rather than the number of release sites), estimation of $n$ could be used to measure the processes of mobilization and quantal docking within the nerve terminal. Such a physiological assay for mobilization and docking would be an important tool for examining the function of presynaptic proteins that may function to guide quanta to the release sites or act as part of the fusion machinery (Greengard et al. 1993; Rothman 1994; Sudhof and Jahn 1991).

To systematically reexamine how the distribution of $m$ and the synaptic parameters $n$ and $P$ depend on the stimulation frequency during facilitation, we have undertaken a statistical analysis of quantal transmitter release with the use of a binomial model. One consideration in applying binomial statistics to the process of neurotransmission is the potentially significant problem of the nonuniformity of $P$ at different presynaptic release sites (Hatt and Smith 1976). Brown et al. (1976) pointed out that if the physiological recordings are made from several release sites simultaneously, each with a different value of $P$, the spatial variance in $P$ due to nonuniformity could result in overestimation, or biasing, of the average value of $P$. In addition, temporal variance in $P$ will also confound binomial analysis. More recently, Miyamoto (1986) suggested an experimental method in which the possible temporal variance in $P$ is reduced by eliminating from analysis any time periods during which quantal content is nonstationary. In addition, Miyamoto (1986) addressed the problem of spatial variance in $P$ by developing a mathematical method for calculating “unbiased” estimates of $P$ with the use of higher moments of the binomial distribution to estimate the magnitude of the spatial variance in $P$. Another approach to estimating average values of nonuniform $P$ includes fitting experimental and nonuniform binomial distributions by expectation-optimization technique (Smith et al. 1991; Wojtowicz et al. 1991). Finally, an approach assuming that the nonuniform probability of release is described by beta distribution has been developed (Dityatev et al. 1992). This method is applicable where the number of active release sites is sufficiently large.

Three different statistical methods (simple binomial, compound binomial, and Miyamoto’s) of quantal analysis were used in this study to test the extent to which both synaptic parameters $n$ and $P$ are frequency dependent.

**METHODS**

**Electrophysiological recording and quantal detection**

Lobster (*Homarus americanus*, Milne Edwards) walking legs were dissected in chilled saline (composition, in mM: 462 NaCl, 16 KCl, 26 CaCl$_2$, 11 glucose, and N-2-hydroxyethylpiperazine-$N'$-2-ethanesulfonic acid buffer, pH adjusted to 7.4). The dorsal surface of the dactyl opener muscle in the propodite segment of the leg was exposed by removing the overlying exoskeleton and muscle. Innervating this muscle are an inhibitory axon and an excitatory axon that run separate courses in the large and small nerves of the propodite segment. After dissection, the preparation was pinned to Sylgard in the bottom of a chamber maintained at 6°C with a refrigerated circulator. To minimize nerve-evoked contractions, the distal cut end of the central apodeme of the dactyl opener muscle was attached to a thread and the muscle was stretched. With the use of this method, it was possible to stimulate at frequencies up to 15 Hz without movement artifacts.

Presynaptic action potentials were elicited by suprathreshold electrical stimulation of the excitatory nerve via a suction electrode. Stimulation trains at frequencies from 2 to 15 Hz were applied in a randomized order and $>600$ stimulus trials were applied at each stimulation frequency tested. Synaptic responses in central regions of the dactyl opener muscle were recorded extracellularly with saline-filled patch pipettes 12–25 μm diam (Stuhmer et al. 1983). Focal recordings were obtained by 1) lowering the patch pipette to the surface of the muscle in regions where fine branches of the nerve could be visualized under a dissecting microscope and 2) applying gentle suction to minimize the leak currents, thus improving the signal-to-noise ratio. Poststimulus currents corresponding to quantal release were amplified by a current-to-voltage converter (Axopatch-1D, Axon Instruments, Foster City, CA). The postsynaptic current responses were not recorded under voltage-clamp conditions. Recordings were digitized and stored on magnetic tape for later computer detection and analysis. To verify that augmentation was not induced by the repetitive stimulation protocols, we confirmed that facilitated quantal release decreased to control values (2-Hz values) within 0.5 s of the cessation of stimulus trains.

An original computer algorithm was used for off-line detection and analysis of quantal events (Bykhovskaya et al. 1996). The detection of a quantum is based on 1) differentiating the recorded signal; 2) excluding from analysis all monotonic regions of the first derivative that are either negative and approaching maximal negativity, or positive and approaching maximal positivity; and 3) examining all other regions of the differentiated signal to identify the peak of each quantal event. The onset of quanta was determined as the derivative became negative and met criteria of amplitude and duration. Peaks of quantal events are detected at the time points on the differentiated signal.
I. A ± C, top traces: regions of synaptic contacts. I. Values of 161 (± ± ±) in a single trial, where events (no infections present) were measured. Superimposition of estimates of C illustrates quantal detection of five successive quanta (Fig. 1). Accurate detection of one (Fig. 1C) by the computer algorithm. In Fig. 1, the signal-to-noise ratio exceeded 20:1.

Statistical analysis of quantal content

Each data set consisted of 600–1,000 stimulus-evoked trials at a constant frequency in which the number of quanta (m) released per trial was counted. The first 10–100 responses at each frequency were discarded and the remaining data were divided into subgroups containing 100 trials, each of which was tested for stationarity (Provan and Miyamoto 1993). Quantal content for each successive trial at two stimulation frequencies is shown in Fig. 2 to illustrate the stationarity of the first subgroups of data as well as the total data sets; these data are representative of all 10 experiments. The criterion for stationarity within a subgroup was that the slope of regression of the value of m as a function of stimulus trial did not differ from zero at a level of significance of 0.01. The criterion for stationarity between subgroups was that the synaptic parameters (see below) determined for each subgroup were not different from the means of the total data set at a level of significance of 0.01. Subgroups that did not meet the above criteria were determined to be nonstationary and were discarded to eliminate data in which quantal parameters might show temporal variance. In almost every experiment one or two subgroups were discarded as nonstationary; in every experiment at least five subgroups for each stimulation frequency passed the test for stationarity.

Three statistical methods were used in this study to be sure that the results obtained are independent of the method employed.

