Regulation of Synaptic Vesicle Recycling by Calcium and Serotonin

Chang Wang and Robert S. Zucker*
Department of Molecular and Cell Biology
University of California
Berkeley, California 94720

Summary

Serotonin, a neuromodulator at the crayfish neuromuscular junction, regulates neurotransmission without changing intracellular calcium levels. However, the mechanism of this regulation remains unclear. By analysis of synaptic depression using a depletion model and measurement of vesicle recycling using the styryl dye FM1-43, we show that serotonin increases the number of vesicles available for transmitter release (total synaptic vesicle pool size). This regulation is due either to an increase in the number of vesicles at each release site or to an activation of previously nonsecreting or silent synapses. We also observed that low calcium medium rendered part of the vesicle pool unavailable for release. These results suggest a new mechanism for regulating synaptic transmission.

Introduction

$[Ca^{2+}]_i$ plays a crucial role in synaptic transmission (Augustine et al., 1987). A large body of literature shows that many neuromodulators operate by regulating Ca^{2+} channels (Mudge et al., 1979; Shapiro et al., 1980; Holz et al., 1989; Edmonds et al., 1990; Heidelberger and Matthews, 1991; Scholz and Miller, 1991a, 1991b; Toth et al., 1993; Yawo and Chuhma, 1993; Fossier et al., 1994; Umemiya and Berger, 1994; Wu and Saggau, 1994, 1995; Huston et al., 1995; Dittman and Regehr, 1996; Huang et al., 1996) or other presynaptic ion channels (Takeuchi and Takeuchi, 1966; Klein et al., 1982; Belardetti et al., 1987; Baxter and Byrne, 1989; Critz et al., 1991; Goldsmith and Abrams, 1992; Fu et al., 1995; McGehee et al., 1995; Gray et al., 1996). A number of other studies implicate a direct action on secretion to explain effects of neuromodulators on synaptic transmission (Hochner et al., 1986; Man-Son-Hing et al., 1989; Dale and Kandel, 1990; Kato et al., 1992; Scholz and Miller, 1992; Silinsky and Solsona, 1992; Klein, 1993; Wu and Saggau, 1994, 1995; Capogna et al., 1995, 1996; Dittman and Regehr, 1996; Hori et al., 1996; Singer et al., 1996). However, none of these studies have identified mechanisms by which a modulator affects neurosecretion directly.

A recent study of adenosine- and $GABA_B$-mediated inhibition of glutamatergic excitatory synapses in cultured rat hippocampal neurons used FM1-43 staining to measure rates of endocytosis and subsequent destaining to measure rates of exocytosis (Isaacson and Hille, 1997). No effects of presynaptic inhibitors on endocytosis or inferred pool size were observed, suggesting

that these modulators alter only the fraction of vesicles released by action potentials, probably by simply blocking Ca^{2+} influx through presynaptic Ca^{2+} channels. A recent study of protein kinase C activators on bovine chromaffin cell secretion suggests that exocytosis is enhanced by an increase in the pool of vesicles available for release (Gillis et al., 1996). Phorbol esters increased the initial rapid phase of secretion evoked by Ca^{2+} released by photolysis of caged Ca^{2+}, the early plateau increase in capacitance to prolonged depolarization, and the average size of paired responses without altering the depression expressed by the ratio of the two responses. All of these results suggest an increase in the number of docked and primed vesicles available for release.

