

Heterogeneity of Release Probability, Facilitation, and Depletion at Central Synapses

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Summary

Previous studies of short-term plasticity in central nervous systems synapses have largely focused on average synaptic properties. In this study, we use recordings from putative single synaptic release sites in hippocampal slices to show that significant heterogeneity exists in facilitation and depletion among synapses. In particular, the amount of paired-pulse facilitation is inversely related to the initial release probability of the synapse. We also examined depletion at individual synapses using high frequency stimulation, and estimated the size of the readily releasable vesicle pool, which averaged 5.0 ± 3.0 quanta ($n = 13$ synapses). In addition, these experiments demonstrate that the release probability at a synapse is directly correlated with the size of its readily releasable vesicle pool.

Introduction

Synaptic strength is dynamically regulated: synapses respond to different patterns of use with short- and long-term changes in their strength. Long-lasting alterations in synaptic strength are thought to underlie learning and memory formation, and have been the subject of intensive research (e.g., reviews by Malenka, 1994; Huang et al., 1996). Synapses also have a form of short-term memory, however, manifested as changes in synaptic strength that occur rapidly and reversibly depending on the input pattern (Magleby, 1987; Zucker, 1989). How this “dynamic gain control,” which may interact with long-term changes, is used to process or store information may yield important insights into mechanisms of neural computation (Markram and Tsodyks, 1996; Abbott et al., 1997).

Certain forms of short-term synaptic plasticity have been well characterized at the neuromuscular junction (Magleby, 1987). Since each of these specialized peripheral synapses contains multiple transmitter release sites (del Castillo and Katz, 1954), quantitative descriptions of plasticity reflect the average properties of these release sites. For central nervous system synapses in the hippocampus, studies of synaptic plasticity have also investigated average properties by recording from populations of cells in the CA1 region (Creager et al., 1980; Wu and Saggau, 1994) or populations of synapses on a single CA1 cell (Hess et al., 1987; Manabe et al., 1993; Debanne et al., 1996). Synapses between hippocampal CA3 cells and CA1 cells, however, mostly contain only a single active zone (Sorra and Harris, 1993; Schikorski and Stevens 1997). Studies of individual hippocampal synapses therefore are mostly investigations of single active zone

properties. These synapses release neurotransmitter only a fraction of the time they are stimulated (Raastad et al., 1992; Allen and Stevens, 1994; Stevens and Wang, 1994; Stevens and Wang, 1995), and considerable variability exists from synapse to synapse in their transmitter release probability (Hessler et al., 1993; Rosenmund et al., 1993; Allen and Stevens 1994; Malinow et al., 1994; Murthy et al., 1997).

In this study, we use stimulation of putative single synapses on CA1 cells in the hippocampus to show that significant heterogeneity exists in facilitation and depletion among synapses. In particular, the amount of paired-pulse facilitation is inversely related to the initial release probability across the population of synapses studied. In addition, studies of synapse depletion were used to measure the size of the readily releasable vesicle pool, revealing that the release probability at a synapse is directly correlated with the size of its readily releasable vesicle pool.

Results

Heterogeneous Responses of Single Synapses to Repeated Stimuli

When a train of high frequency stimuli is applied to hippocampal synapses, the response is complex. Figure 1 shows the average response of a population of synapses to 64 stimuli applied at 10 Hz, repeated again after a 30 s recovery interval. Notice that initially the response size increases, and subsequently it decreases to a low level. This reflects the competing processes of facilitation, which increases the response size, and depletion or depression, which decreases the response size. Figure 1 provides an example of the short-term dependence of synaptic strength on the history of synaptic use. To understand the mechanisms underlying short-term history dependence, it is necessary to understand both of the competing effects, as well as how they interact.

Many studies have examined average changes in synaptic strength in populations of synapses. In this paper, we start by asking the questions: How do individual synapses respond to a high frequency stimulus train? Are they homogeneous or heterogeneous in their responses? Figure 2 shows two typical examples of the average amplitude of the response (including failures of transmitter release) of a single synapse to a 10 Hz stimulus train (these experiments are discussed in greater detail below). In the cell shown in Figure 2A, the synapse is initially facilitated, and the average response amplitude increases; this is followed by a decrease in response size, which approaches a steady value after about 15 stimulations (in these experiments, the $[Ca^{2+}]/[Mg^{2+}]$ ratio is higher, causing the more rapid decrease in the response as compared to Figure 1). In a different example shown in Figure 2B, however, no facilitation is observed; the response decreases monotonically with stimulation to a new plateau value. One of these two patterns was observed in each of 13 cells. Clearly, synapses are heterogeneous in their response to repeated

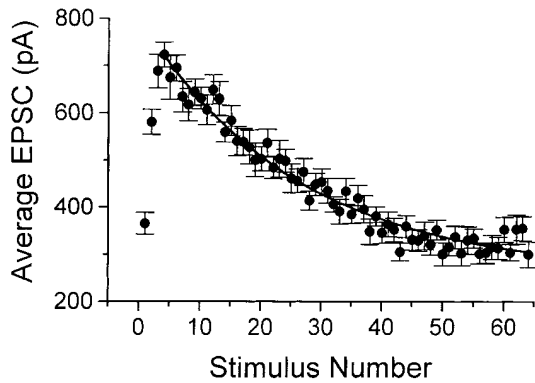


Figure 1. Multisynapse EPSC Response to High Frequency Stimulation Is Biphasic

Peak amplitude of EPSC from CA1 cell initially increases, then decreases during stimulus train at 10 Hz. Mean \pm SE of 18 presentations; train of 64 pulses at 10 Hz with 30 s rest interval between sets. Solid line is the exponential fit to decay phase, $\tau = 25.6$ stimuli. $[Ca^{2+}] = 2.5$ mM; $[Mg^{2+}] = 1.3$ mM.

rapid stimulation. What is the cause of the variability? It appears to be due to a difference in the amount of facilitation present; we investigate this further using pairs of pulses at single synapses.

Facilitation

Amount of Facilitation at Single Synapses Is Highly Variable and Depends on Initial Release Probability

In the previous experiments, facilitation was seen mixed with depression/depletion. To look at facilitation alone, we studied the single synapse responses to pairs of stimuli, separated by 40 ms and repeated at 0.25 Hz. Paired-pulse facilitation has been well-studied in populations of hippocampal CA1 cells; however, here, we study the heterogeneity of paired-pulse facilitation seen at individual synapses. Figure 3 illustrates the results for 38 putative single synapses. For the first pulse (abscissa values, Figure 3A), a wide range of release probabilities was measured from the different synapses in response to the same conditions ($[Ca^{2+}]$, $[Mg^{2+}]$, stimulation). Strikingly, the amount of facilitation (release probability for the second pulse divided by release probability for the first pulse) varies over a wide range (4.2–1), and clearly depends on the release probability on the first pulse (Figure 3A). Synapses with low initial release probabilities facilitate much more than those with higher probabilities, and little facilitation is observed for synapses with a release probability $> \sim 0.4$.

The smooth curve superimposed on the data in Figure 3A is

$$f = \frac{1 - (1 - p)^{u(t)p^{v\beta}}}{p}$$

where f is the facilitation for a synapse whose release probability for the first pulse is p . $u(t)$ depends on the interpulse interval and may depend on the $[Ca^{2+}]/[Mg^{2+}]$. Fitting this equation to the data in Figure 3A (interpulse interval $t = 40$ ms; 2.5 mM Ca^{2+} /1.3 mM Mg^{2+}) gives $u(t) = 1.24 \pm 0.15$, and $v\beta = -0.41 \pm 0.05$ (solid line). An

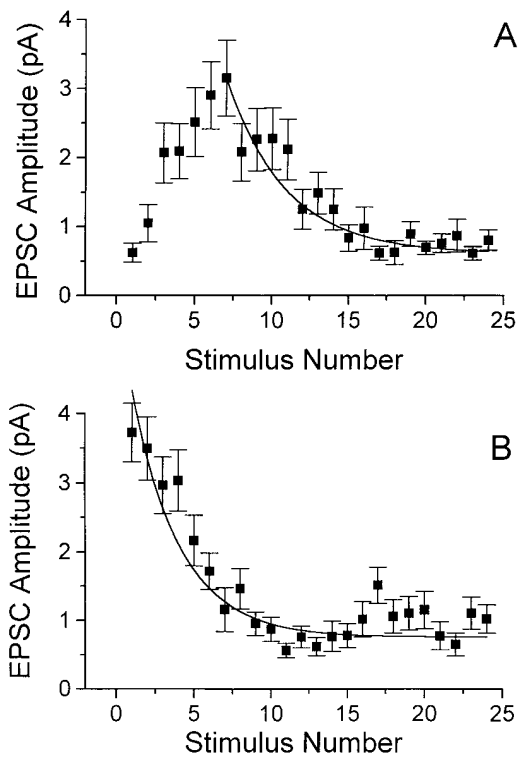


Figure 2. Single Synapse EPSC during High Frequency Stimulation Shows Variable Amount of Facilitation

(A) Average EPSC (including failures) at single synapse shows biphasic behavior. Amplitude rapidly increases, then decreases. Mean \pm SE of 80 presentations; train of 32 pulses at 10 Hz with 20 s rest interval between sets. Solid line is exponential fit to decay phase, $\tau = 3.9$ stimuli. $[Ca^{2+}] = 4.0$ mM; $[Mg^{2+}] = 1.0$ mM.