Simple binomial estimates. The simple binomial estimations (n and P, designated n0 and P0, respectively) were made from the first two moments of the distribution of m

\[ P_b = 1 - (\sigma^2/m(m)) \]  

(1)

and

\[ n_b = (m)/P_b \]  

(2)

Miyamoto’s method. Miyamoto’s method of quantal analysis (Miyamoto 1986) is based on the assumption that the probability of release at different release sites may be nonuniform, with each release site having a different probability of transmitter release. The value of P, assumed to be the average probability of release at all release sites, was estimated for each subgroup as a positive solution of the quadratic equation (Miyamoto 1986)

\[ (P)^2 - 2\gamma(1 - \sigma^2/m(m))P + (2(m) - 3\sigma^2 + M_3)/4(m) = 0 \]  

(3)

where M3 is the third central moment of the distribution of m. In cases where this equation has no real roots, its solution was approximated by the value of P for which the left part of Eq. 3 has the value closest to zero. The assumption underlying Eq. 3 is that the value of the third moment of the distribution of P is small (Miyamoto 1986). The value of n will be

\[ n = (m)/P \]  

(4)

Estimates of m, n, and P were calculated as means of all subgroups that passed the tests for stationarity.

Compound binomial statistics. The “compound” binomial estimations of m and P (designated n and P, respectively) were made by \( \chi^2 \) minimization procedure in space of \((n + 1)\) variables (Cooper et al. 1995; Smith et al. 1991). The values of n were considered in the range from \( n_b \) to \( n_{\text{max}} + 1 \), where \( n_{\text{max}} \) is the maximal quantal content observed in a data set. The initial values of \( P_1 \ldots P_n \) were taken to be equal to \( P_0 \) and then optimized.
to fit the predicted distribution of quanta to that determined experimentally. The predicted frequencies of observations of \( m \) quanta are

\[
P_m = \sum_{j=1}^{i} P_j \frac{m - j}{1 - P_j}
\]  

(5)

The value of \( n \) that gave the best fit of the predicted and experimental quantal content was taken as the compound binomial estimate \( n_c \). The compound binomial estimate \( P_c \) was calculated as an average of the corresponding set of \( P_1 \ldots P_n \)

\[
P_c = \frac{1}{n_c} \sum_{i=1}^{n_c} P_i
\]  

(6)

RESULTS

Frequency dependence of synaptic parameters \( m \), \( n \), and \( P \)

As reported previously by Dudel and Kuffler (1961), \( m \) increases as a function of stimulation frequency. In 10 experiments where quantal events were scored as a function of frequency, the mean value of \( m \) increased from 0.72 ± 0.41 (SD) quanta at 2 Hz to 1.78 ± 0.19 (SD) quanta at 15 Hz.

To test whether the choice of the statistical method is critical for the character of frequency dependence of synaptic parameters, values of \( n \) and \( P \) for the data in experiment I were estimated with the use of three statistical methods (see METHODS). Whereas the absolute values of \( n \) and \( P \) obtained by simple binomial statistics, compound binomial statistics, and the equations of Miyamoto (1986) differ, the character of the frequency dependence is identical with the use of all three methods (Fig. 3, A and B). The observation that compound binomial estimations of \( P \) are lower than Miyamoto’s estimations might suggest that Miyamoto’s assumption that the distribution of individual release probabilities is symmetrical underestimates the variation in \( P \). In addition, both Miyamoto’s and simple binomial estimations of \( n \) are smaller than the maximal number of quanta released in a

FIG. 2. Quantal content is stationary as function of stimulus trial. Data from experiment III. Initial responses collected at each stimulation frequency were discarded before analysis of remaining data; initial 50 responses were discarded from 2-Hz data set, initial 98 responses were discarded from 15-Hz data set. Data are fit by linear regression (—); slopes of regression are not different from 0 at level of significance of 0.5. Note that these data were obtained in an experiment showing strong facilitation and strong increase in value of \( n \) (number of units capable of responding to a nerve impulse; see Fig. 5). A: quantal content for 1st subgroup of 100 trials at 2 Hz; slope of regression (mean ± SD) was equal to \(-6.1 \times 10^{-4} \pm (1.4 \times 10^{-3}) \). B: quantal content for 1st subgroup of 100 trials at 15 Hz; slope of regression was equal to \(2.0 \times 10^{-4} \pm (2.2 \times 10^{-4}) \). C: quantal content for total data set of 700 trials at 2 Hz; slope of regression was equal to \(-2.6 \times 10^{-4} \pm (8.2 \times 10^{-4}) \). D: quantal content for total data set of 500 trials at 15 Hz; slope of regression was equal to \(-1.2 \times 10^{-4} \pm (2.2 \times 10^{-4}) \).

FIG. 3. Frequency-dependent increases in synaptic parameters do not depend on statistical method chosen for estimating \( n \) and average probability of response \( P \). A–C compare results of analyzing quantal content distributions from experiment I with the use of the method of Miyamoto (1986) (■), simple binomial statistics (○), and compound binomial statistics (△). A: increases in \( P \) as function of stimulation frequency \( f \). Values of \( P \) for compound binomial are averages of following individual probabilities \( (P) \): 0.16, 0.16, and 0.41 at 2 Hz; 0.01, 0.18, 0.31, and 0.86 at 4 Hz; 0.99, 0.23, 0.24, and 0.89 at 5 Hz; 0.18, 0.21, 0.25, and 0.92 at 6 Hz; 0.07, 0.10, 0.12, 0.44, and 0.98 at 7 Hz; 0.08, 0.25, 0.26, 0.34, and 0.96 at 10 Hz; and 0.19, 0.19, 0.19, and 0.94 at 15 Hz. B: increases in \( n \) as function of stimulation frequency. C: fit of experimental data to linear equation predicted by model of frequency facilitation. (See Eq. 17 and Fig. 7.) Regression coefficients were 99.7, 99.6, and 99.7 for fits of compound binomial, simple binomial, and Miyamoto’s estimates, respectively.
experiments. The mean value of $P_n$ increased as a function of stimulation frequency. Figure 4 illustrates the average frequency dependence of $P$ for all 10 experiments. The character of the frequency dependence of $P$ varied from experiment to experiment; however, in general $P$ increased as a function of stimulation frequency. Figure 4A illustrates the average frequency dependence of $P$ for all 10 experiments. The mean value of $P$ at 2 Hz is 0.22 ± 0.07 (SD); this value increases significantly between 2 and 4 Hz, and reaches a plateau of ~0.3–0.35 at ~8 Hz. The values of $P$ in different experiments ranged from 0.10 and 0.31 at the lowest stimulation frequency tested to 0.30 ± 0.35 at ~8 Hz. The values of $P$ in different experiments ranged from 0.10 and 0.31 at the lowest stimulation frequency tested to 0.30–0.48 at the highest stimulation frequency tested. As a test of the significance of frequency-dependent changes observed in the values of $P$, the slopes of regression describing the relationship between $P$ and stimulation frequency were examined (Table 1). In 7 of 10 experiments the increase in $P$ was found to be significant at the level of 5%.

