Kinetic Isolation of a Slowly Recovering Component of Short-Term Depression During Exhaustive Use at Excitatory Hippocampal Synapses

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INTRODUCTION

When used repetitively, excitatory chemical synapses exhibit several forms of substantial, but temporary, depression that are collectively known as short-term depression (reviewed in Zucker and Regehr 2002). A key issue for understanding the dynamics and modulation of short-term depression at a molecular level, and also for understanding how synapses function in the context of computational first principles and disease, is to define the underlying elemental processes and the kinetic relationships among them.

Several mechanisms have been shown to contribute to depression at a broad range of synapse types. Mechanisms of depression include presynaptic mechanisms, such as depletion of synaptic vesicle pools and calcium channel inactivation, and postsynaptic mechanisms. Among the presynaptic mechanisms, several kinetic components related to presynaptic vesicle pool depletion and resupply have been reported for CNS synapses as well as for neuromuscular junctions (Dittman and Regehr 1998; Magleby 1973; Rosenthal 1969; Saviane and Silver 2006; Silver et al. 1998; Stevens and Wesseling 1999b; Wu and Betz 1998). The present study focuses on one of the vesicle pool resupply mechanisms that remains poorly understood: a slow kinetic process that seems to be rate limiting for synaptic throughput during exhaustive use.

Quantitative cell biological studies of hippocampal synapses in culture have suggested that rate-limiting steps in the synaptic vesicle exo/endocytic cycle that emerge during heavy use might play important roles in short-term depression under some circumstances. These synapses typically appear to have only between 30 and 45 vesicles available to participate in the cycle (Harata et al. 2001; Murthy et al. 1997). Moreover, at least under some conditions, vesicles take several tens of seconds to complete the cycle (Aravanis et al. 2003; Heuser and Reese 1973; Miller and Heuser 1984; Ryan et al. 1993, 1996), suggesting that the steady-state rate of transmitter release during extended periods of heavy use might be limited to around 1 quantum per second. However, faster vesicle cycling mechanisms have been proposed; which type dominates is presently unknown as is the maximal neurotransmitter release rate during exhaustive use itself (Aravanis et al. 2003; Granseth et al. 2006; Pyle et al. 2000; Sara et al. 2002).

Despite the uncertainties and potential for complexity, however, electrophysiological studies have indicated that at least two mathematically well behaved kinetic mechanisms do exist at excitatory hippocampal synapses that can limit the rate at which vesicles are supplied for release. One of the mechanisms, characterized in cell culture, determines the time it takes for a small, readily releasable pool (RRP) to be replenished with fresh vesicles after being depleted (Rosenmund and Stevens 1996; Stevens and Tsujimoto 1995; Stevens and Wesseling 1998) and was later shown to emerge as a key component of depression during medium-length trains of action potential firing at Schaffer collateral synapses in acute brain slices (Wesseling and Lo 2002). A second mechanism, also identified in cell culture, emerges during more extended stimulation and causes the RRP replenishment time course, measured during subsequent periods of rest, to slow down (Stevens and Wesseling 1999b). However, the functional implications of the second mechanism and its relationship to the short-term depression evident during periods of heavy use remain unknown.

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The present study shows that the second mechanism is operant at Schaffer collateral synapses in acute hippocampal slices and that it can play a dominant role in causing short-term depression during repetitive use. After induction, the depression caused by the second mechanism persists for several minutes and limits steady-state synaptic throughput to a low rate of <1 quantum per second. Strikingly, the onset of depression caused by the second mechanism is delayed, appearing only after the first several seconds of heavy use. This contrasts with key aspects of previous models that describe short-term depression as the summed action of linearly or exponentially fatiguing underlying mechanisms (Zucker and Regehr 2002).

**METHODS**

Animal sacrifice, performed by decapitation, was conducted in accordance with Spanish royal decree 1201/2005. All synaptic responses were measured from patch-clamped neurons held in whole cell voltage-clamp mode with typical pipette resistances of 3–5 MΩ.

**Brain slice preparation**

Experiments were performed on 400-μm-thick transverse hippocampal slices (with area CA3 removed) of 2- to 4-wk-old mice as described previously (Wesseling and Lo 2002). The extracellular recording solution contained (in mM) 120 NaCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 3.5 KCl, 50 μM picrotoxin, and 50 μM D-APV [d(–)-2-aminooxy-5-phosphono-pentanoic acid]; 2.6 mM CaCl₂ and 1.3 mM MgCl₂ were included for all experiments except as noted in RESULTS. The intracellular solution contained (in mM) 130 Cs-glucanate, 5 CsCl, 5 NaCl, 2 MgCl₂, 2 MgATP, 0.2 LiGTP, 1 EGTA, 0.2 CaCl₂, and 10 HEPES.

**Cell-culture preparation**

Isolated hippocampal neurons were grown in cell culture as described previously (Garcia-Perez and Wesseling 2008; Stevens and Wesseling 1998). Briefly, 12-mm glass coverslips were placed in 24-well cell-culture plates and then coated first with agarose (0.15%, type IIa) and then with a particulate mist of substrate solution (1 mg/ml rat tail collagen and 0.05 mg/ml poly-d-lysine) with an airbrush. Tissue from newborn mouse hippocampi was dissected free, dissociated with papain (15 units/ml; Worthington), triturated mechanically with a pipette, and then plated at approximately 3 10⁴ cells (0.5 ml) per well. Cultures were grown for 10 or 11 days in a 5% CO₂ incubator (37°C) before use. The extracellular recording solution contained (in mM) 132 NaCl, 2 KCl, 10 glucose, 15 sorbitol, 10 HEPES, d-APV (50 μM), 2.6 CaCl₂, and 1.3 MgCl₂. Recording pipettes were filled with (in mM) 140 K-glucanate, 9 NaCl, 1 MgCl₂, 2 MgATP, 0.2 LiGTP, 1 EGTA, 0.2 CaCl₂, and 10 HEPES. Both solutions were adjusted to a pH of 7.2 and an osmolarity of 295–305 mOsm.

**Stimulation in brain slices**

Schaffer collateral stimulation was accomplished with a monopolar silver/silver chloride electrode (<±0.1 mA for ±0.1 ms) inserted into a glass pipette (tip diameter >10 μm) and filled with recording solution. Near-minimal stimulator settings were chosen during low-frequency stimulation as the level (both amplitude and duration) needed to evoke successful synaptic transmission about half of the time or less. To ensure that transmission failures did not result from nerve conduction failures arising from fluctuations in the axon threshold, settings were used only if it was possible to both increase and decrease the amplitude over a bracketing range corresponding to 3% of the chosen intensity without noticeably changing the probability of release (Allen and Stevens 1994; Raastad et al. 1992).

**Stimulation in cell culture**

Uncontrolled action potentials were evoked presynaptically by transiently depolarizing cell bodies as described in Bekkers and Stevens (1991). Osmotic shocks were induced with hypertonic solution, consisting of extracellular saline additionally containing 500 mM sucrose, which was applied by picrospritzing from a glass pipette with a tip diameter of 2–3 μm, and was cleared rapidly with a vacuum pipette with a tip diameter of 10–50 μm.

**Experimental design**

It was often possible to repeat several trials of each experiment in individual preparations. To allow the synapses to recover completely between trials, preparations were always allowed ≥4 min of rest before each experiment was initiated. Data were accepted only if the electrode access resistance did not change throughout individual trials and also between paired trials for the experiments documented in Fig. 1 and Supplemental Fig. S1.1 For the experiments with a single experimental variable, the experimental and control trials were alternated. For time courses, the order of trials was shuffled.