Serotonin is an endogenous neuromodulator in vertebrates and in many invertebrate systems. At crustacean neuromuscular junctions, a brief exposure to serotonin produces increased transmitter release that lasts for up to an hour (Glusman and Kravitz, 1982; Dixon and Atwood, 1989a). Serotonin increases neither resting presynaptic $[Ca^{2+}]_i$ nor the influx of Ca^{2+} during an action potential (Delaney et al., 1991). It activates at least two second messenger systems involving phosphatidylinositol and adenylate cyclase. At the crayfish neuromuscular junction, preinjection of either a specific protein kinase A inhibitor (the Walsh inhibitor) or the adenylate cyclase inhibitor SQ22,536 blocks the late phase of serotonin's action (Dixon and Atwood, 1989b). These results suggest a cAMP mediation of the slow phase of serotonin's effects. Presynaptic injection of inositol-1,4,5-triphosphate (IP_3) generated a brief increase in miniature EPSP frequency and an enhancement of EPSP amplitude similar in duration to the early phase of serotonin action (Dixon and Atwood, 1989a). Phorbol ester activation of protein kinase C produced a longer lasting potentiation of EPSP amplitude resembling the late phase of serotonin action. Presynaptic injection of the phospholipase C inhibitor RA 233 blocked all effects of serotonin on synaptic transmission, suggesting that IP_3 causes the early phase and diacyglycerol generates the late phase via downstream action of cAMP (Dixon and Atwood, 1989a). But the targets of these messengers in the synaptic apparatus remain unknown.

Besides its role in triggering transmitter release, calcium has been proposed to affect vesicle recycling and endocytosis in a number of systems (Neher and Zucker, 1993; Ramaswami et al., 1994; Thomas et al., 1994; von Gersdorff and Mathews, 1994). It has also been proposed to influence prerelease steps in vesicle cycling, such as mobilization of vesicles to docking sites (Neher and Zucker, 1993; von Rüden and Neher, 1993).

In this study, we searched for targets of serotonin's action. We used a depletion model of synaptic depression to estimate vesicle pool dynamics and size (Liu and Tsien, 1995). We also used FM1-43 destaining coupled with postsynaptic electrical recording to estimate pool size. FM1-43 is a fluorescent dye that is taken up at terminals by endocytosis following nerve stimulation and thus labels recycled synaptic vesicles. Introduced

*To whom correspondence should be addressed.
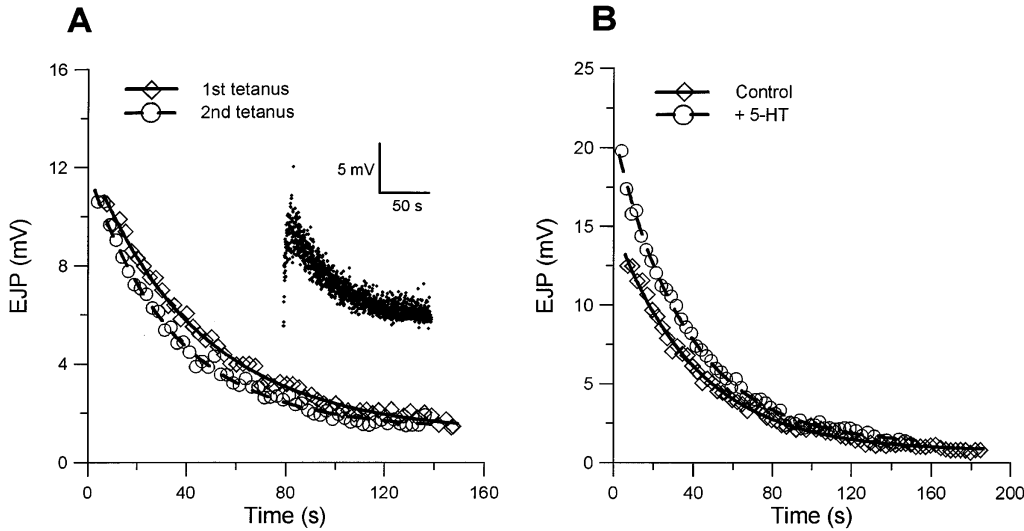
A

B

Figure 1. Estimation of Vesicle Pool Parameters from Synaptic Depression

When a 50 Hz tetanus was applied to the excitor of crayfish claw opener to deplete the pool, EJP amplitudes first increased, due to facilitation and augmentation, and then decreased ([A], inset). Only the depression phase is shown in the main figure, along with fitted exponential curves (smooth lines). Data points were smoothed by a running average of 21 EJPs, and the number of symbols was reduced for clarity.