The results of 10 experiments could be grouped into two categories on the basis of the frequency dependence of quantal content. In the first category (experiments II, IV, VII, and IX), facilitation was weak or quantal release became maximal at stimulation frequencies from 4 to 8 Hz, and stimulation at higher frequencies did not provoke further increases in $m$ (Fig. 5A). In the second category (experiments I, III, V, VI, VIII, and X), the value of $m$ became maximal at frequencies >8 Hz (experiments I and VIII) or increased without reaching a maximum value over the frequency range tested (experiments III, V, VI, and X, Fig. 5D).

When estimates of $n$ were compared in all 10 experiments, these results also could be classified into two categories. In the four experiments with weak facilitation, $n$ shows no statistically significant trend as a function of frequency (Table 1, Fig. 5, B and C). In contrast, in the second category of six experiments, in which facilitation did not reach a maximum value or reached maximum at frequencies >8 Hz, $n$ showed a statistically significant frequency dependence (Fig. 5, D and E, Table 1), increasing by ≥2 at each experiment. The classification of the data into two categories is reflected in the average data in Fig. 5, C and F. The average value of $n$ shows no trend in four experiments in which facilitation was weak or maximal at low stimulation frequencies (Fig. 5C). In contrast, the average value of $n$ increases significantly with stimulation frequency in six experiments in which facilitation did not reach a maximal value, or reached maximum at frequencies above 8 Hz (Fig. 5F).

Surprisingly, the four experiments in which the value of $m$ saturates at low stimulation frequencies demonstrate on average a strong overall increase in $P$, whereas in the six experiments with unsaturated facilitation the average value of $P$ saturates at low stimulation frequencies (Fig. 4B). This result contradicts the suggestion (Wernig 1972; Zucker 1973) that the increase in the release probability is the only factor that determines facilitation.

Our results confirm the previous conclusion (Wojtowitz et al. 1991, 1994) that the frequency-dependent increase in $n$ is an important factor underlying facilitation. In experiments showing a strong frequency dependence of quantal release, $n$ increased directly with stimulation frequency, whereas in other experiments $n$ showed no clear trend. Clearly $n$ cannot be considered to be a frequency-independent constant.

Previous reports that $n$ increases during frequency facilitation have been interpreted to mean that the number of active release sites increases with stimulation frequency (see Wojtowitz et al. 1991, 1994). Instead, we considered the possibility that the number of releasable quanta may be limited by the rates of quantal mobilization and demobilization, and not by a fixed number of release sites, as was previously suggested (Vere-Jones 1966; Zucker 1973). Then, the synaptic parameter $n$ may correspond to the average number of quanta at release sites under the recording pipette that meet two criteria: 1) they are in proper physical relationship to the release site to enable fusion (i.e., docked at the release site) and 2) they have a nonzero probability of releasing neurotransmitter (i.e., are competent to undergo fusion). The number of quanta at release sites meeting these criteria may fluctuate as quanta arrive at and depart from the release sites and become fusion competent. In addition, the number of quanta meeting these criteria may depend on previous presynaptic activity. Thus in steady-state conditions the value of $\langle n \rangle$ will be the average number of quanta docked at release sites, and $\langle n \rangle$ need not be an integer.

The following quantitative model describes how the average number of quanta docked at release sites during frequency facilitation depends on the rates of quantal mobilization and demobilization.

**Kinetic model for quantal mobilization and release**

Our model is based on the following assumptions. 1) The release sites ($N$) are sufficiently numerous that they do not
sites, be described by a rate constant $k_0$ was obtained by comparing $b$ to $s$ with the $t$-distribution. The number of degrees of freedom (df) equals $N – 2$, where $N$ is the number of stimulation frequencies tested in each experiment.

Table 1. Slopes of regression describing the frequency dependent of $n$ and $P$.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>df $(N – 2)$</th>
<th>$b$</th>
<th>$\sigma_b$</th>
<th>$P$, %</th>
<th>$b$</th>
<th>$\sigma_b$</th>
<th>$P$, %</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>0.0013</td>
<td>0.0049</td>
<td>$&gt;10$</td>
<td>0.214</td>
<td>0.032</td>
<td>$&gt;99.9$</td>
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<tr>
<td>II</td>
<td>2</td>
<td>0.0132</td>
<td>0.0040</td>
<td>$&gt;90$</td>
<td>0</td>
<td>0</td>
<td>$&gt;95$</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>0.0224</td>
<td>0.0031</td>
<td>$&gt;99$</td>
<td>0.147</td>
<td>0.055</td>
<td>$&gt;95$</td>
</tr>
<tr>
<td>IV</td>
<td>4</td>
<td>0.0169</td>
<td>0.0051</td>
<td>$&gt;95$</td>
<td>$-0.02$</td>
<td>0.156</td>
<td>$&gt;95$</td>
</tr>
<tr>
<td>V</td>
<td>3</td>
<td>0.0086</td>
<td>0.0026</td>
<td>$&gt;95$</td>
<td>0.341</td>
<td>0.042</td>
<td>$&gt;99.9$</td>
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<tr>
<td>VI</td>
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<td>0.0204</td>
<td>0.0011</td>
<td>$&gt;99.99$</td>
<td>0.214</td>
<td>0.032</td>
<td>$&gt;99$</td>
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<tr>
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<td>0.0054</td>
<td>$&gt;99$</td>
<td>0</td>
<td>0</td>
<td>$&gt;99.9$</td>
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<td>$&gt;95$</td>
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<td>0.027</td>
<td>$&gt;99.9$</td>
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For each of 10 experiments, the slope of regression ($b$) and its SD ($\sigma_b$) was determined for the synaptic parameters $n$ (number of docked quanta available for release) and $P$ (average probability of responding) as a function of the frequency of stimulation. The probability ($P$) that $b$ is different from 0 was obtained by comparing $b/\sigma_b$ with the $t$-distribution. The number of degrees of freedom (df) equals $N – 2$, where $N$ is the number of stimulation frequencies tested in each experiment.