(B) Average EPSC (including failures) at this single synapse shows no initial facilitation, only a decrease in response size. Mean \pm SE of 96 presentations; train of 24 pulses at 10 Hz with 20 s rest interval between sets. Solid line is the exponential fit to decay phase, $\tau = 3.1$ stimuli. $[Ca^{2+}] = 4.0$ mM; $[Mg^{2+}] = 1.0$ mM.

example of how this equation could arise is presented in the Appendix.

Another way of presenting the same data is to plot the release probability on the second pulse as a function of the first pulse release probability, as in Figure 3B. Although the lower probability synapses always have greater facilitation, the release probability for the second pulse is still greater for higher probability synapses than for lower probability synapses. Facilitation would be inversely related to initial release probability if the second pulse release probability was always equal to the same value (e.g., 1.0). This is not the case here.

Paired-pulse facilitation has been shown to be a presynaptic phenomenon (Zucker, 1989); as expected for a purely presynaptic process, it caused no change in the synaptic potency (the average size of the synaptic current when release occurs), as shown in Figure 3C. The average potency for the 38 synapses was 14.1 ± 7.1 pA (range 4.2–29.2 pA). There was no relationship between potency and release probability for either pulse, as shown in Figure 3D.

Although the relatively small sample size ($n = 38$)

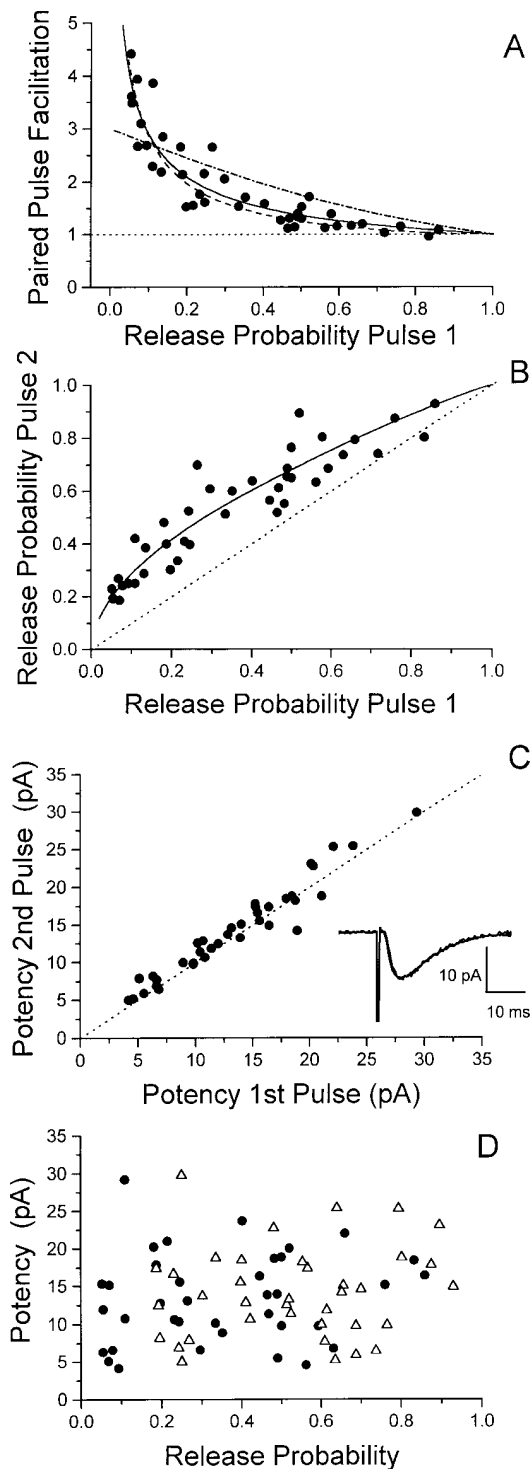


Figure 3. Paired-Pulse Facilitation at Single Synaptic Release Sites (A) Paired-pulse facilitation (ratio of release probability for the second pulse to the first pulse) is greatest for low probability synapses. Pulses in the pair are separated by 40 ms; pairs are repeated with 4 s rest intervals. Dashed and dotted line is fit of the equation $f = \frac{1 - (1 - p)^\varphi}{p}$ to the data, with $\varphi = 3.0$ (f is facilitation; p is release probability on the first pulse; derivation of the equation is in the Appendix). This does not provide a good fit to the data. The solid line is fit to the data of the modified equation $f = \frac{1 - (1 - p)^{u(t)p^\beta}}{p}$

combined with the possibility of selection bias in choosing synapses to record from prevents us from estimating the distribution of release probability across all hippocampal CA3–CA1 synapses, the results in Figure 3 demonstrate that there is a wide range of single synapse release probabilities (the range observed was 0.052–0.859 for the first pulse, 0.187–0.929 for the second pulse). Under our experimental conditions, the initial release probability for most synapses is quite low (average $p_1 = 0.35 \pm 0.23$).

Depletion

We now return to the second part of the phenomenon observed in Figures 1 and 2: the dramatic decrease in the response amplitude produced by application of trains of high frequency stimuli. To examine this in more detail, we next did control experiments repeating the high frequency stimuli in small populations of synapses to assess the contributions of several possible mechanisms.

Decrease in Synaptic Strength Is Not Due to Metabotropic Glutamate Receptors, Desensitization, or Inhibition

Takahashi et al. (1996) demonstrated that at the calyx of Held synapse in rat brain stem slices, transmitter release can be inhibited by metabotropic glutamate receptors (mGluRs) through a reduction in the presynaptic calcium current, causing a decrease in synaptic strength (see also Choi and Lovinger, 1996). To check whether feedback of released glutamate contributes to the decrease in synaptic strength observed in our experiments at hippocampal synapses, we used nonminimal stimulation and measured excitatory postsynaptic current (EPSC) amplitudes during repeated trains of 20–40 stimuli at 10 Hz (depletion protocol) before and during application of either an antagonist or agonist to mGluR receptors. We looked first at the effects of the antagonist MCPG (250 μ M; $n = 3$), which blocks the mGluR pathway

with $u(t) = 1.24 \pm 0.15$, and $v\beta = -0.41 \pm 0.05$. The dashed line is a simplification of the above equation ($u(t) = 1$, $v\beta = -0.5$) to

$$f = \frac{1 - (1 - p)^{\frac{1}{p}}}{p}$$

The dotted line at 1 indicates no facilitation. Data are from 38 single synapses. $[Ca^{2+}] = 2.5$ mM; $[Mg^{2+}] = 1.3$ mM.

(B) Release probability of the second pulse is highest at synapses with high release probability on the first pulse for 38 single synapses from (A). The dotted line indicates unity (no facilitation). The solid line is the same relationship from (A)

$$p_2 = 1 - (1 - p)^{u(t)p^\beta}$$

with $u(t) = 1.24 \pm 0.15$, and $v\beta = -0.41 \pm 0.05$, where p_2 is the release probability on the second pulse, and p is the release probability on the first pulse.

(C) Plot of potency (average amplitude of response when release occurs) on the second pulse versus potency on the first shows that paired-pulse facilitation causes no change in synaptic potency at single synaptic release sites. The dotted line indicates unity (no change in potency). The inset shows an example of average EPSC of releases for the first pulse versus the second pulse.

(D) Potency is not correlated with release probability for synapses in (A) for either the first pulse (closed circles) or the second pulse (open triangles).

(Eaton et al., 1993); the decay in the synaptic response during the train was still present. In separate experiments, we used the broad-spectrum mGluR agonist trans-ACPD (100 μ M; $n = 3$) (Palmer et al., 1989; Baskys and Malenka, 1991) to maximally activate the mGluR pathway and occlude feedback from released glutamate. Again, the decline in synaptic strength was still present. Clearly, the effect we identify as depletion is not caused by inhibitory feedback of released glutamate through presynaptic metabotropic glutamate receptors.

Another possible explanation is that the observed decrease in synaptic response during the high frequency stimulation is actually a desensitization of the postsynaptic glutamate receptors. To test the role of desensitization in our experiments, we repeated the control experiment (nonminimal stimulation) before and after addition of 100 μ M cyclothiazide ($n = 3$), which blocks AMPA receptor desensitization (Bertolino et al., 1993; Zorumski et al., 1993). Blocking desensitization did not eliminate the decrease in response size during trains of fast stimuli.