(A) Control: the first tetanus was applied to estimate pool size (diamonds) and then at least 1.5 hr later a second tetanus was applied to deplete the pool again (circles). Inset shows the same EJP recording as diamonds.

(B) With and without serotonin: the first tetanus was applied to estimate pool size (diamonds) and then at least 1.5 hr later serotonin was added and a second tetanus was applied (circles).

by Betz and colleagues at the frog neuromuscular junction (Betz and Bewick, 1992, 1993; Betz et al., 1992a, 1992b), it has been used to study vesicle cycling at a number of preparations (Ramaswami et al., 1994; Reuter, 1995; Ryan and Smith, 1995; Lagnado et al., 1996; Smith and Betz, 1996). Both methods indicated that serotonin had little effect on the rate of vesicle cycling, but instead increased the number of vesicles available for transmitter release (total pool size).

We also sought to test if this increase in pool size is due to the activation of previously nonsecreting or silent synapses or to an increase in the number of vesicles available for release in active synapses. Using an FM1-43 and FM4-64 dual labeling method (Henkel and Betz, 1995), we found the number of vesicles released and recycled within individual FM1-43-labeled puncta was increased. At this level of resolution, activation of presynaptically silent synapses was not detected.

Finally, we tested effects of reduced extracellular calcium concentration ($[Ca^{2+}]_o$) on vesicle recycling and pool size. We found that low $[Ca^{2+}]_o$ seemed to block release from a part of the vesicle pool and to interfere with recovery of released vesicles into a fully releasable store.

Results

Serotonin's Effect on the Vesicle Pool Measured by Depression

To study if serotonin has any effect on synaptic recycling, we first used a method based on depression (Liu and Tsien, 1995). A train of high frequency stimuli (50 Hz) was applied to the crayfish opener motoneuron, and excitatory junction potentials (EJPs) were measured. As shown in the inset to Figure 1A, EJP amplitude first increased due to facilitation and augmentation (Zucker, 1989; Kamiya and Zucker, 1994) and then gradually decreased. This decrease, which was observed only in central fibers, has a slow time course and is assumed to be due to a depletion of the synaptic vesicles available for release. We used Liu and Tsien's simplified model (1995) to analyze this depression. In their model, all synaptic vesicles are divided into two pools, available or unavailable. The available pool includes both readily releasable and reserve vesicles. Traffic between the pools is governed by forward and reverse transition probabilities. The forward transition (fraction of vesicles released by each action potential, ϕ) incorporates late steps leading up to and including transmitter release, while the reverse transition (fraction of vesicles recycled per interval between action potentials, ρ) represents endocytosis and repriming. Based on this model, if synaptic transmission and recycling occur at a constant rate, then the depletion process can be described by a decaying exponential. If v_0 = initial EJP amplitude, v_∞ = steady state depressed EJP amplitude, q = average quantal amplitude (50 μV, Zucker and Lara-Estrella, 1983; Mulkey and Zucker, 1993), and τ_d = inverse rate constant of EJP decay expressed as number of spikes (i.e., number of EJPs needed for EJP amplitude to drop to 1/e of initial value), then the size of the initial pool available for release (N_a), measured in quanta, is:

$$N_a = v_0^2 \tau_d / q(v_0 - v_\infty). \tag{1}$$

The forward and reverse transition rates can also be calculated:

$$\phi = (v_0 - v_\infty)/ v_0 \tau_d, \tag{2}$$

$$\rho = v_\infty/v_0\tau_d. \tag{3}$$

Table 1. Effects of Serotonin and Low $[Ca^{2+}]_0$ on Vesicle Pool Size and Release and Recovery Rates