This equation predicts that $\langle n \rangle$ should decrease as the stimulation frequency increases. However, at crustacean neuromuscular junctions, $\langle n \rangle$ can increase as a function of stimulation frequency (Table 1, Figs. 5, E and F).

To explain frequency facilitation, we hypothesize that each impulse mobilizes an additional number of quanta $\langle m \rangle$ to depart from the quantal store. The minimal model that makes the fewest number of assumptions is that the number of released quanta $\langle n \rangle$ newly mobilized with each impulse is independent of both the duration of the stimulus train and the stimulation frequency. At the same time, $n_s$ may vary from trial to trial.

All $n_s$ newly mobilized quanta will be docked at release sites if the probability of docking for a single quantum is close to 1, which could happen when $n_s$ is much smaller than the number of unoccupied release sites. The number of newly mobilized quanta $\langle n_s \rangle$ should not greatly exceed the sum of the number of quanta docked at occupied release sites $\langle n \rangle$ and the number of quanta released by an impulse $\langle m \rangle$ where the maximal value of $\langle n \rangle$ is $\sim 2 – 3$ (see Fig. 5, A and D) and $\langle m \rangle$ is $\sim 2 – 7$ (Fig. 5, B and E). The number of release sites $N$ under the macropatch pipette is $\sim 40$ (Cooper et al. 1995; Wojtowicz et al. 1994). Because the number of unoccupied release sites available for accepting the newly mobilized quanta is $N – n$, it is far in excess of the value of $n_s$. The probability $(P_d)$ that a single newly mobilized quantum will be docked can be estimated in the following way.

Let $P_d$ be the probability of docking of a single mobilized quantum to a single release site. Then the probability that a single mobilized quantum will not become docked is $(1 – P_d)^{N – n}$. Therefore the probability of a mobilized quantum to be docked to any of $N – n$ available release sites is

$$P_d = 1 – (1 – P_d)^{N – n}$$

From Eq. 10 it can be seen that even if the probability of docking at any one available site $(P_d)$ is low, the overall probability of docking for each of the available quanta $(P_d)$ will be very close to 1 because the number of available release sites $(N – n)$ is large. (For example, if $P_d = 0.1$ and $N – n = 30$, we obtain $P_d = 0.96$.) In the above reasoning we assumed for simplicity that all the unoccupied release sites and diffuse back to the quantal store. Then the temporal dependence of $\langle n \rangle$ (the average number of docked quanta) will be described by the process of quanta becoming docked minus the loss of docked quanta due to movement back into the quantal store or due to spontaneous release of quanta.

$$d\langle n \rangle/dt = k_0 S - k_d (n) - P_d (n)$$

At steady state, $d\langle n \rangle/dt = 0$ and the number of docked quanta in the absence of impulses will be $(\langle n_0 \rangle)$

$$\langle n_0 \rangle = k_0 S / (k_d + P_d)$$

A single impulse will release a number of docked quanta. Let us define $P$ as the probability of a single docked quantum to undergo fusion. During repetitive stimulation, the number of quanta released per unit time by impulses will be equal to the frequency of stimulation $f$ multiplied by $(\langle n \rangle P)$. If no additional quanta are mobilized to replace those that are released by excitation, the temporal dependence of docked quanta $\langle n \rangle$ during repetitive stimulation then becomes

$$d\langle n \rangle/dt = k_0 S - k_d (n) - P_d (n) - f (n) P$$
sites have equal abilities to accept a newly mobilized quantum; however, the result will be similar if some quanta have higher probabilities of docking than the others. Thus the number of newly docked quanta will be equal to \( f(n_s) \). The temporal dependence of \( n \) (Eq. 9) can be rewritten as
\[
d(n)/dt = k_m S - k_d(n) - P_0(n) - f(n)P + f(n_s) \tag{11}
\]
During long trains of stimuli, transmitter release will achieve the steady-state condition of \( d(n)/dt = 0 \), and the number of docked quanta will be given by
\[
\langle n \rangle = \frac{(k_m S + f(n_s))}{(k_d + P_0 + fP)} \tag{12}
\]
Elimination of the \( k_m S \) term by substituting the values from Eq. 8 gives
\[
\langle n \rangle = \frac{\langle n_s \rangle (k_d + P_0 + fP)}{(k_d + P_0 + fP)} \tag{13}
\]
Note that the value of the parameter \( \langle n_s \rangle \) is very important for determining the frequency dependence of transmitter release. For example, if \( \langle n_s \rangle \) is negligibly small, the number of docked quanta \( \langle n \rangle \) will decrease with frequency as \( 1/f \), leading to synaptic depression (Elmqvist and Quastel 1965). If \( \langle n_s \rangle \) and the frequency of stimulation are relatively large, i.e., if
\[
f(n_s) \gg \langle n_s \rangle (k_d + P_0)\]
\[
\langle n \rangle \approx \frac{\langle n_s \rangle (k_d + P_0)}{(k_d + P_0 + fP)} \tag{14}
\]
Equation 13 can be approximated with
\[
\langle n \rangle = f(n_s)(k_d + P_0 + fP) \tag{15}
\]
In this case \( \langle n \rangle \) will increase as a function of stimulus frequency. Multiplying both sides by \( P \) and taking the reciprocal gives the linear form of Eq. 15
\[
1/(m) = 1/(n_s) + [(k_d + P_0)/(n_s)](1/fP) \tag{16}
\]
where average quantal content \( \langle m \rangle \) is equal to \( \langle n \rangle P \). Because the probability of spontaneous release is negligible at each release site (\( \langle n \rangle \) is equal to \( \langle n_s \rangle \) in Eq. 16 can be approximated as
To test whether the proposed stimulus-dependent mobilization model accurately describes facilitation, the experimental data were plotted in the form of $1/(m)$ as a function of $1/fP$. For eight experiments, the resulting plots were linear over the entire frequency range tested (Fig. 7A, Table 2). For two experiments (VII and X) plots were linear only for values of frequency ($f$) $> 2$ Hz (Fig. 7B). In these two cases, the quantal content $(m)$ was sufficiently high at 2 Hz that the corresponding data point deviated from the straight line described by data points at higher stimulation frequencies. This situation may be explained if condition given by Eq. 14 is unsatisfied at low stimulation frequencies (for example, if $k_0$ or $(n_0)$ is relatively large). If the condition given by Eq. 14 is not satisfied, the frequency dependence of $(n)$ will be described by Eq. 13 and thus $(n)$ and $(m)$ will be higher than predicted by Eq. 15 and 16. Thus the model accurately describes the experimental results in the frequency range $f > 4$ Hz for all 10 experiments, as well as the results in the frequency range $f > 2$ Hz for 7 of the 10 experiments. This linear relationship between the reciprocal value of quantal content and the reciprocal product of the stimulation frequency and the probability of release is not altered by the choice of the statistical method for estimation of $P$ (Fig. 3C).