**Measurements**

For experiments documented in Figs. 1, B and F, synaptic responses were measured as the current integral of (binned segments of) the entire digitized recording. For Fig. 1, B and F, paired traces obtained in the presence of either 5 or 10 μM DNQX [6,7-dinitroquinoxaline-2,3-(1H,4H)-dione] were subtracted first and, to make the 20-Hz trials directly comparable to those at 40 Hz, 4-ms time windows aligned to match the stimulation artifacts during the 40-Hz trials were excluded. Synaptic strength was also measured for individual responses for the experiments documented in Fig. 1, A–D, and is documented in Table 1. Because individual responses took >25 ms to decay completely at room temperature, current integral and peak measurements of individual synaptic responses recorded at 40 Hz were adjusted for corresponding changes in the tail current obtained from the 20-Hz experiments (<10% of total measure, Fig. 1D and Table 1 only); because responses decayed more quickly at near-body temperatures, this was not necessary for the 40-Hz experiments documented in Fig. 9. For each experiment in Fig. 1F, three trials were conducted for each condition and the raw data were averaged before further analysis. For near-minimal stimulation experiments, responses were measured as the peak amplitude. In all other cases, synaptic response sizes were measured as the current integral after the stimulus artifact.

**Analysis**

Fractional RRP replenishment values (Figs. 5–7 and 9) were estimated in three steps as in previous studies (Garcia-Perez and Wesseling 2008; Stevens and Wesseling 1999b; Wesseling and Lo 2002): 1) a raw fractional recovery ratio was calculated by dividing the size of the sum of the first 60 excitatory postsynaptic currents during the second train by the corresponding size of the sum during the first train (raw traces were summed before measurement, but using instead the sum of measurements of the individual responses yielded indistinguishable results); 2) the raw fractional recovery ratio was then adjusted to start at 0 by subtracting the raw fractional recovery ratio for experiments where the time between trains was nominally 0 s (i.e., 50 ms when stimulation was at 20 Hz); 3) the adjusted fractional recovery measure was then normalized, so that complete recovery

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1 The online version of this article contains supplemental data.
would have a value of 1.0, by dividing by 1 minus the raw fractional recovery ratio for the 0-s time interval. The estimates for RRP recovery after only 20 pulses documented in Fig. 7 or 40 pulses in Fig. 9 required an additional correction because trains <60 pulses do not empty the RRP. To achieve the additional correction, the size of the sum of responses during 20- or 40-pulse trains was scaled, before further analysis, by the quotient of the sizes of the sums of the first 60 and first 20 or 40 responses of longer trains that were available from other experiments conducted on the same preparation (scaling was generally between 1.2 and 1.6).

**RESULTS**

The synapses between Schaffer collaterals and CA1 pyramidal neurons in hippocampal slices were chosen for the present study because: 1) short-term plasticity can be studied in isolation here by pharmacologically blocking longer-lasting types of plasticity such as long-term potentiation and depression; and 2) short-term plasticity is presynaptic—postsynaptic elements of short-term depression do not seem to play a role, at least not when long-term changes are blocked with N-methyl-d-aspartate (NMDA)—receptor antagonists, as they were in the present study (Wesseling and Lo 2002; also see following text).

The first two figures summarize the determination and validation of stimulation protocols effective at driving neurotransmission maximally over extended periods at Schaffer collateral synapses. We have previously shown that several seconds of 20-Hz stimulation is sufficient to empty the RRP and, thereafter, to drive the exocytosis of newly supplied vesicles as quickly as they are made available, at least for 1 or 2 s after the RRP has been exhausted (Garcia-Perez and Wesseling 2008; Wesseling and Lo 2002). However, the moderate-length stim-

**FIG. 1.** Stimulation at 20 Hz drives transmitter release at a maximal rate over extended periods. A–D: 20- vs. 40-Hz stimulation: Schaffer collaterals were stimulated for 60 s, while postsynaptic responses were recorded from patch-clamped pyramidal neurons. A: matched example traces after subtraction of the stimulus artifacts recorded independently in 5 μM DNQX. Scale is 50 pA × 20 s; B: binned-integrated measurement of responses vs. time: complete current integral during 1-s bins of first 35 s of digitized traces after subtracting matching traces recorded in DNQX. The value of each bin was normalized by the bin value corresponding to the 4th s of stimulation at 20 Hz (means ± SE; n = 5/8 matched experiments from 5 preparations, 3 matched experiments when expressing results because of slow drift in the recording baseline that was evident after stimulation). Note that after the first several seconds, response sizes at both 20 and 40 Hz decreased with similar time courses before achieving the same low steady state; when expressed as the fractional change after the 4th s, response sizes decreased to 0.37 ± 0.03 (20 Hz) vs. 0.36 ± 0.04 (40 Hz) when measured during the 30th–35th s of stimulation. C: individual responses: traces were averaged across all 8 trials. Scale is 200 pA × 40 ms; i: responses 1–3 at 20 Hz (top) and 1–6 at 40 Hz (bottom); ii: responses 81–83 at 20 Hz (top) and 81–86 at 40 Hz (bottom); iii: responses after 30 s of continuous stimulation: numbers 601–603 at 20 Hz (top) and 1,201–1,206 at 40 Hz (bottom). D: averaged traces after subtracting the stimulus artifacts (20 and 40 Hz) and tail currents (40 Hz) as described in METHODS (all 8 trials). E: overlay average of responses i–20, responses 61–80, and all responses during last 30 s of stimulation at 20 Hz. Scale is 20 pA × 5 ms, and also applies to ii.iii: same for 40-Hz trials. iii: overlay of all 6 averages in i and ii after scaling so that the peaks match. Scale bar is 5 ms. Note that no systematic changes in the time course of individual synaptic responses were detected during continuous stimulation or at 20 vs. 40 Hz; thus the peak amplitude and current integral are equivalent measures of synaptic strength. However, in contrast, substantial changes were detected in the time course of individual responses during similar types of experiments at the lowest extracellular Ca²⁺ concentrations (standard is 2.5 mM Ca²⁺/3.0 mM Mg²⁺; high is 5.0 mM Ca²⁺/0.5 mM Mg²⁺; all stimulation was at 20 Hz). Responses were measured as the current integral of individual responses, normalized by the mean size during the 4th s of stimulation in standard Ca²⁺ (dashed line), binned into groups of 20, and plotted vs. time (n = 5/8 paired trials, 3 preparations; normalized steady-state response sizes were 0.30 ± 0.05 in standard Ca²⁺ vs. 0.32 ± 0.04 in high Ca²⁺). Note that although there is a difference in response size during the first several seconds, the difference disappears after the 3rd s. Also note that the time base is 90 s compared with 35 s in B and that the onset of depression is similar in the 2 panels. Inset: average of the first responses across all trials; scale is 100 pA × 5 ms. Note that the first response in high Ca²⁺ is approximately twice that in standard Ca²⁺; F: frequency-jump experiments: Schaffer collaterals were stimulated at 20 Hz for 30 s followed immediately by an additional 3 s at either 40 or 20 Hz; as in A, stimulus artifacts were recorded independently in DNQX and subtracted prior to analysis. i: high Ca²⁺ (5.0 mM Ca²⁺/0.5 mM Mg²⁺); plot of the binned-integrated response (200-ms bins) during the last 1 s before the frequency jump and the first 1 s afterward. The response did not change detectably, indicating that 20-Hz stimulation drives release at a maximum rate in high Ca²⁺ (response size was 1.02 ± 0.13 during the first 1 s of 40-Hz stimulation compared with the last 1 s at 20 Hz; n = 6 preparations for experimental trials, n = 3 for controls, where stimulation was maintained at 20 Hz throughout). ii: positive control in low Ca²⁺ (0.63 mM Ca²⁺/1.3 mM Mg²⁺); note the robust increase (1.41 ± 0.08-fold, n = 6 preparations, P < 0.02, n = 6 experimental trials, n = 5 controls), as expected, because 20-Hz stimulation is not sufficient to deplete the readily releasable pool (RRP) in low Ca²⁺. This positive control verifies that changes in stimulation frequency are effective at changing repetition rates of action potential initiation and conduction. No increase was detected in controls when stimulation was continued at 20 Hz throughout (0.92 ± 0.06-fold, n = 5).
Confirmation of conditions for driving synaptic release at maximal rates: stimulation frequency

As a first test that 20-Hz trains continue to drive exocytosis as quickly as vesicles are supplied for release over longer periods, we verified that higher-frequency stimulation over longer periods does not elicit transmitter release at a higher rate or a larger aggregate postsynaptic response.