In all of our experiments, picrotoxin and bicuculline methiodide were present to block inhibitory GABA_A synapses. GABA_B responses were unlikely to be present, especially in the single synapse experiments, due to the low stimulation levels used (typically 20–80 μ A). To verify that the observed decrease in synaptic strength was not due to GABA_B, we repeated the control experiment before and after addition of 200 μ M hydroxysaclofen, a GABA_B antagonist. For this control, high stimulus levels were used (200–500 μ A) to activate GABA_B currents. Although hydroxysaclofen eliminated the GABA_B response, the depletion was still present.

Reduction in Response Size with High Frequency Stimulation Is a Decrease in Release Probability

As seen in Figures 1 and 2, the average response size for a population of synapses decreased due to repetitive stimulation, either immediately or after the first few stimuli. When a population of synapses is being studied, it is not directly evident whether the decrease in response size resulted from a presynaptic change in transmitter release or from a diminished size of the postsynaptic response.

At these synapses, transmitter release due to the arrival of a nerve impulse is probabilistic at individual synapses, and does not always occur. We therefore wanted to determine both release probability and synaptic potency (average response size, when a release occurred) as a function of stimulus number in a high frequency train. For a single synapse, a train of action potentials will give a sequence of releases and failures. By applying many repetitions of the stimulus train, then counting the fraction of the trains that give a release for the first action potential in the train, for the second action potential, etc., we estimated the release probability for each stimulus in the train. We also can measure the potency for each stimulus in the train. These experiments used a high calcium/low magnesium perfusion solution (4 mM Ca²⁺/1 mM Mg²⁺) to minimize the effects of facilitation (Manabe et al., 1993), as well as to promote transmitter release and enable more rapid depletion.

For these experiments, we have used the following

stimulation protocol: a train of stimuli (each stimulus epoch consists of 15–40 stimuli presented at 10 Hz) was used to cause the decrease in synaptic strength, and a 20–30 s rest period was given to permit the synapse to recover; then another train was given, etc. The number of stimulus epochs presented to a particular synapse was usually \sim 120 (ranging from 27–280). For each synapse, the subset of stimulus epochs for which release properties were stable was identified (average: 50; range: 23–106) and used for analysis. For each stimulus in each epoch, the amplitude of the response was measured, and release failures were tallied. Then the fraction of the stimuli that produced a release and the potency were computed for each stimulus in the epoch.

The results of one such experiment are illustrated in Figure 4A. Release probability started at \sim 0.43 and declined approximately exponentially to a steady level of \sim 0.05. At the same time, potency remained unchanged through the stimulus train as is illustrated in Figure 4B. Thus, this decrease in synaptic strength is manifest as a decline in release probability without a change in the synaptic strength. This also directly demonstrates that desensitization is not occurring during these high frequency stimulus trains. We interpret this as a presynaptic effect, which we refer to from now on as depletion.

For some synapses (particularly those whose initial release probability was quite high), the release probability followed a simple, nearly exponential decline (for example, the synapse illustrated in Figure 2A). Other synapses, usually those with a lower initial release probability, exhibited a response pattern like that illustrated in Figure 4C where the release probability increased for the first few stimuli and then declined approximately exponentially. In this case as well, potency remained unchanged through the course of the stimulus train (see Figure 4D), demonstrating again that depletion is manifest through release probability and not as a change in potency. One of these two patterns was observed in 13 recordings from cells stimulated at single synaptic release sites.

Decrease in Release Probability Is Due to the Depletion of a Readily Releasable Vesicle Pool

We propose that the observed decrease in release probability during rapid stimulation reflects the depletion of the vesicles available to be released (Thies, 1965; Mallart and Martin, 1968; Bennett et al., 1976). A pool of readily releasable vesicles has been defined for cultured hippocampal synapses (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996) and retinal amacrine cell synapses (Borges et al., 1995). In cultured hippocampal cells, the readily releasable pool size can be estimated by either counting the quanta released per synapse in response to the application of hypertonic solution (Stevens and Tsujimoto, 1995), or by rapidly evoking release by action potentials and counting the number of quanta released before depletion (Rosenmund and Stevens, 1996). In the single synapse depletion experiments above, counting the number of quanta released before the release probability decays to zero provides a way to measure the readily releasable vesicle pool for single synapses in hippocampal slices.

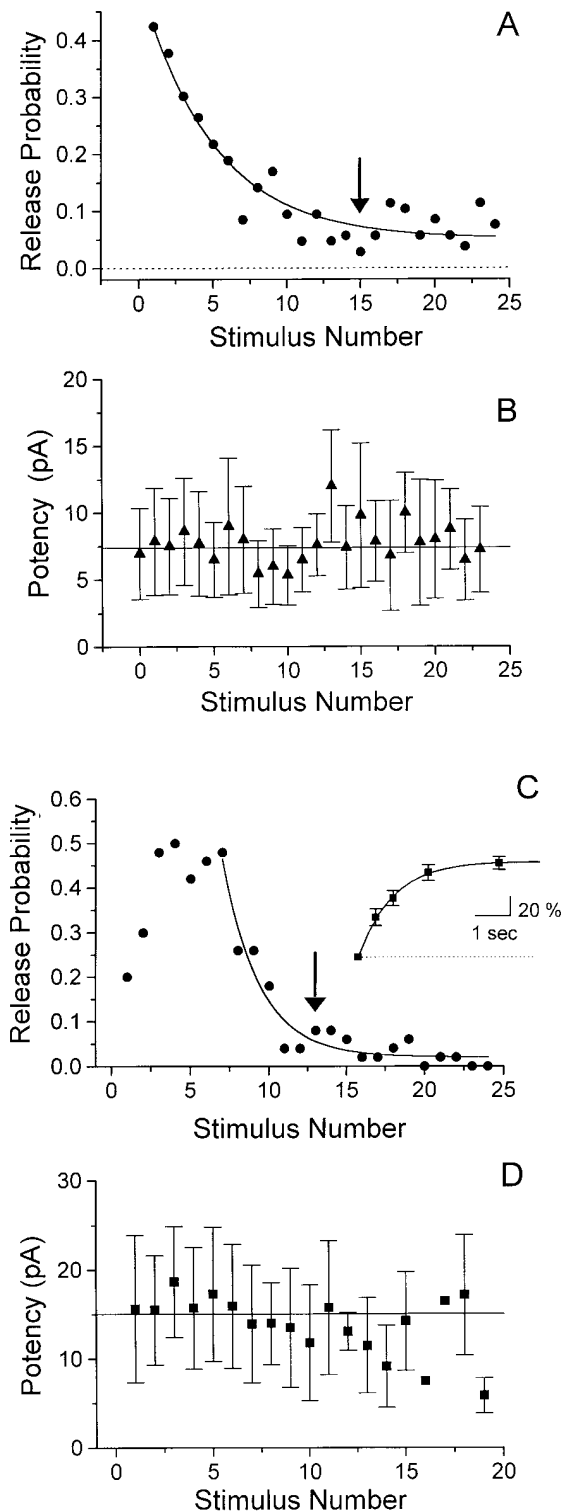


Figure 4. Change in Synaptic Response during High Frequency Stimulation Is Due to Modulations of Release Probability, with No Change in Potency of Responses at Single Synapses

(A) Release probability versus stimulus number during trains of 24 pulses at 10 Hz. In this example, release probability is initially high (0.43) and declines approximately exponentially. The solid line is the exponential fit to decay phase ($\tau = 4.9$). The pool is considered depleted when release probability has declined by 95% (3 decay

Size of the Readily Releasable Pool

How large is the readily releasable pool for the synapses we have studied in slices? For each of the 13 synapses, the number of stimuli needed to produce depletion was calculated from the estimate of release probability versus stimulus number (see Figures 4A and 4C). The decay phase of the curve was fit with a single exponential; the synapse was considered depleted when the release probability had declined by 3 exponential decay constants (95%) from its maximum (indicated by an arrow in Figures 4A and 4C). The number of stimuli to reach depletion, N_d , averaged 15.2 ± 2.6 (range 12–20) at 10 Hz stimulation. For each stimulus epoch, the number of releases in the first N_d stimuli was counted. The average number of quanta released during the approach to a low steady state we refer to as the “functional” pool size; for 13 putative single synapses, the functional pool size ranged from 1.30–11.9 quanta with an average of 5.0 ± 3.0 . Note that this pool size was determined for repeated epochs in which the average pool size was not varying systematically over time. This value is comparable to the results obtained in cultured cells, where the size of the readily releasable pool was found to average 15.7 (Stevens and Tsujimoto, 1995).

The refilling of empty sites in the readily releasable pool occurs at a low rate; the time constant for refilling has previously been estimated to be ~ 10 s (Stevens and Tsujimoto, 1995). It is possible, however, that the refilling rate depends on the rate of stimulation and/or the extracellular $[Ca^{2+}]$. We estimated the refilling rate under our experimental conditions by giving a 16 stimulus depleting train, followed after a variable recovery time interval by another 16 stimulus train to measure the amount of refilling. An example of the time course of refilling is indicated in the inset to Figure 4C. The time constant for refilling was 2.8 ± 2.0 s ($n = 6$). In our experiments, the synapses depleted rapidly, and the pool size was measured in 1–2 s (12–20 stimuli at 10 Hz). The refilling rate of 2.8 s measures the refilling of the pool from the completely empty state; during the depletion experiments, the pool is still partially filled for much of the time, during which refilling should be slower. In addition, the release probability decayed to zero or nearly zero at all of the synapses (0.090 ± 0.132 ; $n = 13$), indicating that refilling was slow compared to the depletion rate. Taken together, these results suggest that the amount of refilling likely to have occurred during

constants) from its maximum. In this example, N_d , the number of stimuli to deplete the synapse, was 15 (indicated by the arrow).