The correlation coefficients for linear regressions fit to the experimental data exceed 93% for all 10 experiments; for 7 experiments the value of the correlation coefficient exceeds 98% (Table 2). The slopes $(k_d/(n))$ of the linear regressions vary significantly, whereas the ordinate intercepts $(1/(n))$ are remarkably similar. For eight experiments (all except IV and VIII), the mean value of $(n)$ was 2.02 ± 0.26 (SE), suggesting that usually an average of two additional quanta become docked as a function of each stimulus. Thus the observed variability of the frequency dependence of the parameter $n$ (Table 1) can be explained by variability in the rate constant of quantal demobilization $(k_d)$.

Equation 17 predicts that the higher the value of $P$, the lower the frequency at which $(m)$ will approach its maximal value $(n)$. This prediction is in agreement with the observation (Fig. 4B) that the six experiments with unsaturated $(m)$ have on average lower values of $P$ than the four experiments showing saturation of $(m)$ at low stimulation frequencies.

It is important to note that the variability in the number of docked quanta $n$ from trial to trial will affect the distribution of quantal content and therefore may bias the estimations of the synaptic parameters. The relative error in the estimations of $(n)$ and $P$ with the use of binomial statistics will be $\sigma \sqrt{n}/(n)$ (see APPENDIX). As far as $\sigma \sqrt{n}/(n)$ is likely to be relatively small (see APPENDIX), the derived values for the parameters $n$ and $P$ will be reasonable approximations for the average number of releasable quanta and the probability of release.

**Discussion**

Facilitation may result from increases in both $n$ and $P$

The results of this study demonstrate that facilitation of transmitter release is a presynaptic process in which the increase in $m$ may be due to an increase in both the synaptic parameters $n$ and $P$. The synaptic parameter $n$, therefore, cannot be considered to be frequency independent. One reason for the discrepancy between this finding and that of Zucker (1973) is that the earlier study focused largely on the release statistics of paired pulse stimulation, and estimates of $n$ are unreliable for the unfacilitated (1st) responses, resulting in large values of SE of $n$. In three experiments in which repetitive trains of stimuli were delivered (Zucker 1973), SEs in the estimate of $n$ became smaller, but changes in the parameter $n$ could not be measured against the large variability in unfacilitated responses. Another reason for the discrepancy is that the present experiments were designed to test several stimulation frequencies $> 5$ Hz in a single experiment, and changes in $n$ generally are more pronounced at higher frequencies. In the study by Zucker (1973), only one experiment was performed at a frequency $> 5$ Hz.

Our finding that $n$ is frequency dependent during frequency facilitation is in agreement with results reported by Wernig (1972) and Wojtowicz et al. (1994). However, nei-
neurosecretion is limited by the rates and synaptic morphology (Wojtowicz et al. 1994).

Parameters of the stimulus-dependent mobilization model and synaptic morphology

Wojtowicz et al. (1994) and Cooper et al. (1995) have shown that ~40 synapses are present under a macropatch recording pipette in crustacean neuromuscular preparations, and that each synapse contains thousands of presynaptic vesicles. We have proposed that the synaptic parameter $n$ corresponds to the number of activated and docked quanta under the recording pipette. Our results show (Fig. 5, B and E) that the value of $n$ is never >7; therefore average $n$ is far smaller than either the number of synapses under the recording pipette or the thousands of presynaptic vesicles that comprise the reserve quantal store ($S$). Thus these morphological data are in agreement with our suggestions that 1) the number of release sites ($N$) does not limit the number of docked quanta ($n$) and 2) the reserve store of quanta ($S$) is sufficiently large to be treated as constant, in agreement with the recent finding (Smith and Betz 1996) that during prolonged stimulation, rates of exocytosis and endocytosis are equal. It follows that most of the release sites present under the tip of the electrode do not have a docked quantum,