Synaptic responses were monitored in paired trials from patch-clamped CA1 pyramidal neurons during continuous 20- and 40-Hz Schaffer collateral stimulation (Fig. 1A); a train duration of $\geq 60$ s was chosen because responses finally settle to a steady-state size after about 30 s of stimulation (600 pulses at 20 Hz) in the pilot experiments. To avoid systematic errors in measuring the smallest responses, occurring when the synapses were maximally depressed, the contribution of electrical stimulus artifacts to the postsynaptic response was determined from additional trials conducted in the presence of 5 or 10 $\mu$M DNQX, which, in combination with NMDA-receptor blockers already in our standard solution, blocks synaptic transmission at these synapses.

Data analysis was accomplished by integrating the entire digitized postsynaptic recordings over 1-s time bins, after subtracting the matched traces recorded in DNQX (Fig. 1B). This type of measurement, referred to throughout the present report as the “binned-integrated measurement,” incorporates all synaptic responses including any asynchronous components (Hagler Jr and Goda 2001) and would be in units of charge, but were instead normalized by the average of bin values during the 4th s of 20-Hz stimulation to facilitate comparison between experiments from different preparations. The bin value during the 4th s was chosen for normalization because it is thought to be proportional to the maximum rate of exocytosis that can still be elicited from Schaffer collateral terminals immediately after the RRP has been exhausted, when only newly supplied transmitter is being released, and thus does not change when release parameters are changed, such as by changing the level of extracellular $\text{Ca}^{2+}$ (Wesseling and Lo 2002).

The binned-integrated measurement was initially higher during the 1st s of stimulation at 40 Hz (Fig. 1B), as expected from previous reports, because the vesicles that were available for immediate release when the experiment began (i.e., those constituting the RRP) would be triggered to undergo exocytosis faster at 40 Hz. However, the difference between the binned-integrated measurement for trials at 20 versus 40 Hz disappeared after the 3rd s of stimulation (Fig. 1B), after the induction of a substantial amount of depression, as expected if the first 60 pulses at either stimulation frequency are enough to

Table 1. Comparison of peak and integral measurements of individual responses

<table>
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[See Table 1 for the full table content here.]

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drive the RRP into a nearly empty steady state where the rate of new vesicle supply to the RRP is balanced by the release rate (Molder and Mennerick 2005; Wesseling and Lo 2002). The binned-integrated measurement remained matched during the following 30 s of stimulation at the two stimulation frequencies, confirming that 40-Hz stimulation does not elicit more neurotransmitter release than 20-Hz stimulation, and consistent with the hypothesis that, after initial RRP exhaustion, 20-Hz stimulation continues to drive transmitter release as soon as it is supplied to the RRP over durations lasting tens of seconds. (As expected, individual responses were smaller at 40 Hz; see Fig. 1C, ii and iii.) In spite of the match at 20 and 40 Hz, the binned-integrated measurement, at both frequencies, declined about threefold over the next 20 s.

**No change in time course of individual responses**

Although not our objective when designing the experiment, we noticed that the time courses of individual responses did not change substantially either with stimulation train duration or with stimulation frequency (Fig. 1, C and D), which was surprising because time courses of individual responses do change during rapid stimulation at excitatory hippocampal synapses grown in cell culture (Hagler Jr and Goda 2001); the absence of a substantial change in the time courses is subsequently confirmed with near-minimal stimulation experiments (see Fig. 3D). Thus measurements of the peak amplitude, or the current integral of individual traces, and the binned-integrated measurement described in the preceding text, all yielded comparable estimates of relative changes in response size as a result of the induction of short-term plasticity. The absence of a change in the time courses of individual responses allowed us to simplify the design of subsequent experiments by omitting the matched control experiments performed in the presence of DNQX.

Table 1 verifies that the individual response sizes during the 30th–35th s of stimulation at 20 Hz were reduced by an amount that was similar to the decrease in the binned-integrated measurement documented earlier. Individual responses at 40 Hz were close to one half the value at 20 Hz (see also Fig. 1C), confirming that the overall transmission rates were equivalent because there were twice as many responses per unit time at 40 Hz. Responses during the 35th–60th s of stimulation were not detectably more depressed than they were during the 30th–35th s, confirming that 30 s of 20-Hz stimulation is enough to drive Schaffer collateral synapses to a depressed steady state.

**Confirmation of conditions for driving synaptic release at maximal rates: extracellular Ca\(^{2+}\)**

As a second test that 20-Hz trains continue to drive exocytosis as quickly as vesicles are supplied for release over tens of seconds, we compared postsynaptic responses during extended stimulation under standard conditions (2.5 mM Ca\(^{2+}\)) and in high extracellular Ca\(^{2+}\) (5 mM) because raising extracellular Ca\(^{2+}\), increases the fraction of readily releasable vesicles that are triggered for release by individual action potentials, but should not affect release when already being driven at the maximal rate (Wesseling and Lo 2002). (The total divalent ion concentration was kept constant by lowering extracellular Mg\(^{2+}\).) At the beginning of the 20-Hz trains, the responses were twice as large at the higher compared with the standard Ca\(^{2+}\) concentration (Fig. 1E, inset), as expected, because the RRP would start off in the full state, but the difference disappeared by the 60th pulse (Fig. 1E). The sizes of responses remained matched during the remaining stimulation at the two Ca\(^{2+}\) levels. Nevertheless, responses depressed extensively between the 60th and 600th pulses, with matched time courses of decline at the two Ca\(^{2+}\) concentrations (both matching Fig. 1B), reaching a steady-state size that was about threefold less than that during the 4th s of stimulation.