(B) Potency versus stimulus number for experiment (A). Potency does not change despite the changes in release probability.

(C) Release probability versus stimulus number for a different synapse. In this example, the release probability is initially low (0.20) and increases during the first few stimuli, after which it declines approximately exponentially. The solid line is the exponential fit to decay phase ($\tau = 2.2$). The pool is considered depleted when release probability has declined by 95% (3 decay constants) from its maximum; in this example, $N_d = 13$ (indicated by the arrow). The inset shows an example of the time course of recovery from depletion.

(D) Potency versus stimulus number for experiment (C). Again, synaptic potency does not change despite the changes in release probability.

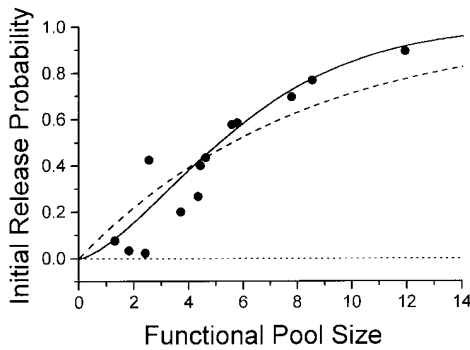


Figure 5. Initial Release Probability versus Functional Pool Size
The initial release probability (for the first stimulus in the train) is related to the functional pool size (average number of releases until the synapse is depleted) for a population of synapses. The pool is considered depleted when release probability has declined by 95% (3 decay constants). The solid line is the fit of the equation

$$p(n) = 1 - e^{-kn^3/2}$$

where p is release probability, n is pool size, and $k = 0.06$ (derivation of the equation is in the Appendix).

the measurement of pool size should be small; what refilling did occur will cause the measured pool sizes to be an overestimate of the actual pool sizes.

Release Probability Is Directly Related to the Readily Releasable Pool Size

We have demonstrated that depletion is manifest as a decrease in release probability. Implicit in our discussion is the notion that release probability is determined in part by pool size: as the size of the readily releasable pool decreases, the probability of a release also decreases. One way to check this suggestion is to examine the relationship between pool size (as determined in the previous section) and release probability for a single stimulus (the first stimulus of a train). This relationship is presented for 13 putative single synapses in Figure 5 where the release probability is plotted as a function of the readily releasable pool size; here, the functional, rather than maximal (defined later), pool size has been used. Clearly, the relationship is monotonically increasing and orderly. The smooth curve is the fit of the equation

$$p(n) = 1 - e^{-kn^3/2}$$

where $p(n)$ is the release probability for a synapse with a functional pool size of n , and $k = 0.06$. A possible derivation of this equation is given in the Appendix.

Pool Size Varies from Trial to Trial

Interestingly, the pool size (determined by the number of quanta released by a synapse in response to a train) varies from stimulus epoch to stimulus epoch. An example of the distribution of quanta released per epoch is illustrated in Figure 6: the average number was 8.5, but number released varied from 5–13 for this particular synapse. As discussed above, little refilling of the readily releasable vesicle pool is likely to have occurred during measurement. The maximum number of vesicles released therefore should be the maximal size of the readily releasable pool. For the synapse represented in Figure 6, that maximal pool size would be 13; for the 13

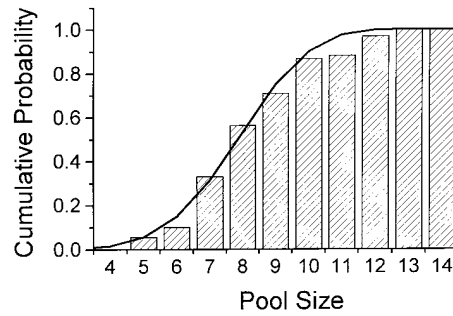


Figure 6. Distribution of Pool Size Estimates for Multiple Trials at a Single Synapse

Pool size is the number of releases counted in the first 14 stimuli (number needed to deplete pool) of the 10 Hz stimulus train. Average pool size is 8.5 ± 1.9 . The bars represent measured data points; the solid line is the binomial fit to the data ($N = 13$; $p = 0.639$).

putative single synapses, the maximal pool size averaged 8.8 ± 3.3 (range: 4–16). Calculation of the serial correlation coefficient reveals that the pool size varied at random from one stimulus epoch to the next.

Is this truly a variation in the pool size from trial to trial? One alternative possibility is that some fraction of the vesicles are released asynchronously, in between stimuli or after the stimulus train is over. If a significant number of vesicles were released asynchronously, they could contribute to depleting the pool without getting counted (in experiments of Figures 5 and 6, only quanta whose release was synchronized by the stimulus were counted). Another possibility is that the pool is not actually completely depleted during each trial. This would cause an underestimate of the total pool size, and could cause it to appear to vary from trial to trial. We investigated these two possibilities below.

Pool Size Measurement Is Not Affected by Asynchronous Component of Release

We checked whether quanta were released asynchronously during the experiments to measure pool size; the above measurements of pool size assume that all quanta are released immediately after action-potential stimulation. However, an asynchronous component of release has been demonstrated at these synapses that is normally very small (Goda and Stevens, 1994). If high frequency stimulation were to potentiate this asynchronous component, some vesicle release might have occurred and not been included in the count of quanta used to measure pool size. To examine this possibility, we measured all asynchronous events during 2 s of stimulation at 10 Hz and for at least 1 s after the end of stimulation. Most of these events were likely to be spontaneous releases from other synapses on the cell and had different EPSC shapes. We therefore counted the resting rate of spontaneous releases for 1 s before each stimulation, and subtracted this rate from the asynchronous rate. We observed no remaining asynchronous component to release during these experiments ($n = 5$); this suggests that the asynchronous release does not contribute additionally to pool depletion. One possibility is that the asynchronous release rate per synapse is too low to be detected at a single synapse under these experimental conditions. Another possibility is

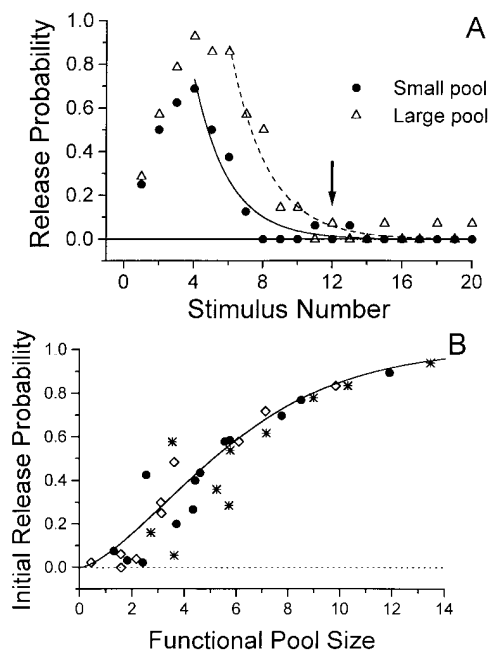


Figure 7. Depletion at a Single Synapse: Comparison of Subset of Trials for Which the Pool Size Was Less than the Median Number ("Small" Category) and the Other for Which the Pool Size Was the Median Number or Greater ("Large" Category)

(A) Release probability versus stimulus number for the "small" pool subset of stimulus epochs (closed circles), which had 4 or fewer releases in the first 12 stimuli (average 3.1). The solid line is the exponential fit for small pool data (A) with $\tau = 2.0$ stimuli. Release probability versus stimulus number for the "large" pool subset (open triangles), which had 5 or more releases in the first 12 stimuli (average 5.7). The dashed line is the exponential fit for large pool data (B) with $\tau = 2.2$ stimuli.

(C) Initial release probability versus functional pool size for small (hollow diamonds) and large (*) pool subsets. Of the 13 synapses in Figure 5, 10 had enough trials to break into subsets of large pool and small pool. Combined data for all trials (from Figure 5) is shown with closed circles. The solid line is the equation

$$p(n) = 1 - e^{-kn^3}$$

where p is release probability, n is pool size, and $k = 0.06$ (derivation of the equation is in the Appendix).

that the synchronous and asynchronous release mechanisms draw from the same pool of vesicles that is completely depleted by the synchronous component of release. In either case, counting only the synchronously evoked quanta should provide an accurate estimate of the total released quanta (pool size) under our experimental conditions.