$k_d$ are means ± SE. $1/m$, reciprocal value of quantal content; $1/P$, reciprocal value of product of stimulation frequency and probability of release; $R$, regression coefficient.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Stimulation Frequencies, Hz</th>
<th>$R$</th>
<th>Slope ($k_d/\langle n_0 \rangle$)</th>
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<tbody>
<tr>
<td>I</td>
<td>2, 4, 5, 6, 7, 10, 15</td>
<td>0.998</td>
<td>0.462 ± 0.013</td>
<td>0.415 ± 0.011</td>
<td>2.41 ± 0.06</td>
<td>1.11 ± 0.06</td>
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<tr>
<td>II</td>
<td>2, 4, 8, 10</td>
<td>0.999</td>
<td>0.187 ± 0.006</td>
<td>0.577 ± 0.006</td>
<td>1.73 ± 0.02</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>III</td>
<td>2, 4, 8, 10, 15</td>
<td>0.998</td>
<td>0.908 ± 0.031</td>
<td>0.523 ± 0.072</td>
<td>1.91 ± 0.26</td>
<td>1.74 ± 0.30</td>
</tr>
<tr>
<td>IV</td>
<td>2, 4, 5, 6, 8, 10</td>
<td>0.932</td>
<td>0.185 ± 0.036</td>
<td>0.320 ± 0.027</td>
<td>3.13 ± 0.27</td>
<td>0.58 ± 0.16</td>
</tr>
<tr>
<td>V</td>
<td>2, 4, 8, 10, 15</td>
<td>0.930</td>
<td>0.893 ± 0.023</td>
<td>0.573 ± 0.283</td>
<td>1.75 ± 0.86</td>
<td>1.56 ± 1.12</td>
</tr>
<tr>
<td>VI</td>
<td>2, 4, 5, 6, 7, 8, 10</td>
<td>0.990</td>
<td>0.505 ± 0.031</td>
<td>0.561 ± 0.041</td>
<td>1.78 ± 0.13</td>
<td>0.90 ± 0.12</td>
</tr>
<tr>
<td>VII</td>
<td>4, 5, 6, 7, 8</td>
<td>0.987</td>
<td>0.936 ± 0.087</td>
<td>0.515 ± 0.060</td>
<td>1.94 ± 0.23</td>
<td>1.82 ± 0.38</td>
</tr>
<tr>
<td>VIII</td>
<td>4, 5, 6, 7, 8, 9, 12</td>
<td>0.982</td>
<td>1.779 ± 0.152</td>
<td>0.151 ± 0.106</td>
<td>6.64 ± 4.67</td>
<td>11.82 ± 9.31</td>
</tr>
<tr>
<td>IX</td>
<td>2, 5, 6, 7, 8</td>
<td>0.988</td>
<td>0.466 ± 0.042</td>
<td>0.445 ± 0.045</td>
<td>2.25 ± 0.23</td>
<td>1.05 ± 0.20</td>
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<tr>
<td>X</td>
<td>4, 5, 6, 7, 8, 10, 15</td>
<td>0.973</td>
<td>0.650 ± 0.069</td>
<td>0.422 ± 0.041</td>
<td>2.37 ± 0.23</td>
<td>1.54 ± 0.31</td>
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The two models of facilitation described above differ in three important respects. The first fundamental difference relates to whether quantal release is limited by the physical number of release sites or by the rates of quantal mobilization and demobilization. A second difference is that a model postulating the recruitment of silent synapses necessitates the assumption that the values of $P$ at different release sites are nonuniform, with inactive release sites having extremely low values of $P$. The stimulus-dependent mobilization model of facilitation makes no assumption about whether the values for $P$ are uniform or nonuniform. (Note that if the assumption of nonuniformity is introduced into Eq. 7–17 describing our model, the term $P$ will represent the average probability of release). Finally, the stimulus-dependent mobilization model postulates that quanta that are mobilized from the reserve store simply become releasable from any unoccupied release site, without making assumptions regarding the spatial positions of newly docked quanta relative to active or “previously silent” release sites.

In the stimulus-dependent mobilization model of facilitation, we propose that neurosecretion is limited by the rates and synaptic morphology of docked quanta (Hubbard 1963) and that the rate of mobilization is frequency dependent (Elmqvist and Quastel 1965).

<table>
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Two models of frequency facilitation

Previously, it has been hypothesized that quantal release is limited by the number of active release sites (Korn et al. 1982; Zucker 1973), and that facilitation may result from an increase in the probability of release at the active release sites. In addition, it has been suggested that complex synapses (those with >2 dense bodies) are responsible for transmitter release at low stimulation frequencies, and that the number of complex synapses increases at higher stimulation frequencies because of the activity-dependent recruitment of previously silent or inactive simple synapses (Wojtowicz et al. 1991, 1994). The conversion of simple to complex synapses is hypothesized to be related to increases in $P$ at individual synapses, such that synapses where $P$ was equal to zero become active when $P$ increases, thereby increasing the value of $n$, the number of complex synapses or “responding units” (Wojtowicz et al. 1994).

In the stimulus-dependent mobilization model of facilitation, we propose that neurosecretion is limited by the rates of quantal mobilization and demobilization; thus we consider $n$ to be a measure of the number of releasable quanta under the recording pipette. Our kinetic model describes the frequency dependence of quantal release in terms of two parameters that do not depend on frequency: $\langle n_0 \rangle$ (the average number of quanta newly mobilized to release sites and docked as a function of each stimulus) and $k_d$ (the rate constant for demobilization of docked quanta off docking the recording pipette). Our results show (Fig. 7), suggesting that the model is reasonable. It should be noted that the stimulus-dependent mobilization model suggests a set of measurable parameters by which facilitation can be described, and is in agreement with previous suggestions that facilitation results from a dominance of mobilization over depletion during repetitive stimulation (Hubbard 1963) and that the rate of mobilization is frequency dependent (Elmqvist and Quastel 1965).

The two models of facilitation described above differ in three important respects. The first fundamental difference relates to whether quantal release is limited by the physical number of release sites or by the rates of quantal mobilization and demobilization. A second difference is that a model postulating the recruitment of silent synapses necessitates the assumption that the values of $P$ at different release sites are nonuniform, with inactive release sites having extremely low values of $P$. The stimulus-dependent mobilization model of facilitation makes no assumption about whether the values for $P$ are uniform or nonuniform. (Note that if the assumption of nonuniformity is introduced into Eq. 7–17 describing our model, the term $P$ will represent the average probability of release). Finally, the stimulus-dependent mobilization model postulates that quanta that are mobilized from the reserve store simply become releasable from any unoccupied release site, without making assumptions regarding the spatial positions of newly docked quanta relative to active or “previously silent” release sites.
and therefore have a probability of releasing a quantum equal to zero.