**FIG. 3.** No changes in quantal size during continuous stimulation. A near-minimal number of Schaffer collaterals was stimulated at 20 Hz for 90 s. A: traces of recordings when synapses were rested (top trace) and after 50 s of repetitive stimulation (bottom trace); the stimulus artifacts are blanked in these examples; the arrows indicate stimulus times. Scale is 20 pA \(\times\) 100 ms. B: probability of release vs. time during the first 3 s of stimulation (i, bins of 3, \(n = 14\) trials, 4 preparations) and during the entire 90 s of stimulation (ii, bins of 100; each data point is the average probability of release during a 5-s interval). C: no change in quantal size after the first 1 s; cumulative histograms of successful response sizes during the 2nd–5th (squares), and 50th–90th s (circles) of stimulation; there was a small decrease during the first 1 s (not shown), but this likely resulted from the decreased occurrence of simultaneous release of multiple quanta because it is not typically seen in other studies when a special effort is made to limit stimulation to single afferent synapses. Inset: averaged traces of segregated responses judged to be successes and failures. The traces to the left are the averages during the first 5 s of stimulation; to the right are the averages from the 50th to 90th s. Scale is 10 pA \(\times\) 20 ms. D: no change in timing of release during extensive stimulation. Event latencies were measured as the time between the stimulation artifact and peak response for all successful responses. The black line is a density histogram of latencies (1-ms bins) during the first 5 s of stimulation; the gray line is during the 50th–90th s; a baseline incidence rate of 0.36/s (0.00036/bin, measured during the 5 s prior to stimulation) was subtracted from the frequency histograms before normalization to construct the density histograms.
Additional test to show the RRP is maintained in a nearly empty steady state

Both sets of results in the preceding text are consistent with the hypothesis that continuous 20-Hz stimulation drives neurotransmitter release fast enough to maintain the RRP in a nearly empty steady state because, if not, one would expect more transmitter release at the higher stimulation frequency or at the higher Ca\(^{2+}\) concentration. We next conducted a set of “frequency-jump” experiments to test the hypothesis with more precision.

That is, we attempted to drive exocytosis harder still by doubling the stimulation frequency from 20 to 40 Hz in high Ca\(^{2+}\), after the maximum amount of depression had already been achieved with 30 s of 20-Hz stimulation (diagram above Fig. 1F). The release rate did not change (Fig. 1Fi) when the stimulation frequency was doubled or, as expected, in control experiments where the stimulation frequency was maintained at 20 Hz throughout (also Fig. 1Fi). As a positive control for the effectiveness of doubling the stimulation frequency on increasing transmitter release rates when the RRP is not empty, we repeated the entire experiment, except at a low extracellular Ca\(^{2+}\) level where 20-Hz trains are no longer effective at depleting the RRP. As expected, and in marked contrast to experiments in high Ca\(^{2+}\), doubling the stimulation frequency to 40 Hz under low Ca\(^{2+}\) conditions did result in a robust increase in the postsynaptic response (Fig. 1Fi), whereas, as expected, no increase was detected in the corresponding control trials when stimulation was maintained at 20 Hz throughout (also Fig. 1Fi).

Taken together, the frequency-jump experiments strengthen the hypothesis that, at standard Ca\(^{2+}\) levels or higher, the RRP is maintained in a nearly empty steady state during extended trains of 20-Hz stimulation (for similar frequency-jump experiments after the 4th s of stimulation, see Fig. 2C of Garcia-Perez and Wesseling 2008). In contrast, the results do not seem to be consistent with alternative presynaptic explanations for the late phase of depression, such as: 1) Ca\(^{2+}\) channel inactivation (or some other type of fatigue of the release machinery) because, if so, newly supplied readily releasable vesicles that were not released would accumulate in the RRP, and therefore increasing the stimulation frequency would be expected to increase the release rate (but it did not; see Fig. 1Fi); or 2) a declining maximum repetition rate for action potentials in Schaffer collaterals because, if so, increasing the stimulation frequency in the low Ca\(^{2+}\), positive control experiments would have failed to increase the release rate (contrary to the positive control in Fig. 1Fi).

Experiments documented later show that response size recovers completely over subsequent 4-min-long rest intervals, identifying all phases of depression as short-term phenomena. In addition, several sets of control experiments, also documented later, argued strongly against postsynaptic mechanisms making a significant contribution to the depression. Our working hypothesis is therefore that the several phases of depression reflect the serial emergence of multiple rate-limiting steps in the synaptic vesicle exo/endocytic cycle, each one slower than the previous one. Indeed, the late phase of depression induced between the 60th and 600th stimulus pulses (after the RRP had already been emptied) was anticipated from an earlier cell-culture study demonstrating that extensive use results in a substantial slowing of the rate at which synaptic vesicles are supplied to the RRP, as judged by measurements of the RRP replenishment time course during subsequent rest intervals (Stevens and Wesseling 1999b).

Cell-culture test to confirm the plausibility of the working hypothesis

We next sought to confirm the plausibility of our working hypothesis with experiments on isolated hippocampal neurons in cell culture (i.e., autapses), where it is possible to test the extent of RRP fullness immediately after electrical stimulation with a technique that altogether avoids complications such as action potential failures and Ca\(^{2+}\) channel inactivation (i.e., with osmotic shocks; Rosenmund and Stevens 1996). In matched experiments, we evoked either 80 or 600 presynaptic action potentials (4 or 30 s at 20 Hz), and then immediately afterward applied a first osmotic shock to assess the remaining contents of the RRP, followed 3 min later by a second osmotic shock to determine RRP contents when completely full (see diagram at top of Fig. 2A). Trains of both lengths reduced the size of the response elicited by the first osmotic shock to a similar fraction (Fig. 2A; to 21 ± 2% after 80 action potentials vs. 23 ± 2% after 600; the standing steady-state fullness of the RRP during electrical stimulation was likely less; see legend of Fig. 2A). Because osmotic shocks are thought to release the entire contents of the RRP, these results: 1) confirm that the RRP is substantially depleted by the first 80 action potentials in a 20-Hz train; and 2) that the RRP does not refill appreciably during the 26 s it takes to stimulate 520 more times, confirming that 20-Hz stimulation is fast enough to maintain the RRP in a nearly empty steady state. Because osmotic shocks trigger release via a Ca\(^{2+}\)-independent mechanism, the observations are not susceptible to contamination with Ca\(^{2+}\) channel inactivation or action potential failure.

In spite of the absence of significant transmitter accumulation within the RRP during the final 26 s of stimulation (80th–600th action potentials), we did observe a clear decrease in the synaptic responses to the action potentials, which appeared to be comparable to the decrease seen in the slice preparation over the same interval. The decrease of the already substantially depressed synaptic responses was difficult to quantify, however, because of the overlapping nonsynaptic currents of the presynaptic action potentials, which were unavoidable because of the simultaneous presynaptic and postsynaptic roles of autapses. Therefore short-term depression during 30 s of stimulation (20 Hz, 600 action potentials) was assessed at the synapses between pairs of neurons, grown on similar isolated islands of substrate (Fig. 2B, inset). The entire digitized postsynaptic traces were analyzed with the binned-integrated measurement (Fig. 2B) but, because ephaptic electrical stimulation artifacts are miniscule in the postsynaptic neuron of pairs, matched experiments in DNQX were not required.

Multiple phases of depression were evident and were similar to depression in the slice preparation. Synapses depressed 5.4 ± 0.6-fold (i.e., to 23 ± 4%) during the first 80 pulses in cell culture, which was similar to the 5.4 ± 0.3-fold decrease in slices. Thereafter, the response depressed another 4.3 ± 1.8-fold in culture, which was similar to the 3.1 ± 0.4-fold further depression in slices over the same period.
We calculated that the equivalent of 3.2 ± 0.6 RRPs of neurotransmitter were released between the 80th and 600th action potentials (i.e., while the RRP was maintained in a nearly empty state); the quantity was calculated by dividing the integral of all responses between the 80th and 600th action potentials by the integral of the first 40 responses because the first 40 action potentials are thought to release the equivalent contents of a single RRP (Stevens and Williams 2007). Thus a great majority of the transmitter released by the 80th–600th action potentials must have come from vesicles that were newly supplied to the RRP during stimulation.