Variability in Measured Pool Size Is Not Due to Incomplete Pool Depletion

We next investigated the possibility that some or all of the variability in the measured pool size was due to incomplete depletion of the readily releasable pool during the first N_d stimuli used to count quanta. If this were true, trials for which the number of released quanta was small should have a higher release probability after the N_d stimuli than trials with a higher measured pool size.

We tested this hypothesis as follows. For the 10 data sets that were large enough, we divided single synapse

responses to stimulus epochs into two categories, one for which the pool size was less than the median number ("small" category) and the other for which the pool size was the median number or greater ("large" category). The average pool size for 10 experiments for the "small" category was 3.9 ± 2.9 quanta (range: 0.44–9.8) and, for the "large" category, was 6.6 ± 3.4 quanta (range: 2.7–13.4). For each synapse, release probability versus stimulus number was calculated separately for the two subsets of "small" pool and "large" pool data.

A typical result is illustrated in Figure 7A. For the "small" case (closed circles), the measured pool size averaged 3.1 releases in the first 12 stimuli, while in the "large" case (open triangles), it averaged 5.5 releases in the first 12 stimuli. However, for the "small" pool case in Figure 7A, the release probability decays to zero earlier (fit to solid line) than in the large pool case (fit to the dashed line). Clearly, the smaller measured pool sizes are not due to incomplete emptying of the pool. These same observations have been made in nine other synapses where we had sufficient data to divide into two categories.

As seen in Figure 7, the initial release probability also differs for the "large" pool and "small" pool subsets. Is the relationship between release probability and pool size the same as in Figure 5? Figure 7B shows the data points for the "small" pool subset (open diamonds), the "large" pool subset (asterisks), and the entire data set (closed circles) for each synapse. All three sets of data are well fit by the same relationship between initial release probability and average pool size.

Depletion versus Depression

The phenomenon we have designated "depletion" is sometimes also called "depression." Since release occurs so rapidly after calcium influx (Borst and Sakmann, 1996; Sabatini and Regehr, 1996; Almers and Tse (1990) have argued that only vesicles from a special fusion competent pool already docked to the membrane could be released in the time available. Analysis of the proteins involved in docking and exocytosis suggests a similar picture (see Südhof, 1995). The physiologically defined readily releasable pool is, then, naturally identified with the special fusion competent vesicle pool. Our depletion therefore would occur by using up vesicles in this pool. But one can imagine quite different mechanisms for depression whose properties might seem much like depletion. The pharmacological controls presented above have ruled out desensitization of the receptors, inhibitory feedback through presynaptic mGlu receptors, and build up of GABA_B inhibition. Other possible mechanisms, such as an accumulation of calcium channel inactivation or of some other unknown inhibitory factor, could decrease the release probability and appear formally like depletion.

Although purely electrophysiological experiments cannot decide between various formally equivalent physical mechanisms, there is a distinction between "depression" and "depletion" classes of mechanisms that we can make: for depression due to the accumulation of an inhibitory factor, the state of the synapse depends on how many releases have occurred or how many stimuli have been given, whereas for depletion, the state of the synapse depends on how much of the pool (or, more

generally, of the resource that can be depleted) remains. That is, we can distinguish between depletion-class and depression-class mechanisms by determining whether the state of the synapse depends on how many have been released or on how much (of the depletable resource) is left. This discrimination is possible in our case because the functionally defined readily releasable pool fluctuates in size from stimulus epoch to stimulus epoch, as we described above.

If a depression-class mechanism holds, then release probability should follow the same initial time course independent of the number of quanta eventually released because each stimulus (or perhaps each release) would produce the accumulation of the depressive factor. Alternatively, if the mechanism falls into the depletion class, the responses to early stimuli in the train should be different, but the release probability should decline in a similar way once any facilitation is maximal and the pool has depleted to a certain size. The decay should be the same shape, then, for the "large" and for the "small" classes of records, but should be delayed for the "large" class relative to the "small." As is apparent from the data presented in Figure 7 for one experiment, our data fall into the depletion rather than the depression class. This same observation was made for all 10 synapses where sufficient numbers of trials were measured in order to analyze large and small pools separately.

Discussion

Synapses are not all alike. Prior experiments using several different techniques have indicated that hippocampal synapses are heterogeneous in release probability. Rosenmund et al. (1993) concluded that release probabilities were nonuniform at cultured hippocampal synapses from analysis of the progressive block of synaptic currents by the use-dependent blocker MK-801. Allen and Stevens (1994) found a broad distribution of the failure probabilities of synapses on CA1 pyramidal cells in slice when stimulated by minimal stimulation. Using optical detection of Ca^{2+} transients through NMDA receptors, Murphy et al. (1995) showed that synapses on the same CA1 cells in hippocampal slice differed in their probability of response to stimulation. And in hippocampal cultured cells, Murthy et al. (1997) report, based on optical measurements of the fluorescent membrane probe FM1-43, that most synapses in hippocampal cultures have a low release probability, and that the distribution of release probabilities of synapses made by a single neuron is broad. Our results again demonstrate nonuniform release probabilities that vary over a wide range, both at normal $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentrations (2.5 mM $\text{Ca}^{2+}/1.3$ mM Mg^{2+}) and with high extracellular Ca^{2+} to enhance release (4.0 mM $\text{Ca}^{2+}/1.0$ mM Mg^{2+}).

Earlier experiments have raised the possibility that facilitation is not homogeneous across synapses, even synapses between the same types of cells (Malinow et al., 1994; Debanne et al., 1996). Our results, however, directly demonstrate significant heterogeneity in paired-pulse facilitation within a subpopulation of synapses. We quantitate this effect, and find that the amount of facilitation is directly related to initial release probability.

Low probability synapses exhibit much more facilitation than do high probability synapses, making measurements of average facilitation across populations of synapses harder to interpret. Estimates of facilitation using optical techniques independently demonstrate that in cultured hippocampal neurons, the amount of paired-pulse facilitation depends strongly on release probability (Murthy et al., 1997). Note that the relationship between release probability and facilitation is in quantitative agreement for the two different methods (compare our Figure 3 with Figure 4 of Murthy et al., 1997). The observation that the success probability for the second pulse depends on that for the first pulse (Figure 3B) is different than that observed previously in CA1 cells using minimal stimulation (Stevens and Wang, 1995), where the probability was about 0.9, independent of the initial probability. Although slightly different stimulus protocols were used (stimulus rate, interpulse interval), they do not seem to account for the difference. We are not sure why the result is different; the synapses presented in the earlier paper seem to be atypical in that respect. Possibly the sample was inadvertently biased toward synapses with unusually high paired-pulse facilitation.

Enhancing the synaptic release probability by raising extracellular Ca^{2+} concentrations has been shown to reduce or eliminate paired-pulse facilitation measured in populations of synapses (Creager et al., 1980; Manabe et al., 1993). However, when looking at single synapses recorded with 4.0 mM $\text{Ca}^{2+}/1.0$ mM Mg^{2+} , significant facilitation was still present at low probability synapses (e.g., Figure 4C). High probability synapses have little or no facilitation, and may exhibit paired-pulse depression, as seen in Figure 4A; these high probability synapses dominate the population average.

Trains of high frequency stimuli cause a reduction in the size of EPSCs for a population of synapses. By measuring the response at single synaptic release sites, we confirm that this is a presynaptic phenomenon caused by a reduction in release probability; this agrees with results obtained by Larkman et al. (1991) using quantal analysis. In addition, the number of stimuli needed to deplete the synapse varies across synapses. We measured the number of vesicles released prior to depletion as an estimate of the size of this readily releasable pool. This measurement depends on being able to deplete the pool before significant vesicle recycling can take place to replenish it. By using high frequency stimulus trains and high extracellular $[\text{Ca}^{2+}]$, synapses were depleted in ~ 1 s. In cultured hippocampal synapses, the readily releasable pool refills with a time constant of ~ 10 s (Stevens and Tsujimoto, 1995); we measured the refilling rate at 2.8 ± 2.0 s. This slow rate of refilling suggests that our estimates of pool size were not seriously influenced by refilling.

The single synapses studied here have a small pool of readily releasable vesicles, and as this pool is depleted by repeated stimulation, the release probability declines. Thus, release probability appears to depend on the size of the readily releasable pool. We find, indeed, that pool size (estimated by the number of quanta needed to deplete the pool) is related to release probability across the population of synapses studied. And by subdividing the data from each synapse into trials

with "small" versus "large" pool sizes, we also show that release probability is related to pool size at each individual synapse.