Method	Parameter	Control (Mean ± SE)			Serotonin (Mean ± SE)			$[Ca^{2+}]_0$ (Mean ± SE)		
		Control 1	Control 2	N	Without	With	N	Control	Low Ca^{2+}	N
Depression method	v_0 (mV)	13.4 ± 2.0	11.2 ± 2.0	8	12.0 ± 1.6	19.0 ± 2.3[a]	9	10.9 ± 1.6	5.58 ± 1.02[a]	5
	v_∞ (mV)	1.00 ± 0.19	1.07 ± 0.27	8	1.10 ± 0.14	1.81 ± 0.35	9	0.84 ± 0.26	0.15 ± 0.06[a]	5
	τ_d (10^3 action potentials)	2.9 ± 0.3	2.8 ± 0.5	8	3.4 ± 0.4	2.6 ± 0.3	9	4.54 ± 0.54	4.35 ± 0.62	5
	Pool size (10^3 vesicles)	840 ± 130	740 ± 190	8	920 ± 180	1140 ± 240[a]	9	1090 ± 140	500 ± 80[a]	5
	$\phi \times 10^{-4}$ (per pulse, at 50 Hz)	3.4 ± 0.5	4.0 ± 0.9	8	3.1 ± 0.5	3.9 ± 0.5	9	2.0 ± 0.2	2.3 ± 0.3	5
	$\rho \times 10^{-5}$ (per pulse, at 50 Hz)	3.2 ± 0.8	6 ± 2	8	4.4 ± 1.4	4.8 ± 1.4	9	1.8 ± 0.4	0.6 ± 0.2[a]	5
FM1-43 destaining method	V (mV)	5.1 ± 0.9	5.3 ± 1.3	4	5.1 ± 0.5	10.6 ± 1.2[a]	5	6.4 ± 0.2	1.8 ± 0.3[b]	4
	τ (s)	329 ± 36	324 ± 20	6	278 ± 13	235 ± 31	7	289 ± 16	401 ± 21	4
	Pool size (10^3 vesicles)	570 ± 100	650 ± 170	4	490 ± 20	720 ± 80[a]	5	720 ± 20	260 ± 37[b]	4
	$F \times 10^{-4}$ (per pulse, at 20 Hz)	1.7 ± 0.2	1.6 ± 0.1	4	1.9 ± 0.1	2.3 ± 0.4	7	1.8 ± 0.1	1.4 ± 0.1	4
FM1-43 intensity	Percent increase	27 ± 18		4	120 ± 20[b]		5	not measured		

[a] Indicates $p < 0.05$ compared to controls; calculated for percentage change.
[b] Indicates $p < 0.01$ compared to controls.
v_0, v_∞, τ_d, V, and τ are measured; pool size, ϕ, ρ, and F are calculated.

In this model, v_0 refers to the EJP amplitude after full development of facilitation and augmentation, but before it decays due to depression. This procedure for extracting the effects of depression alone on EJP amplitude is valid so long as facilitation and augmentation develop more quickly than depression (tens of seconds versus minutes; see Zucker, 1974; Delaney and Tank, 1994), and the processes responsible for facilitation and augmentation (activated by residual Ca^{2+} acting at specific sites; see Kamiya and Zucker, 1994) are independent of those responsible for depression (depletion of a releasable pool). Effects of posttetanic potentiation and long-term facilitation are neglected (see Discussion). The value of v_0 is estimated by extrapolating the exponentially decaying EJP amplitudes back to time zero.

This model is able to describe the decay of the EJP amplitudes, as shown in Figures 1A and 1B. The parameters of the fitted curves are summarized in Table 1. At the crayfish neuromuscular junction, $N_a = 840,000 \pm 130,000$ (mean ± SEM) vesicles (n = 8; Table 1).

We also estimated the forward (ϕ) and reverse (ρ) transition rates. At 50 Hz, ϕ was 0.00034 ± 0.00005 (Table 1). This means each action potential released 0.034% of the total synaptic vesicle pool. The reverse transition rate was 1.6×10^{-3} s^{-1} (0.000032 ± 0.000008 per interval between action potentials; Table 1), which means that it took about 10 min for 67% of the vesicles to reenter a releasable pool.