Wojtowicz et al. (1994) have proposed a different definition of the synaptic parameter $n$: that the value of $n$ corresponds to the number of complex synapses (those with $\geq 2$ dense bodies) under the recording pipette, each of which is a responding unit. These authors report that the number of complex synapses increases following a stimulation protocol that induces long-term facilitation (20 Hz for 10 min), suggesting that long-term stimulation can provoke ultrastructural modifications of synaptic morphology. A similar morphological change was not observed after shorter stimulation protocols. In our experiments, the highest-frequency stimulation protocol involved 15-Hz stimulation for 40 s (600 trials); this protocol is insufficient to induce long-term facilitation and any associated morphological changes (Wojtowicz et al. 1994). Thus it is likely that the ultrastructural changes observed after long-term facilitation (which decays over tens of min) represent a different mechanism for potentiating transmitter release than that which underlies frequency facilitation (which decays within s).

Wojtowicz et al. (1994) also report that a short-term stimulation protocol induces a decrease in the number of readily releasable vesicles, defined as those within 0.05 $\mu$m of a synaptic membrane. It is unclear whether this observation means that the number of docked quanta decreases with repetitive stimulation, because the spatial positions of vesicles relative to a synaptic dense bar may or may not be indicative of whether a quantum is activated or fusion competent. In addition, the processes of quantal mobilization and demobilization are likely to be fast and not to be captured on the time course with which histological fixation occurs.

**Comparison with other models of neurosecretion**

Vere-Jones (1966) considered a model of neurosecretion in which 1) the number of release sites $(N)$ limits the number of quanta released, 2) each unoccupied site has a fixed probability of being occupied by a quantum in the interval between two impulses, and 3) this probability is independent of what occurs at other sites and of the past history of the process. This set of assumptions would be consistent with the docking process being a binomial sampling from the number of release sites $N$. In contrast, our model assumes that 1) there are $N$ sites and $n$ quanta are docked to them, 2) between successive impulses stochastic mobilization and demobilization of quanta take place with associated rate constants $k_m$ and $k_d$, 3) each impulse causes additional docking of an average of $n$, quanta (with some variability) with a probability equal to 1, and 4) under our experimental conditions, $N$ is always much larger than $n$. It follows that the average number and variance of docked quanta depends only on the biochemical processes of activation and docking, and does not depend on the number of release sites $N$.

In some respects, our model of neurosecretion is similar to a model originally developed by Elmquist and Quastel (1965), in which they describe the transfer of quanta between stored and releasable pools. These authors suggested that the pool of releasable quanta was partly refilled between each pair of impulses, which allowed the derivation of a linear dependence between the reciprocal value of $m$ and stimulation frequency. This model accurately explained the phenomenon of synaptic depression observed at human skeletal neuromuscular junctions. The main difference between our model and that of Elmquist and Quastel (1965) is that we suggest an additional number of quanta $(n_s)$ is mobilized with each excitatory nerve impulse, leading to the observed facilitation of transmitter release as a function of stimulation frequency at the crustacean neuromuscular junction.

The stimulus-dependent mobilization model for frequency facilitation is also similar in some respects to a model proposed by Maeno and Edwards (1969) for the mobilization of transmitter at the amphibian neuromuscular junction. Both models assume that quanta are mobilized from a store to become docked at release sites, and that the demobilization process by which quanta disengage from release sites $(k_d)$ is independent of stimulation frequency. However, facilitation at amphibian synapses was expressed as an exponential relation between quantal content and stimulation frequency (Maeno and Edwards 1969), whereas in the present study we find that quantal content approaches saturation at high frequencies and therefore there is no exponential relation. At the crustacean neuromuscular junction we find a linear relation between reciprocal values of quantal content $(1/(m))$ and reciprocal values of the product of stimulation frequency times probability of release at that frequency $(1/fP)$, Table 2). Therefore, in contrast to the model of Maeno and Edwards (1969) in which the rate of mobilization was proposed to be related exponentially to frequency, our data and model describe mobilization as a linear function of stimulation frequency, with a rate constant equal to $(k_m + f(n_s))/S)$.

**Limits to facilitation**

Neurosecretion became maximal at low frequencies in some experiments (Fig. 5A), whereas in other experiments neurosecretion did not reach a maximum at frequencies up to 15 Hz (Fig. 5D). It is therefore of interest to consider which physiological processes might limit the maximal number of quanta released and 2) determine whether facilitation at a given synapse is strong or weak. Previously, it had been assumed that the value of $n$ was small and fixed (Vere-Jones 1966; Zucker 1973); therefore facilitation would become maximal when the value of $P$ became equal to 1.0 (Zucker 1974). This contradicts our observation that the highest estimate of $P$ never exceeded 0.48 (see paragraph 3 in RESULTS), even in experiments in which quanta release became maximal at low frequencies (for example, experiment IV).

Our model for frequency facilitation suggests another possibility, that the maximum quantal content is limited not by $P$ but by the average number of quanta that become docked with each excitatory impulse $(n_s)$, because Eq. 15 predicts a hyperbolic dependence of $(m)$ on $(fP)$. This hyperbolic dependence determines $(n_s)$ to be the upper limit of $(m)$ (limit as $f \to \infty$). Support for the hypothesis that $(n_s)$ limits quantal content comes from the following observations: 1) the average number of released quanta $(m)$ never exceeded 2–3 per stimulus even at high frequencies (Fig. 5, A and D); 2) fits of experimental data by the kinetic equations show that $\sim 2–3$ quanta, on average, become newly mobilized and docked...
in response to a single stimulation \((n_s, \text{Table 2})\); and 3) \(n\) is \(\sim 2-3\) at low stimulation frequencies (Fig. 5E), suggesting that \(\langle n \rangle\) is approximately equal to \(\langle n_s \rangle\) in the absence of facilitation. Thus the value of \(\langle n_0 \rangle\) may be vanishingly small in the absence of stimulation, and the arrival of an excitatory impulse may transiently increase \(\langle n \rangle\) to a value of 2–3.