Our interpretation of these experiments rests on the notion of a vesicle pool that is depleted, and our conclusions are consistent, given our operational definition of pool size. The main outstanding problem, however, is the physical interpretation of our "readily releasable pool." Several lines of evidence suggest that this pool corresponds at least roughly to the morphologically defined pool of docked vesicles. First, nerve impulse release draws on the readily releasable pool and must also draw on the pool of docked vesicles. This implies that our readily releasable pool is a subset of the docked vesicles. Second, the size of the readily releasable pool varies from synapse to synapse but averages 8.8 (for the maximal pool size), whereas the morphologically defined docked vesicle pool, which also varies from bouton to bouton, averages 10 vesicles (Schikorski and Stevens, 1997). Third, the distribution of release probabilities matches the distribution of docked vesicle pool size (Schikorski and Stevens, 1997). Finally, in a single preliminary experiment (Jacobs et al., unpublished data), the readily releasable pool size equaled the docked vesicle pool size when the same synapse in culture was studied physiologically and then reconstructed from electron micrographs. Although the identification of the readily releasable pool with the morphologically docked vesicles is plausible, this identification is far from established. What is required is a manipulation of the readily releasable pool size and a morphological demonstration that the docked vesicle pool varies correspondingly. From a functional point of view, however, the physical mechanism is immaterial: synapses behave as if they have a readily releasable pool of a small size that can be depleted, and the synapse's release probability is partly determined by the size of this pool. Thus, synaptic strength varies with the history of synapse use in the same manner it would if "depletion" actually corresponds to a using up of docked vesicles.

We must stress that although the mechanism studied here falls into the depletion class rather than the depression class (as described above), this does not imply physical depletion of vesicles or some other limited resource. Specifically, these classes are defined according to whether the state of the synapse depends on the history of use (release or occurrence of action potentials) or on the present content of the operationally defined pool. One can imagine mechanisms that do not involve depletion of a resource that would fall into the depletion class. Our result, however, excludes at least the simplest version of likely depression-class mechanisms like inhibitory feedback through released glutamate or an accumulation of calcium channel inactivation with repeated stimulation.

In summary, we have presented a consistent picture of synapse function in which a small pool of docked vesicles are drawn upon for release, and release probability depends on the size of this pool. Continued use of the synapse depletes this pool after a handful of releases, and release probability declines correspondingly. Thus, synaptic strength varies with the history of synapse use to provide an "automatic gain control",

which could be used to keep the signal in an appropriate operating range. The strength of the synaptic "gain" depends in large part on the number of vesicles in the readily releasable pool.

Experimental Procedures

Experimental Preparation

Transverse hippocampal slices were prepared from Long Evans rats (ages P11–P18, mostly P13–P15). Rat was anesthetized by the volatile anesthetic Metofane, and then quickly decapitated. The brain was removed and rapidly cooled by immersion in ice-cold solution composed of (in mM): NaCl, 120; KCl, 3.5; CaCl₂, 0.7; MgCl₂, 4.0; NaH₂PO₄, 1.25; NaHCO₃, 26; and glucose, 10. After the cerebellum was removed, the resulting flat surface was glued to the pan of a DSK Microslicer vibratome using cyanoacrylate glue. Slices (400 μm) were cut by the vibratome, then the hippocampus was carefully dissected out of each slice. All of the above dissection was done in ice-cold solution (composition above) with high [Mg²⁺] and low [Ca²⁺] to prevent synaptic release and minimize injury to the cells. Slices were then transferred to a holding chamber where they were stored submerged in room temperature (~22°C) solution (composition described above), which was bubbled with 95% O₂/5% CO₂. Slices were stored in the holding chamber for >1.5 hr prior to recording.

During the experiment, slices were perfused with solution composed of (in mM): NaCl, 120; KCl, 3.5; CaCl₂, 2.5; MgCl₂, 1.3; NaH₂PO₄, 1.25; NaHCO₃, 26; and glucose, 10. Picrotoxin (50 μM) and 50 μM bicuculline methiodide were added to block inhibitory responses; to prevent recurrent excitation, the CA3 region was surgically removed from each slice prior to use. The solution also contained 100 μM D-APV in order to block possible LTP or LTD. The solution was bubbled with 95% O₂/5% CO₂, and the pH was adjusted to 7.35 using NaOH. All recordings were done at room temperature (~22°C). Mg²⁺ and Ca²⁺ concentrations were modified in some experiments as specified below.

MCPG ((+)-α-Methyl-4-carboxyphenylglycine), trans-ACPD ((1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid), (-)-Bicuculline methiodide, 1(S), 9(R), and 2-Hydroxysaclofen were obtained from Research Biochemicals International. (+)-APV ((+)-2-Amino-5-phosphonopentanoic acid) was obtained from Tocris Cookson. All other chemicals were from Fisher Scientific or from Sigma.

Experimental Protocol

Whole-cell voltage-clamp recordings were made from CA1 pyramidal cells using 2–4 MΩ pipettes. Pipettes were filled with a solution containing (in mM): K gluconate, 170; HEPES, 10; NaCl, 10; MgCl₂, 2; EGTA, 1.33; CaCl₂, 0.133; MgATP, 3.5; GTP, 1.0 (pH 7.2). The CA1 cell membrane potential was held at –60 mV; access resistance was monitored, and only cells with stable access resistance during the experiment were included in the analysis. The Schaeffer collateral pathway was stimulated with 100 μs duration pulses via a tungsten bipolar electrode, and EPSCs were recorded at 5–8 KHz.

In the experiments shown in Figure 1 and in control experiments where indicated, a moderate amplitude stimulus was used to stimulate many axonal fibers and a population of synapses.

In the remaining experiments (Figures 2–7), the technique of minimal stimulation (Raastad et al., 1992; Allen and Stevens, 1994; Raastad 1995; Stevens and Wang, 1995) was used to putatively stimulate one axonal fiber making one synapse on the CA1 cell recorded from. Pulse pairs (40 ms interval) were applied at 0.25 Hz while adjusting stimulus strength to find conditions of minimal stimulation. Our criteria for minimal stimulation as well as examples and discussion of analyses are described below.

For paired-pulse facilitation experiments (Figure 3), pulse pairs (40 ms interpulse interval) were applied at 0.25 Hz. The extracellular solution contained 2.5 mM CaCl₂ and 1.3 mM MgCl₂.

For the depletion experiments (Figures 4–7), stimuli were presented as trains of 16–40 pulses at 10 Hz, which were repeated many times separated by rest intervals of 16–30 s. Success probability and average potency were computed independently for each stimulus in the train by averaging across the multiple presentations of the

train. The depletion experiments were done with high extracellular Ca^{2+} (4 mM CaCl_2 and 1 mM MgCl_2) to minimize the amount of facilitation (Manabe et al., 1993).

Minimal Stimulation Technique and Analysis

The technique of minimal stimulation has been frequently used since its introduction by Raastad et al. (1992). Different people have used the term "minimal stimulation" to refer to a variety of stimulation conditions, assessed by different criteria. This has led to some confusion. We describe here in greater detail what we mean by minimal stimulation, showing examples of raw data and elaborating on the method of analysis.

Our goal in using minimal stimulation is to stimulate only one synapse (with one synaptic release site) on the postsynaptic cell. Our criteria for minimal stimulation are that (1) the average size of the response and the failure probability be independent of stimulus intensity over a range of $\sim\pm 5\%$ from the stimulus intensity used for gathering data; (2) the latency and shape of the individual synaptic currents be invariant for repeated stimuli (although the amplitude varies); (3) the quantitative characteristics of the synaptic response (release probability, average response size, latency and shape of synaptic current) remain invariant, under standard stimulation conditions, throughout the period of data collection; and (4) turning the stimulation down to lower intensities leads abruptly to a sudden and complete failure of responses. We have found no instances for which these criteria are met, and the mean response size, excluding release failures, exceeds the average amplitude of a single quantum ($< \sim 0.5$ nS for slices). We sometimes find cells for which criterion (1) is met (no change in response characteristics with changes in stimulus intensity), but at least two different response latencies and/or shapes are seen from stimulus to stimulus (criterion [2] is not met); we have excluded these cells from our analysis. We also sometimes find cells for which criterion (2) is met, but criterion (1) is not. These also were excluded. Criterion (3) is also very important, and many cells that met the first two criteria were discarded because response properties changed over the course of the experiment.

Figure 8A shows eight individual traces from a typical recording of a putative single synaptic release site to paired-pulse stimulation. These eight traces were recorded from consecutive stimulations, and illustrate that it is quite possible to separate failures of transmitter release from successful release events. For this synapse, the initial release probability was quite low (0.109 ± 0.028 ; 128 stimulus pairs were used to estimate release probabilities); note that in these eight traces, it failed to release transmitter on all of the first pulses. For the second pulse, the release probability was higher (0.421 ± 0.044), showing clear paired-pulse facilitation. It is possible to easily classify most of the second pulses as either clear releases (marked with *) or clear failures (not marked). For the four events marked as releases, note the similarity in latency and shape, as well as the variability in amplitude. The first trace (marked with a ?) is less clear; it is representative of the small fraction of traces ($\approx 2\%$ – 3%) in each experiment that are harder to classify. To make the classification procedure as objective as possible, all classification was done using an automated analysis program as described below. For all minimal stimulation experiments, each EPSC record was examined and classified as either a failure or release; for releases, the peak amplitude was recorded. Release probability and average potency (amplitude of the response of the releases) were computed.