In principle, ρ can also be measured from the time course of recovery of infrequent EJPs following a tetanus (Liu and Tsien, 1995). This time course was complicated by recovery from facilitation and augmentation and especially from posttetanic potentiation, which obscured the recovery from depression (data not shown). Potentiation was not expressed during the tetanus (Figure 1), apparently due to saturation of transmitter release (Zucker, 1974; see Discussion). But after the tetanus, potentiation was large and decayed with a rate similar to recovery from depression, obscuring the latter's recovery.

We then tested whether serotonin changes release or recovery rates or the initial pool size. We first gave a tetanus to measure all the parameters in normal solutions. Since the long tetanus needed to observe depression sometimes induces long-term facilitation (LTF) (Atwood and Wojtowicz, 1986), we waited for at least 1.5 hr before giving a second tetanus to allow for decay of LTF and the released vesicles to recycle. If the EJP returned to its initial control level, we added serotonin, waited 10 min, and repeated the same sequence. As shown in Figure 1B, in 5 μM serotonin, the EJP amplitude reached a higher maximum compared to that in the absence of serotonin in the same muscle fiber. However, the time constant of depression changed very little (41 s in control versus 37 s in serotonin). Equation 1 suggests that an increase in the initial vesicle pool size has occurred.

To confirm that this increase is due to serotonin rather than an aftereffect of the first tetanus, we performed control experiments where the experimental procedures were the same except that serotonin was not added during the second tetanus. In this case, the maximum EJP amplitude was slightly smaller in the second tetanus, while the decay rates were similar (Figure 1A). Therefore, after the first tetanus, the vesicle pool size appeared to decrease. On average, pool size increased 25% in serotonin, while in the corresponding control experiments the pool size decreased by 12%.

In order to test for significance in the effect of serotonin, we determined the percentage change in estimated pool size in each preparation and compared the distributions of changes in serotonin and control (two tetani in normal solution) experiments. In serotonin, the change in pool size was about 40% greater than the change in pool size during a second control tetanus, and this difference was significant ($p < 0.05$). As summarized in Table 1, there was no statistically significant difference
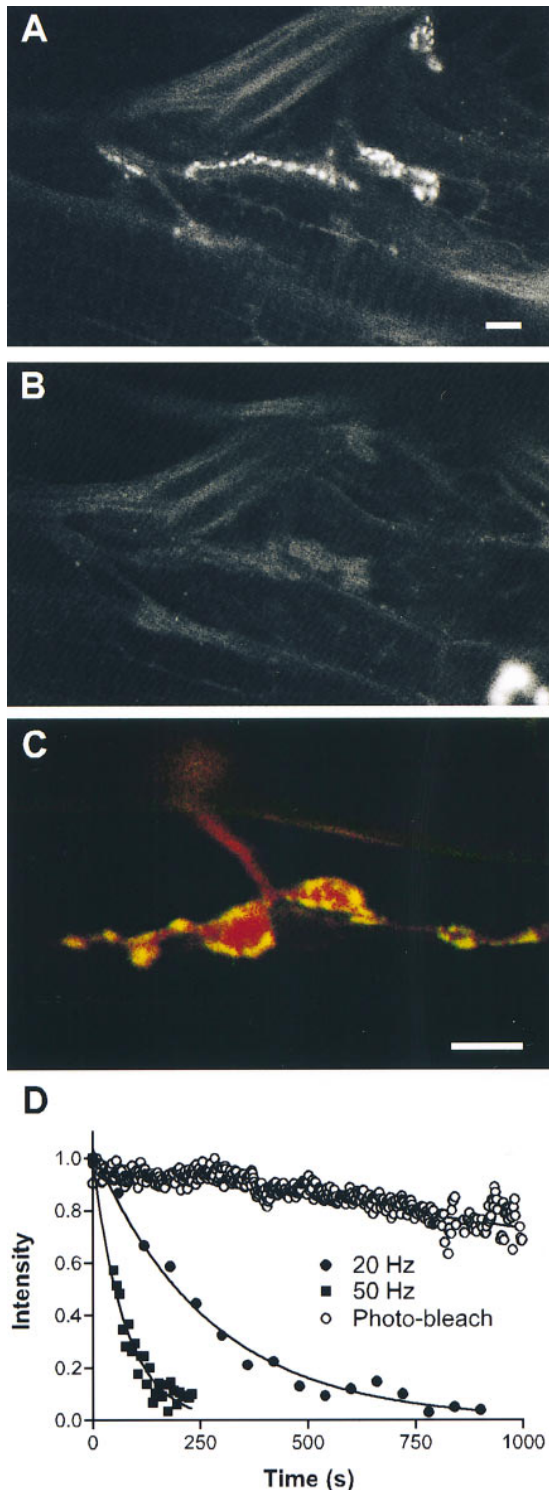
(t test) between the release and the recovery rates in the presence and the absence of serotonin. Nonparametric statistics also revealed no significant change, because there was no consistent trend toward an increase or decrease in ϕ or ρ in individual experiments.