Conversely, if no synaptic depression had occurred between the 80th and 600th action potentials, the equivalent contents of 7.6 ± 0.5 RRPs would have been released, a difference of 4.4 ± 0.3 RRPs. Thus we reasoned that if the RRP resupply rate had not slowed down concurrently with the depression elicited between the 80th and 600th action potentials, then the equivalent contents of multiple RRPs of unreleased transmitter would have been available for replenishing the RRP, but this possibility was excluded because no accumulation of transmitter within the RRP was observed (see Fig. 2A).

Notably, the cell culture results are not compatible with alternatives to our working hypothesis that would ascribe the depression evident after the 80th pulse to presynaptic mechanisms acting downstream of vesicle supply into the RRP, but upstream of release from the RRP, such as Ca\(^{2+}\) channel inactivation or action potential failures, because the alternatives would predict substantial RRP replenishment between the 80th and 600th action potentials, which was not seen. The cell culture results thus provide a plausibility test for our working hypothesis that the later phase of depression seen during extended use at Schaffer collateral synapses is caused by a use-dependent decrease in the rate at which vesicles are supplied for release. We note, however, that our results do not exclude the possibility that Ca\(^{2+}\) channel inactivation and action potential conduction failures happen at all, only that such mechanisms are not likely significant (i.e., rate-limiting) for transmitter release during continuous 20-Hz stimulation (under standard divalent ion conditions).

**Presynaptic mechanism for late phase of short-term depression**

A postsynaptic mechanism for the late phase of depression seemed unlikely because declines in receptor sensitivity would be driven by transmitter binding or receptor activation, and no such decline was seen in our previous study during the first several seconds of stimulation when the overall neurotransmitter release rate was much higher (Wesseling and Lo 2002). However, to confirm a presynaptic locus, we compared all phases of depression in the presence and absence of 300 μM kynurenic acid (KYN). As expected, KYN reduced all responses equally (to 33 ± 5% of control) and thus had no effect on any phase of depression (Supplemental Fig. S1). The absence of an effect of KYN on depression argues strongly against a postsynaptic mechanism because KYN effectively lowers the amount of time receptors are bound to transmitter (Diamond and Jahr 1997) and would thus be expected to lessen, or delay, induction of depression driven by postsynaptic mechanisms.

To exclude postsynaptic mechanisms more directly, an additional series of experiments was conducted under conditions where the sizes of quantal responses could be resolved. Single or small numbers of afferents were activated with near-minimal stimulation intensities at 20 Hz for 90 s (Fig. 3A). A stimulation intensity was chosen that set the initial probability of release close to 0.5, but after 600 pulses it had depressed to 0.045 (Fig. 3B) for a total depression of 89 ± 1.4%, which matched the depression in response sizes (92 ± 0.8%) observed during 20-Hz stimulation at ordinary stimulation strengths. As expected of a presynaptic mechanism, the average size of the successful postsynaptic responses did not decrease during the 4th–90th s of stimulation (Fig. 3C), arguing against receptor desensitization and other types of postsynaptic mechanisms that would cause graded decreases in quantal postsynaptic response sizes. In addition, no change was seen in the timing of release events during near-minimal stimulation (Fig. 3D), explaining the absence of detectable changes in the time courses of individual synaptic responses during ordinary-strength stimulation (see Fig. 1D).

The near-minimal stimulation experiments, together with the KYN experiments, argue against significant contributions to any of the phases of depression from postsynaptic mechanisms, consistent with our working hypothesis that the later phase of depression, induced after the 4th–90th s of stimulation, likely corresponds to the emergence of one or more rate-limiting vesicle trafficking step(s) that determine(s) the supply side of the steady-state content of the RRP. We have thus provisionally termed the later phase of depression "supply-rate depression."
regression analysis of responses 61–110 yielded a slope of close to 0 (i.e., $-0.59 \pm 1.4\%/s$) compared with a slope of $-5.8 \pm 1.1\%/s$ for responses 151–200 (Fig. 4B, $P < 0.01$, $n = 138$ trials from 20 preparations; see Fig. 7 for an independent confirmation).

**Stereotyped double-exponential RRP replenishment time courses**

The working hypothesis predicts that recovery should take longer after the induction of supply-rate depression because it would take longer to replenish the RRP when the supply rate is slower. In fact, as noted earlier, a previous study in cell culture has already shown that extensive use does indeed slow the overall time course of RRP replenishment (Stevens and Wesseling 1999b). However, although the previous study and at least two others (Garcia-Perez and Wesseling 2008; Wesseling and Lo 2002) included kinetic information about RRP replenishment at synapses in hippocampal slices, replenishment time courses after multiple stimulation protocols have never been analyzed at high enough resolution for the analysis documented in the following text.

Therefore we conducted a more detailed analysis of RRP replenishment time courses after stimulation trains consisting of 80, 150, 200, or 600 pulses (Fig. 5A; see diagram at top); replenishment was measured after experimentally varied rest intervals with a second train of $\geq 60$ pulses, which were necessary to ensure complete RRP depletion during the second train, and key to avoiding contaminating estimates of RRP replenishment with residual Ca$^{2+}$-dependent enhancement mechanisms (Garcia-Perez and Wesseling 2008).

As expected, RRP replenishment time courses (see METHODS) were slower after the longer trains; despite the differences, however, all time courses were well fitted with the same general double-exponential function, with component time constants of 7 s and 1 min (Fig. 5B). The slower time courses were slower because they had a lower value for the weighting parameter ($w$) in the following equation

$$s(t) = w(1 - e^{-t\tau}) + (1 - w)(1 - e^{-t\tau})$$

where $s(t)$ is the fraction of the RRP that has replenished after a rest interval of time $t$ and $\tau_i$ and $\tau_s$ are the time constants. The fits to Eq. 1 are significant in the context of the earlier report of Stevens and Wesseling (1999b) because RRP replenishment time courses in the earlier report could also be fitted with $Eq. 1$, with similar, invariant values for $\tau_i$ and $\tau_s$ and a wide range of values for $w$, even though the time courses in the earlier study were measured with postsynaptic responses to osmotic shocks instead of electrical stimulation ($w$ was denoted by $f$ in the earlier report). Because the possible invariance of the two component time constants is key for the present study, a detailed analysis was performed to detect possible changes in $\tau_i$ or $\tau_s$ that might coincide with the differences in the overall time courses (Fig. 6), but none was detected (see legend of Fig. 6).

**Supply-rate depression accurately predicts increases in RRP replenishment time**

Because the parameters $\tau_i$ and $\tau_s$ in $Eq. 1$ are at least close to invariant over the full range of RRP replenishment time courses, the analysis so far implies that changes in the time course of RRP replenishment—induced during repetitive stimulation, but measured during subsequent periods of rest—can be characterized with a single parameter (i.e., $w$ in $Eq. 1$). Therefore to determine a quantitative relationship between supply-rate depression and changes in the time course of RRP replenishment measured subsequently, a series of experiments was conducted to measure the weighting parameter $w$ as a function of stimulus train length.
Synapses were probed with pairs of 20-Hz trains similar to those documented earlier, except that the length of the first train of each pair was varied experimentally, whereas the intertrain interval was fixed at 20 s (see diagram at top of Fig. 7). Values for \( w \) could then be extracted from the estimates of RRP replenishment because \( w \) is the only free parameter in Eq. 1 (compare left and right \( y \)-axes in Fig. 7A).