The automated analysis program to classify trials as releases or failures did the following: first, a preliminary template trace was created, which was the average of many traces in which the integral (for a window of 20–30 ms after the first stimulus) was greater than a threshold value. Then, the preliminary template was fit to each trace, and a subset containing all traces with the same shape and latency was averaged to form the final template. In most cases, the final template was indistinguishable from the preliminary template; however, if any trials had release events with different shape and latency (due to spontaneous events from other synapses), these events were not included in the final template. Next, all trials were compared to the final template to be classified as releases or failures. First, the maximum amplitude was measured within the selected window; traces with amplitudes less than the threshold of

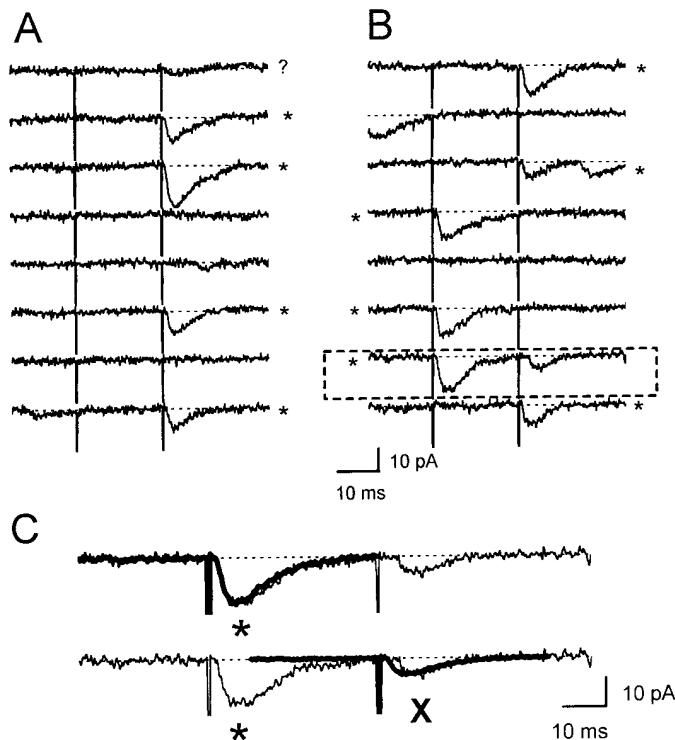


Figure 8. Individual Traces (EPSC versus Time) from a Typical Recording of a Putative Single Synaptic Release Site

Stimulation was applied as pulse pairs (40 ms interpulse interval) at 0.25 Hz. For this synapse, the initial release probability was quite low (0.109 ± 0.028 ; 128 stimulus pairs were used to estimate release probabilities). For the second pulse, the release probability was higher (0.421 ± 0.044), showing clear paired-pulse facilitation.

(A) Eight consecutive recordings from a single synapse. Note that in these eight traces, the synapse failed to release transmitter on all of the first pulses. It is possible to easily classify most of the second pulses as either clear releases (marked with *) or clear failures (not marked). The first trace (marked with a ?) is less clear; it is representative of the small fraction of traces ($\approx 2\%$ – 3%) in each experiment that are harder to classify.

(B) Eight additional consecutive recordings from the same synapse. Releases are marked with *; failures are not marked. Analysis of a trace indicated by the dotted box is illustrated in (C).

(C) Fit of template (scaled, thick line) to response (thin line) for the first pulse and the second pulse for the trace indicated in (B) by the dotted box. Note that template fit is excellent for the first pulse; this is automatically detected by the analysis program as a

release. For the second pulse, the same template (thick line) is shifted by 40 ms and scaled to peak amplitude of response. Notice that initially the two curves are quite different; the latency of the response is greater than the template. This response is classified as an asynchronous event or spontaneous release from another synapse, and therefore as a failure of evoked release from this synapse (marked with X).

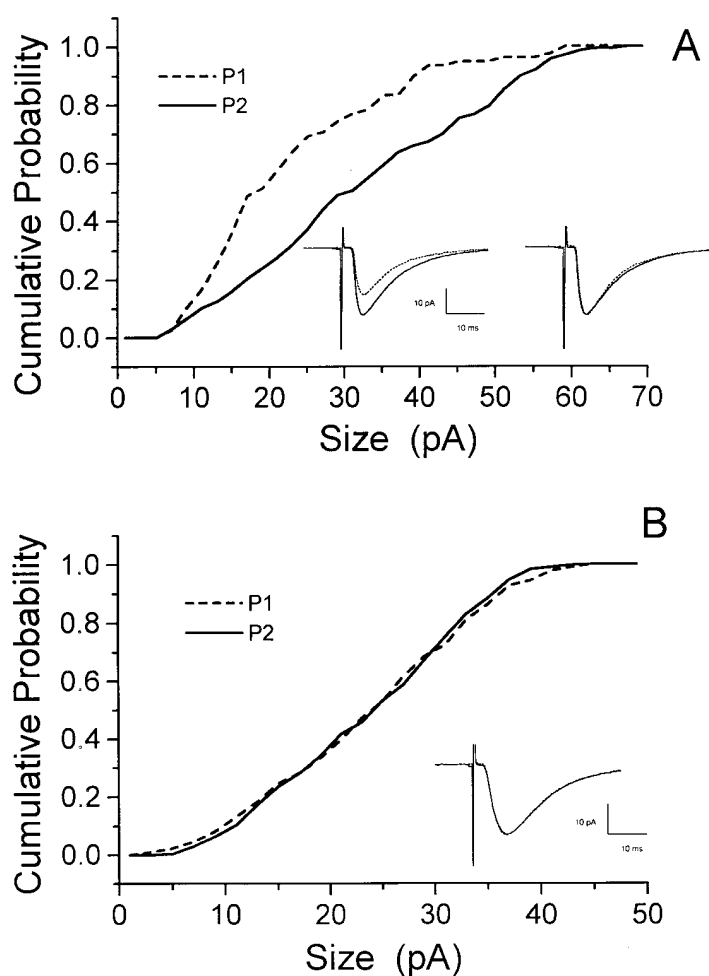


Figure 9. Comparison of Cumulative Amplitude Histogram for the First Pulse and the Second Pulse of a Putative Single Synapse (A) Cumulative amplitude curve for the second pulse (solid line) is clearly shifted to the right (larger amplitudes) as compared to the first pulse (dashed line). Release probability increased from 0.203 ± 0.021 for the first pulse to 0.356 ± 0.024 for the second pulse (384 trials). The inset to the left shows that average EPSC versus time for successes is much larger for the second pulse (solid line, 32.9 ± 15.5 pA) than for the first pulse (dashed line, 23.1 ± 12.6 pA). The inset to the right shows the EPSC averages scaled to the same maximum amplitude; the shapes of the curves are not different. Because of the increase in amplitude of the second pulse due to paired-pulse facilitation, this recording is believed to be of a compound synapse with more than one release site. Data from experiments that seemed to contain more than one release site were not included in our results. (B) Example of a synapse that seems to have only one release site. Release probability increased from 0.299 ± 0.019 for the first pulse to 0.476 ± 0.020 for the second pulse (608 trials). Cumulative amplitude curve for the second pulse (solid line) overlies that for the first pulse (dashed line). The two curves are not statistically different according to the Komolgorov-Smirnov test. The inset shows that the average EPSC versus time for successes for the first pulse (dashed line, 24.5 ± 10.0 pA) is the same as for the second pulse (solid line, 24.4 ± 9.3 pA).

2.5 pA were automatically classified as failures. For traces with amplitudes >2.5 pA, the final template trace was scaled to a peak value of the measured amplitude and displayed on screen superimposed on the original trace. (For the second pulse, both the analysis window and the template to fit are shifted over by the duration of the interpulse interval). The mean squared error was calculated for the difference between the data trace and the scaled template; if the difference was less than a preset threshold (traces were basically identical except for noise), the analysis program automatically classified it as a release. Usually, $>80\%$ of the traces were automatically sorted by the analysis program. When the mean squared error exceeded the threshold value (some significant difference existed between the data trace and the scaled template), the analysis program paused and asked the user to manually classify the trace based on visual inspection. Usually, these were cases of a spontaneous release event from another synapse that fell within the analysis window.

Figure 8B shows eight more sample data traces from the same synapse in Figure 8A. These are all classified as releases (*) or not using the analysis program. Figure 8C illustrates the template matching procedure for one of the traces. The scaled template provides an excellent fit to the first pulse; it is automatically classified as a release. Then the template is shifted by 40 ms and scaled to fit the second pulse. In this case, the fit is not as good; this trace, while clearly containing mini EPSC, has a longer latency and is therefore not an evoked release from the stimulated synapse. It is classified as a failure.