Maximum quantal content is determined by the balance between the frequency-independent parameter \(k_d\) and the frequency-dependent parameter \(P\). From Eq. 15 it follows that \(\langle m \rangle\) will approach its upper limit at low stimulation frequencies when the value of \(k_d\) is small. This is illustrated by the results from two experiments showing weak facilitation, experiments II and IV (Fig. 5A), for which the lowest values of \(k_d\) were obtained \(k_d \text{ for experiments II and IV is } \sim 0.3-0.6, \text{ whereas in all the other experiments it is } \approx 0.9, \text{ see Table 2}\). Note that whereas in both these experiments the value of \(\langle m \rangle\) approaches its maximal value \(\langle n_s \rangle\) at low \(f\), and the value of \(\langle n \rangle\) shows a weak overall dependence on \(f\) (Fig. 5B). In contrast, a high value of \(k_d\) predicts that at low \(f\) much of the quanta will disengage from the release sites between two sequential impulses, and therefore the value of \(\langle n \rangle\) at low \(f\) will be relatively small. As \(f\) increases, fewer quanta will disengage from the release sites between two sequential impulses and therefore \(\langle n \rangle\) will increase. Such a scenario may explain the results obtained in six experiments in which the value of \(\langle m \rangle\) did not saturate at low \(f\) and \(\langle n \rangle\) increased with stimulation frequency (Fig. 5, E and F).

From the hyperbolic relation between \(\langle m \rangle\) and \(\langle fP \rangle\) it follows that the relation between \(\langle m \rangle\) and \(f\) can be rather complicated because \(P\) itself increases with stimulation frequency \(f\) (Fig. 4). Therefore, if \(P\) increases strongly with \(f\), \(\langle m \rangle\) will approach its upper limit at low \(f\). This tendency can be clearly seen in experiment VII, which shows weak facilitation (Fig. 5A). This experiment is characterized by a pronounced increase in \(P\), as indicated by the finding that its regression slope \(b\) for the relationship between \(P\) and \(f\) is the highest among all the experiments (Table 1). Because the term \(P\) appears only in the denominator of the relation between \(\langle n \rangle\) and \(f\) (Eq. 13), a strong increase in \(P\) can suppress any frequency-dependent increase in \(n\), as was observed in the results from experiment VII (Fig. 5B, Table 1).

In summary, the stimulus-dependent mobilization model of facilitation predicts that neurosecretion will be maximal when the value of \(\langle m \rangle\) approaches the value of \(n_s\), and that strong frequency facilitation will be observed in cases where the value of \(k_d\) is relatively high.

**Appendix**

**Variability in the number of docked quanta**

Let \(n\) represent the number of quanta docked at release sites just before an excitatory impulse. If \(n\) varies from trial to trial, the variability in \(n\) will affect the distribution of quantal content. Therefore the derived estimations of synaptic parameters \(n\) and \(P\) may be biased. In this case, the correct estimation of \(P\) will be (Brown et al. 1976; Vere-Jones 1966)

\[
P = P_b/(1 - \sigma^\prime(n))
\]

where \(P\) is the probability of release, \(P_b\) is the binomial estimation of \(P\), \(n\) is the average number of docked quanta, and \(\sigma^\prime(n)\) is the temporal variance in \(n\). Thus the relative error in the estimations of \(n\) and \(P\) obtained with the use of binomial statistics will be \(\sigma^\prime(n)/n\). If \(\sigma^\prime(n)\) is relatively small, the obtained values for the parameters \(n\) and \(P\) will be reasonable approximations for the average number of releasable quanta and the probability of release.

Below, we illustrate how \(\sigma^\prime(n)\) may be estimated in the stimulus-dependent mobilization model of neurosecretion. The probability for a quantum not to be released with the arrival of the first impulse and thus to remain at the docking site for the arrival of a second impulse is \(1 - P\). The probability for the nonreleased quantum not to be demobilized back into the quantal store, and thus to remain for the second impulse, is \(\exp(-k_d/f)\). Thus the probability \(P_b\) for each of \(n\) docked quanta to remain at release sites and undergo fusion with the arrival of the second impulse is

\[
P_b = (1 - P) \exp(-k_d/f)
\]

In addition, \(n\), quanta with variance \(\text{var}(n_s)\) will be added to the pool of docked quanta because of stimulus-dependent mobilization triggered by the second impulse. Therefore the variance in the number of docked quanta will be

\[
\alpha^\prime(n) = n_P(1 - P) + \text{var}(n_s)
\]

which can be approximated by

\[
\alpha^\prime(n) = \langle n \rangle P_b(1 - P) + \text{var}(n_s)
\]

Thus

\[
\alpha^\prime(n) = P_b(1 - P) + \text{var}(n_s)/\langle n \rangle
\]

Examination of this equation leads to several predictions. 1) The value of the term \(P_b(1 - P)\) will always be less than 0.25, because \(P_b \leq 1\). 2) Because \(P\) is a product of \((1 - P)\) and \(\exp(-k_d/f)\) and these terms behave in opposite ways when the frequency of stimulation increases \([i.e., \text{as } f\text{ increases, the term } 1 - P \text{ decreases, and the term } \exp(-k_d/f) \text{ increases}], the dependence of \(P\) on the frequency of stimulation is likely to be weak. Consequently, the frequency dependence of \(\alpha^\prime(n)\) is likely to be weak. 3) If \(\text{var}(n_s)\) is comparable with \(\langle n_s \rangle\), the process of docking would be close to Poissonian, and thus the statistics of quantal release would also be close to Poissonian (Vere-Jones 1966), even at high frequencies of stimulation. Such a result was never observed, which can be clearly seen from large values of the binomial parameter \(P\) at high frequencies of stimulation \(0.30-0.48\), see paragraph 3 in RESULTS. Thus, although we do not possess sufficient information to evaluate the magnitude of \(\text{var}(n_s)\), we can infer that it is sufficiently smaller than \(\langle n_s \rangle\), and therefore sufficiently smaller than \(\langle n \rangle\) to justify the assumption that the variance to mean ratio of the number of docked quanta is small.

Therefore the main findings reported in this study (that \(n\) is frequency dependent and the frequency dependence of \(m\) can be predicted by the stimulus-dependent mobilization model) are not strongly affected by the possible bias in the estimation of synaptic parameters due to the variability in \(n\).

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