Figure 7A shows that \( w \) maintained a steady value of close to 1.0 during recovery intervals following stimulation trains consisting of as many as 120 pulses, but was progressively less.
during recovery intervals after longer trains, reaching a value of around 0.3 after trains of ≥1,000 pulses. A linear regression analysis of the decline in \( w \) induced between the 20th pulse and the 120th yielded a slope of close to 0 (\(-1.0 \pm 0.8\%/s\)), compared with a significantly steeper slope of \(-4.4 \pm 0.7\%/s\) for the decline between the 120th pulse and the 200th (\( P < 0.01 \)), indicating that the decline in \( w \) was delayed in a fashion that was similar to the delay before the onset of the late phase of depression documented in Fig. 4B.

In fact, a direct comparison between the supply-rate depression induced during the first train and \( w \) yielded a one-to-one, linear relationship (Fig. 7B). The linear relationship suggests that supply-rate depression is mechanistically equivalent to the change in the time course of RRP replenishment measured over much longer rest intervals. The linear relationship is a striking result because, when the RRP is maintained in a nearly empty steady state, measurements of synaptic strength would be proportional to the amount of transmitter supplied to the RRP during the 50-ms interpulse intervals of 20-Hz stimulation (Wesseling and Lo 2002). The linear relationship thus suggests that the time course of even the minuscule amount of RRP replenishment occurring over very short rest intervals follows Eq. 1 and can thus accurately predict the entire RRP replenishment time course taking more than three orders of magnitude longer. Furthermore, the mathematical simplicity of Eq. 1 suggests that supply-rate depression is caused by the emergence of only a single rate-limiting vesicle trafficking step that may follow first-order kinetic rules (see DISCUSSION).

To complete the analysis of RRP replenishment time courses, values for \( w \) are plotted again, in Fig. 7C, this time against the cumulative synaptic response during the first stimulus train of each pair, because changes in \( w \) have elsewhere been reported to depend specifically on exocytosis, as opposed to other effects of repetitive stimulation that depend on activity (i.e., in Stevens and Wesseling 1999b, where \( w \) was denoted by \( j \)).

\textbf{Quantal measurements of recovery}

For simplicity, we have assumed thus far that decreases in the RRP resupply rate, measured either during or after stimulation, are caused by decreases in the rate at which fresh vesicles are supplied for release, but another explanation would be that incompletely filled vesicles are among those supplied to the RRP, which could lead to a decrease in the quantal size if loading vesicles with neurotransmitter were the rate-limiting step characterized by \( \tau \), in Eq. 1. Although no decrease in quantal size was seen during continuous stimulation in the near-minimal stimulation experiments documented in the preceding text (see Fig. 3C), a decrease might have gone undetected if incompletely filled vesicles were themselves nearly empty. Therefore to investigate the possibility that incompletely filled vesicles release an undetectably small amount of neurotransmitter during continuous stimulation, additional near-minimal stimulation experiments were conducted after 20-s-long rest intervals allowed for partial recovery.

The probability of release remained partially depressed during 60-pulse trains initiated 20 s after 200 or 600 pulses of near-minimal stimulation (Fig. 8, A and B), as expected, if supply-rate depression was caused by a decrease in vesicle supply rates. In contrast, no significant changes in the sizes of the quantal responses were detected (Fig. 8C). Therefore these results argue against vesicle filling per se as the rate-limiting mechanism underlying supply-rate depression because 20 s would be enough time for empty vesicles to fill >25% (assuming a time constant of 70 s), which would have been detected in Fig. 8C as an overall decrease in the quantal size.

\textbf{No evidence for a delayed response enhancement}

Experiments thus far suggest that a second phase of short-term depression, induced after initial RRP depletion, is due to a single component of depression in the RRP resupply mechanism, which is induced with a delayed onset during extensive use. In contrast, a recent paper has reported a form of enhancement, also with a delayed onset, that appeared only at elevated temperatures (Jensen et al. 2007). We thus checked to see
whether our analysis would continue to apply at elevated temperatures or whether an additional, enhancing component of RRP resupply would appear.

Similar to experiments at room temperature, multiple phases of depression were evident at 33–35°C during extensive stimulation at both 20 Hz (not shown) and 40 Hz (Fig. 9A).

A first set of experiments was designed to measure RRP replenishment time courses after stimulation trains of several durations and were similar to those documented in Figs. 5 and 6, except stimulation was at 40 Hz instead of at 20 Hz. Similar to results at room temperature: 1) recovery was slower after more extensive stimulation (Fig. 9B); 2) all recovery time courses followed \( \text{Eq. 1} \) with invariant values for \( \tau_s \) and \( \tau_f \) (Fig. 9B, i and ii); and 3) the value of the weighting parameter \( w \) was decreased after more extensive stimulation, from 1.0 after 80 pulses to 0.25 after 1,200 (Fig. 9Bi). The experiments thus failed to detect a delayed onset component of enhancement in RRP replenishment time courses, at least when measured over long rest intervals (see following text). Nevertheless, values for \( \tau_s \) (43 s, Fig. 9Bi) and \( \tau_f \) (4 s, Fig. 9Bii) were less than those at room temperature, suggesting that both kinetic components of RRP replenishment are faster at the elevated temperature.

A second set of experiments at the elevated temperature measured the relationship between depression in synaptic responses during ongoing stimulation and changes in the time course of RRP replenishment measured afterward (similarly to Fig. 7). Similar to results at room temperature, we found a one-to-one linear relationship between the second phase of depression induced during continuous stimulation (i.e., beginning after the 60th pulse) and \( w \) in \( \text{Eq. 1} \) (Fig. 9C), indicating that the constraints on RRP replenishment that apply during long recovery intervals also apply to RRP resupply during repetitive stimulation, when recovery intervals are limited to very short interstimulus intervals (i.e., 25 ms). The elevated-temperature experiments thus failed to detect a delayed onset enhancement in RRP resupply rates measured over time intervals of any length. Nevertheless, further analysis showed that, similar to results at room temperature, the onset of the second component of depression was apparently delayed at the elevated temperature (Fig. 9D).

**DISCUSSION**

This study finds a one-to-one correspondence between 1) a component of short-term depression that emerges during extended periods of heavy synaptic use; 2) use-dependent depression in the rate at which vesicles are supplied to the RRP during extensive stimulation; and 3) increases in the time required for RRP replenishment during subsequent rest intervals (see Figs. 7 and 9). The three related phenomena are not caused by RRP depletion because their induction proceeds with a delayed onset only after several seconds of heavy use, by which time the RRP has already been driven to a nearly empty state (see Figs. 1 and 2). To our knowledge, the observation of a delay before the decrease in the rate of RRP resupply (Figs. 4 and 7) is novel and may have important implications for limiting total synaptic throughput in hippocampal circuits and others that are prone to epileptiform behavior (see following text).