Although $\sim 30\%$ of Schaeffer collateral fibers have been shown to make multiple synaptic contacts on some CA1 cells (Sorra and Harris, 1993), only recordings of cells with putative single synaptic

contacts (only one EPSC shape and latency, criterion 2) were included here. It is possible that compound synapses with multiple release sites were stimulated, which would have EPSCs with very similar latency and shape that would not be excluded by any of the above criterion. These were detectable by a shift in the amplitude histogram to higher values during paired-pulse facilitation. We did not include these in our analysis, as we were interested in single release sites. Figure 9A shows an example of a recording that was interpreted as not containing a single release site despite satisfying criteria 1–3. For a pair of pulses 40 ms apart, the release probability increased from 0.203 ± 0.021 for the first pulse to 0.356 ± 0.024 for the second pulse (384 trials). The cumulative amplitude plot was clearly shifted to the right for the second pulse of the pair (statistically different, Komolgorov-Smirnov test, $P < 0.001$). The inset shows EPSC for average of successes; again, the second pulse is much larger (32.9 ± 15.5 pA for the second pulse; 23.1 ± 12.6 pA for the first pulse). For comparison, Figure 9B shows the same analysis for a synapse that also satisfied criteria 1–3 above and which appears to be a single release site. For a pair of pulses 40 ms apart, the release probability increased from 0.299 ± 0.019 for the first pulse to 0.476 ± 0.020 for the second pulse (608 trials). The amplitude histograms of the successes are not statistically different, and the average currents are also the same for both pulses (24.5 ± 10.0 pA for first pulse; 24.4 ± 9.3 pA for the second pulse).

Appendix

The purpose of this Appendix is to derive relations between (1) the readily releasable pool size and release probability, shown in Figures 5 and 7B, and (2) between release probability and facilitation, shown

in Figure 3A. These derivations show how the equations we have used could arise, and are not intended to represent a definite or complete theory for the relations under investigation.

I. Pool Size and Release Probability

Let $q(t)$ be the probability that no release has occurred up to t ms after a nerve impulse has arrived at the bouton. We assume, following Barrett and Stevens (1972), that release occurs according to a Poisson process with a Poisson rate $\alpha(t)$ for each vesicle; the readily releasable pool contains n vesicles, and we assume that they behave independently up to the time of the first fusion event. As soon as the first vesicle starts to fuse, we propose that the energy barrier for other fusions is raised sufficiently that another exocytosis would be very unlikely (Stevens and Wang, 1995). The equation that governs $q(t)$ is obtained by the standard method for a Poisson process. To first order, the probability of no release up to time $t + \Delta t$ is equal to the product of the probability $q(t)$ of no release up to time t , and the probability $(1 - n\alpha(t)\Delta t)$ of no release in the ensuing Δt seconds:

$$q(t + \Delta t) = q(t)(1 - n\alpha(t)\Delta t);$$

note that here we used the Poisson assumption that the probability of a release in Δt seconds is $n\alpha(t)\Delta t$. When this equation is rearranged and the limit taken as Δt approaches zero, we obtain the differential equation

$$\frac{dq(t)}{dt} = -n\alpha(t)q(t),$$

which has the solution:

$$q(t) = e^{-n \int_0^t \alpha(x) dx}.$$

The period during which a release is considered to occur is the interval $[0, T]$; after T , release is considered to be asynchronous. The probability $q = q(T)$ that a nerve impulse arrival will result in a release failure is therefore:

$$q = e^{-n \int_0^T \alpha(x) dx} = e^{-na},$$

where a , the fusion probability, is defined to be $a = \int_0^T \alpha(x) dx$. The release probability p we measure is $p = 1 - q$, so

$$p = 1 - e^{-na} \quad \text{Equation 1.A}$$

This equation predicts a simple relationship between release probability and pool size. If we assume that a is a constant and fit this equation to the data in Figure 5, the best fit is shown with the dashed line ($a = 0.125$). This does not provide a very good fit to the data, however. We next look at modifications of Equation 1.

Active Zone Area and Fusion Probability

We identify the readily releasable pool with the vesicles docked at an active zone, and the active zone area A varies considerably from synapse to synapse. Since the resting pool size is proportional to the active zone (Schikorski and Stevens, 1997), it could be that the fusion probability α depends on active zone area and thus on pool size n . Rewriting the equation to include this possibility yields

$$p = 1 - e^{-n\alpha(n)}.$$

Next, we consider the limiting cases for the dependence of a on pool size n .

If each fusion site on an active zone had its own private calcium channels (producing a microdomain with a single release site), then we would expect a to be constant (k) as long as all calcium channels were equivalent, giving our original equation,

$$p = 1 - e^{-kn}.$$

As described above, this did not provide a very good fit to the data.

If, on the other hand, calcium channels were in higher density at the active zone than in the surrounding membrane and if calcium were well mixed across the entire active zone, then the calcium concentration at all release sites would depend on the total calcium influx and thus would be proportional to the active zone

area (A). Since fusion probability should, according to the Dodge and Rahamimoff equation, vary as a power of the calcium concentration, a would be proportional to A^v and thus would vary as the v th power of the resting pool size, giving

$$p = 1 - e^{-kn^{v+1}}.$$

Neither of these limiting cases appears likely (Regehr et al., 1994), so we need to treat an intermediate case for which calcium channels that surround a release site would cooperate to determine the local calcium concentration but mixing across the active zone would not be complete. Suppose that the density of calcium channels is greater in the active zone than in the surrounding presynaptic membrane and further suppose that the calcium that enters is partially but not completely mixed across the active zone on the relevant timescale. This would mean that release sites near the center of the active zone would have a calcium concentration determined by the influx in the region entirely surrounding it, whereas a release site near an active zone edge would have a lower calcium concentration because it would receive a smaller calcium influx from the adjacent nonactive zone membrane. The average probability for the entire active zone would then depend on the area A since a smaller fraction of the release sites would be adjacent to nonactive zone membrane in a large active zone. The average fusion probability then would vary with the area to circumference ratio:

$$a \sim \frac{A}{\sqrt{A}} = \sqrt{A} \sim \sqrt{n}$$

and, therefore,

$$p = 1 - e^{-kn^{\frac{3}{2}}}, \quad \text{Equation 1.B}$$

for some constant k . This is the equation used in Figures 5 and 7 where $k = 0.06$.

II. Paired-Pulse Facilitation and Release Probability

Next, we turn to a consideration of how of the relationship between facilitation and release probability (Figure 3A) might arise. The probability of a release by a second action potential t ms after a first action potential has arrived at a bouton is denoted p^* and is, as above, given by

$$p^* = 1 - e^{-na\varphi}$$

where φ is the facilitation factor for each vesicle and depends on t and perhaps on n . Facilitation f is defined, for a particular t , by

$$f = \frac{p^*}{p} = \frac{1 - e^{-na\varphi}}{1 - (1 - p)^\varphi} \quad \text{Equation 2.A}$$

where we have used $e^{-na} = 1 - p$ to simplify the numerator in the preceding. The assumption that φ is independent of n (for a fixed t) is shown by the dashed and dotted line in Figure 3A, which only approximately accounts for the data. We therefore have sought to include a dependence of φ on n .

φ as a Function of n

We emphasize that the following is not intended to constitute a theory for the facilitation but rather to suggest the type of theory that might be appropriate. Schikorski and Stevens (1997) find that active zone area A is proportional to the bouton volume V as well as to the docked vesicle pool size n . We assume that φ is related to a power of the residual calcium concentration (Ca_{res}), giving $\varphi \sim Ca_{res}$. Suppose that the majority of the calcium influx is through calcium channels associated with the active zone. Since A is proportional to V , Ca_{res} would be independent of bouton and active zone size if the fraction of the internal bouton volume occupied by organelles were fixed. One might suppose, however, that smaller boutons would have a larger fraction of their internal volume occupied by mitochondria and other organelles than would larger boutons, and this would give a smaller effective volume and thus a larger Ca_{res} . Suppose that the fraction of the bouton volume that is excluded by the presence of organelles is related to a power of the bouton volume. This gives

$$Ca_{res} \sim V^\beta,$$

for some constant β ; we anticipate that this number would be negative so that the residual calcium concentration would be higher for smaller boutons. Since $V \sim A$ and $p \sim n \sim A$ (at least for the lower probability synapses), this gives

$$Ca_{res} \sim p^\beta,$$

where β will vary with time as Ca_{res} is reduced by diffusion and transport.

Since we are assuming $\varphi \sim Ca_{res}$, this means that φ is related to p by the equation

$$\varphi = u(t)p^{v\beta},$$

where $u(t)$ is the time dependent function that relates φ to $p^{v\beta}$. This gives the equation

$$f = \frac{1 - (1 - p)^{u(t)p^{v\beta}}}{p} \quad \text{Equation 2.B}$$

Fitting this equation to the data in Figure 3A (interpulse interval = 40 ms) gives $u(t) = 1.24 \pm 0.15$ and $v\beta = -0.41 \pm 0.05$ (solid line in Figure 3A).

If we use the approximations $u(t) \approx 1$, and $v\beta \approx -0.5$, this gives

$$\varphi = \frac{1 - (1 - p)}{\sqrt{p}}$$

and we have the relation shown by the dashed line in Figure 3A:

$$f = \frac{1 - (1 - p)^{\frac{1}{\sqrt{p}}}}{p}.$$

This is the equation used in Figure 4 of Murthy et al. (1997).

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