The underlying kinetic processes seem to be mathematically well behaved. After induction of depression, subsequent RRP

![FIG. 9](http://jn.physiology.org/)

Near body-temperature measurements. A: short-term plasticity during 30 s of 40-Hz stimulation. Mean response sizes were normalized by the mean size of responses to pulses 61–80 to match with Fig. 4A. Insert: averaged traces of responses 1–10, 61–120, and 600–1,200. Scale bar is 100 pA × 10 ms. B: RRP replenishment during long rest intervals continues to follow \( \text{Eq. 1} \). RRP replenishment time courses were measured after trains of 60, 80, 200, or 1,200 pulses (40 Hz), with a second train consisting of ≥60 pulses initiated after experimentally varied time intervals. \( \tau_s \) complete replenishment time courses after 200 or 1,200 pulses plotted on semilog plots, similar to Fig. 6. The dashed lines are the single exponential, \( y(t) = (1 - w)(1 - e^{-\tau_f}) + w \), with \( \tau_s = 43 \) s and \( w \) as indicated. \( \tau_f \): exponential peeling; plotted is the first part of the RRP replenishment time courses after 200 or 1,200 pulses (symbols as in i) derived by first subtracting the slower exponential component fitted in i, along with the entire recovery time course after 60 or 80 pulses (open squares represent the time course after 60 or 80 pulses, and were taken from a data set that was partially documented previously in Garcia-Perez and Wesseling (2008)). The dashed line is the single-exponential function \( y(t) = (1 - e^{-\tau_s}) \), with \( \tau_s = 4 \) s. C: RRP replenishment during the shortest rest intervals continues to follow \( \text{Eq. 1} \). Compare with Fig. 7B. Synapses were activated with pairs of 40-Hz stimulus trains separated by fixed 20-s rest intervals. The length of the first train of each pair was varied experimentally and the fractional RRP replenishment over subsequent 20 s of rest was measured as for Figs. 5–7 and B. The value was then used to calculate the contribution of the faster exponential component to the overall recovery time course (i.e., \( w \) from \( \text{Eq. 1} \), assuming \( \tau_f = 4 \) s and \( \tau_s = 43 \) s). Displayed is the plot of depression at the end of the first train vs. \( w \). The filled circle represents trials with first trains consisting of only 40 pulses and, as expected, deviates from the line because 40 pulses are not enough to exhaust the RRP. D: replot of \( w \) vs. the cumulative sum of synaptic response sizes during the first train. The cumulative sum (y-axis) was normalized by the size of the equivalent response of the contents of a single RRP, which was estimated, without free parameters, according to a kinetic model described in Wesseling and Lo (2002).
replenishment time courses were always well fitted by a stereotyped double-exponential equation (Eq. 1), characterized by well-separated, invariant time constants of about 7 s and 1 min (at room temperature; see Figs. 5 and 6). Therefore the slower RRP replenishment time courses apparent after heavier use could be characterized by varying a single parameter—\( w \) in Eq. 1—that represents the fractional weighting of the faster of the two exponential components. The value of \( w \) decreased in register with the rate of RRP resupply during stimulation (see Figs. 7B and 9C), suggesting that even the miniscule amount of RRP replenishment occurring during 25- or 50-ms-long interstimulation intervals follows Eq. 1, which is a striking result because, at times when synapses were in the most depressed state, 25 or 50 ms was enough time only to partially replenish the RRP by \(<0.5\%\). The result implies that the same kinetic rules determine rates of vesicular mobilization and functional recycling during periods of heavy use and during subsequent periods of rest. The term “supply-rate depression” is proposed to reference the phenomenon because the term connotes a decrease in the rate at which vesicles are supplied to the RRP for subsequent release.

**Nonrate-limiting types of short-term depression**

Our study was designed to focus on components of depression that are caused by rate-limiting mechanisms of neurotransmitter mobilization. The conclusions are based on experiments indicating that continuous 20-Hz stimulation drives release quickly enough to maintain the RRP in a nearly empty steady state (see Figs. 1 and 2). Because the RRP never gets a chance to replenish, positive or negative regulators of mechanisms involved in triggering exocytosis of readily releasable vesicles, such as short-term enhancement (Garcia-Perez and Wesseling 2008; Hsu et al. 1996; Kalkstein and Magleby 2004; Lou et al. 2005; Stevens and Wesseling 1999a; Zucker 2003) and Ca\(^{2+}\) channel inactivation (Inchauspe et al. 2004; Xu and Wu 2005), are not likely to complicate the analysis. However, it is anticipated that such other mechanisms play important roles in short-term synaptic plasticity at Schaffer collateral synapses and at other similar synapse types, during moderate use, including in physiological situations.

We emphasize that the RRP replenishment time courses measured over rest intervals in this report were extracted from the summed responses of trains made up of \( \geq 60 \) pulses because previous studies have determined that shorter trains are not sufficient to fully exhaust the RRP (Molder and Mennerick 2005; Wesseling and Lo 2002). In fact, recovery time courses calculated from shorter trains, or single pulses, were substantially faster and did deviate substantially from Eq. 1; i.e., they were often nonmonotonic and, in some cases, even transiently overshoot baseline values. A separate study shows that the different character of recovery time courses calculated from responses to short trains is likely due to residual Ca\(^{2+}\)-dependent enhancement of the release machinery and thus likely does not reflect rate-limiting steps in vesicle trafficking (Garcia-Perez and Wesseling 2008). However, it is not clear how the present analysis would apply to other synapse types where the RRP resupply rate may be substantially faster and dynamically modulated by stimulation frequency during heavy use (Crowley et al. 2007; Hermann et al. 2007; Sakaba 2008).

**No evidence for a delayed response enhancement**

The onset of supply-rate depression at Schaffer collateral synapses was delayed (see Figs. 4, 7, and 9D), but we found no evidence for a delayed-onset element of enhancement in the supply rate (see RESULTS), which has been suggested previously, specifically for elevated temperatures (Jensen et al. 2007). However, the delayed onset of supply-rate depression can account for most, but not all, of the delayed response enhancement reported by Jensen et al. That is, Jensen et al. quantified delayed response enhancement as the large deviation from a smoothly decaying exponential fit of the decreases in synaptic strength measured during repetitive stimulation, but the sigmoid shape of the induction time course for supply-rate depression (Figs. 7A and 9D) would provide an alternate explanation for much of the deviation.

In some experiments, Jensen et al. (2007) additionally observed a small, delayed increase in the absolute size of responses during continuous stimulation that began after the induction of a substantial amount of depression, a phenomenon that we have not observed. However, multiple differences in how the experiments were conducted could account for the differences between the studies. For example, the measurements of Jensen et al. showing delayed increases in the absolute sizes of responses were made with extracellular field recording electrodes, which would record conjoint synaptic activity at multiple classes of synapses, including facilitating synapses onto local inhibitory interneurons (Sun and Dobrunz 2006). In contrast, delayed increases in the absolute sizes of responses were absent from the intracellular postsynaptic recordings of CA1 pyramidal neurons reported by Jensen et al., consistent with our results.

**Mechanism: inhibition versus reserve pool depletion**

Since the progression of first-order kinetic processes, such as chemical reactions rate limited by a single type of enzyme, follow exponential time courses, the double-exponential form of Eq. 1 would be consistent with the serial emergence of two elementary rate-limiting steps in the synaptic vesicle cycle. However, conclusions such as these must be drawn with caution because double-exponential equations can often be used to fit kinetic data arising from more complicated combinations of underlying mechanisms. Nevertheless, the apparent invariance of the two component time constants in Eq. 1 in the face of a fourfold range of weighting values (i.e., \( w \) ranges from 1.0 to 0.25; see Fig. 9D) provides a more stringent constraint that continues to be consistent with the possibility that the synaptic vesicle cycle is rate limited only by two first-order biochemical processes [i.e., two in addition to the fusion step(s), which is rate limiting during periods of rest and during low-frequency use].

The molecular bases of rate-limiting synaptic vesicle trafficking steps are not currently known. The RRP resupply rate has variously been ascribed to synaptic vesicle docking; priming; recycling of the enzymes involved in catalyzing exocytosis, such as SNAREs (soluble N-ethylmaleimide–sensitive factor attachment protein receptors); or the time it takes to clear the residual membrane of spent vesicles from the active zone. To date, however, none of these mechanisms has been proven to be rate limiting for synaptic vesicle trafficking at intact...
synapses (Zucker and Regehr 2002). Even activation of second-messenger pathways linked to metabotropic receptors cannot be excluded at present because, although these pathways are thought to often target Ca\textsuperscript{2+} channels, there has been at least one report of an additional link to mechanisms that control vesicle trafficking rates (Sakaba and Neher 2003).

In a series of classic studies at a variety of synapse types, it was suggested that the speed of RRP resupply might be related to the number of vesicles contained in a reserve pool, according to mass action laws (Birks and McIntosh 1961; Elmqvist and Quastel 1965). However, although mass action models would account for the general observation that RRP resupply slows down with extensive use, the models also predict that even small amounts of reserve pool depletion would quickly cause a proportionate decrease in the RRP resupply rate and are thus not compatible with the delayed induction of supply-rate depression reported here (see Figs. 4, 7, A and C, and 9D). The double-exponential time course of RRP replenishment is also incompatible with mass action models, for reasons described previously, in the APPENDIX of Stevens and Wesseling (1999b).

Alternative, “inhibition” models (discussed in Stevens and Wesseling 1999b) that would ascribe supply-rate depression to a decrease in the capacity of the RRP to accept new vesicles, instead of reserve pool depletion, can explain most qualitative observations equally well and can also provide quantitative explanations for the delayed onset and the double-exponential RRP replenishment time course. Nevertheless, there are notable similarities between the results documented here and quantitative cell biological observations from cell culture, which, taken together, suggest that depletion of a reserve store of vesicles may indeed play a role in short-term depression under some circumstances. For example, the 600 pulses of 20-Hz stimulation needed to drive exocytosis and recycling of almost all reserve and readily releasable vesicles at least one time (Ryan et al. 1996) match the 600 needed to drive synaptic strength to the most depressed steady state in the present study. Moreover, estimates of the total size of a recycling pool at individual synapses seem to match the number of quanta recorded electrophysiologically; the synaptic terminals activated by near-minimal stimulation released a mean of 123 quanta during 90 s of stimulation, which could correspond to three rounds of exocytosis of a cycling pool (the initial pool plus two recycling rounds) consisting of 41 vesicles, each taking 45 s to travel through the exo/endocytic cycle one time, which is quantitatively consistent with independent measures of the size (Harata et al. 2001b) and recycling kinetics (Ryan et al. 1993) of the cycling pool of vesicles measured for individual synapses grown in cell culture. If depletion of a reserve pool is involved in short-term depression, however, the kinetic framework relating depletion to supply-rate depression will require further resolution.

A clear distinction between reserve pool depletion models and inhibition of the RRP capacity for accepting new vesicles will likely require additional investigation that may be aided by molecular biological tools. Particularly relevant in this regard, several synaptic vesicle proteins have been implicated in controlling the speed of recovery from depression under various experimental conditions (Cabin et al. 2002; Deak et al. 2006; Greengard et al. 1993; Sakaba and Neher 2003; Schluter et al. 2006). To date, none has specifically been shown to be rate limiting for synaptic vesicle trafficking at intact synapses, but neither has this possibility been excluded. We anticipate that the quantitative framework presented here will be useful for future work, involving these molecules and others, focused on resolving the molecular and cell biological identity of rate-limiting steps in the synaptic vesicle cycle.

**Confidence intervals for \( \tau_s \)**

For example, the time course for endocytosis often takes tens of seconds, which puts it in a range that is similar to the slow phase of RRP replenishment. However, identifying endocytosis of spent synaptic vesicle membranes with \( \tau_s \) alone would not seem to be consistent with reports indicating that endocytosis at synaptic terminals slows down progressively with extended use (Sankaranarayanan and Ryan 2000; but see Ryan et al. 1996). For this type of comparison, it is important to note that although use-dependent changes in \( \tau_s \) might have gone undetected, any such effects would likely be small (see legend of Fig. 6). Moreover, apparent invariance of \( \tau_s \) and \( \tau_r \) has now been documented in two separate reports, in which measurements were made with notably different types of techniques: i.e., the present study and Stevens and Wesseling (1999b), in which Ca\textsuperscript{2+}-independent osmotic shocks were used to trigger transmitter release from synapses in cell culture. In any case, the analyses documented in Figs. 7 and 9, C and D are tolerant to small changes in \( \tau_s \) as long as the faster phase of recovery mostly runs its course within 20 s (see Figs. 6 and 9Bii).

**Low steady-state quantal release rate**

Regardless of mechanism, our results are consistent with the possibility that slow synaptic vesicle recycling rates measured previously (Ryan et al. 1996; Sankaranarayanan and Ryan 2001) play an important role in determining synaptic output during periods of heavy use at Schaffer collateral synapses. Steady-state quantal release rates can be extrapolated from the amount of depression observed during exhaustive use, both in the ordinary and near-minimal stimulation strength experiments. During ordinary-strength stimulation, responses recorded at the standard divalent ion concentrations depressed to a mean of 9.4% of their original value during 20-Hz stimulation (see Fig. 4A). The average probability of release of individual resting Schaffer collateral terminals has been measured at about 35% with several independent types of techniques in comparable preparations under comparable conditions (Allen and Stevens 1994; Hessler et al. 1993; Huang and Stevens 1997). Thus the average probability of release likely depressed to about 3.3% (obtained by multiplying 0.35 by 9.4%), which would correspond to a quantal release rate of 0.66/s (calculated by multiplying 3.3% by 20 Hz). The steady-state quantal release rate detected in the near-minimal stimulation experiments was only slightly higher (0.90 ± 0.01/s) and the small difference could have resulted from the likelihood that our near-minimal stimulation technique sometimes evoked responses from more than a single afferent synapse. Our results thus support the hypothesis that the maximum sustainable rate of quantal synaptic transmission at individual Schaffer collateral terminals is indeed less than one event per second (as predicted previously by optical tracer techniques; see also Awatramani et al. 2007). However, our measurements do not exclude the possibility that...
some vesicles may recycle by much faster routes some of the time (Pyle et al. 2000).

**Short-term depression as a native antiepilepsy device**

The value of <1 quanta per transmission event per second per synapse during exhaustive use is consistent with the idea that information is encoded sparsely in neural circuits (Marr 1971; McNaughton and Nadel 1990). Indeed, Schaffer collaterals in awake and behaving animals typically fire short, sporadic bursts of action potentials, but rarely discharge for long periods at high frequency (McNaughton et al. 1983; Ranck Jr 1973), as would be required for induction of the supply-rate depression documented here. The firing pattern seen in vivo would elicit transmitter release at a higher rate, if only briefly, and appears to be well matched to the kinetics of the vesicle trafficking machinery at these synapses. In contrast, 20-Hz stimulation is far from sufficient for driving neurotransmission maximally at calyx of Held synapses, but such a difference makes sense in light of the extremely different physiological requirements at the calyx of Held (Hermann et al. 2007).

It has been suggested elsewhere that the substantial amount of depression that occurs during long episodes of intense activity at small excitatory synapses that are typical throughout many brain regions, including the hippocampus, might serve as a native antiepilepsy device that would shut down neural networks when pathologically active (Galarreta and Hestrin 1998; Kaplan et al. 2003). The delayed onset of supply-rate depression at Schaffer collateral synapses reported here could add an important feature to this hypothesis. Incipient seizure activity might be intense enough to substantially depress the RRP resupply rate, switching synapses over into a slowly recovering depressed state that could serve as a potent break on the initiation and propagation of epileptiform activity. Pharmacological reagents that selectively lower the threshold for induction of supply-rate depression at excitatory synapses might thus prove useful for treating epilepsy in a way that avoids the onerous side effects associated with currently available medicines that dampen ordinary function in addition to epileptiform activity (Panayiotopoulos 2002).

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