Serotonin's Effect on the Vesicle Pool Measured by FM1-43 Destaining

A different approach to detecting changes in the number of vesicles involved in synaptic transmission uses FM1-43 staining (Ryan et al., 1996). In the simplest application of this method, all synaptic vesicles are loaded with FM1-43, and the total intensity is measured. A second long tetanus then destains the fluorescence, and residual nonspecific fluorescence is measured. The difference in intensities is proportional to the total pool size. However, at the crayfish neuromuscular junction, a long tetanus that totally loads the synaptic vesicle pool usually induces LTF. Since LTF would complicate our study of the function of serotonin, we used a different method to estimate the pool size. This method combines FM1-43 destaining and postsynaptic electrical recording to estimate synaptic vesicle pool size (see Appendix).

We first checked whether FM1-43 is incorporated into synaptic vesicles during endocytosis at the crayfish neuromuscular junction. We used conventional methods to load FM1-43 into the nerve terminals. Figure 2A shows the FM1-43 staining. After a second long tetanus, the presynaptic fluorescent staining disappeared, leaving only faint nonspecific staining (Figure 2B).

Presynaptic staining was correlated with transmitter release. After normal ringer was changed to a solution in which Ca^{2+} was replaced with Co^{2+} (13.5 mM), an ion that blocks Ca^{2+} channels and therefore inhibits transmitter release (Mulkey and Zucker, 1991), the fluorescent labeling could not be destained by stimulation. If we replaced Co^{2+} with Ca^{2+}, the FM1-43 labeling could again be destained (data not shown). These results show that the destaining of FM1-43 labeling was Ca^{2+} dependent. When we injected Texas red into the crayfish excitor axon and then loaded FM1-43, we found that the FM1-43 staining colocalized with presynaptic boutons labeled with Texas red (Figure 2C). These results indicate that the FM1-43 puncta label clusters of presynaptic vesicles at active zones in boutons.

The time courses of FM1-43 destaining at two stimulation frequencies are shown in Figure 2D. These time courses fit nicely to simple exponential decays. At 20 Hz stimulation, FM1-43 fluorescence decayed fairly slowly, with a time constant of 329 ± 36 s (n = 6). If FM1-43-labeled vesicles mix well with unlabeled vesicles, then

Figure 2. FM1-43 Loading and Destaining

(A) Fluorescence image of crayfish neuromuscular junction where FM1-43 (10 μM) was loaded with a tetanus of 3600 pulses at 20 Hz, followed by rinsing for 40 min in dye-free saline.

(B) The same field as in (A) after FM1-43 labeling was destained by firing a train of 20,000 action potentials, showing almost all fluorescent dye has been released.

(C) A fluorescent image of FM1-43 colabeled with Texas red that was microinjected into the axon. FM1-43 is shown as green and Texas red as red. When the images are overlapped, the color becomes yellow. Scale bar = 10 μm.

(D) Time course of FM1-43 destaining and photobleaching. Each point in photobleach trace represents the time a frame was collected or each time the shutter was opened (every 2 s). Each point in the FM1-43 destaining trace represents a saved image, while the rate at which frames were collected was the same as in the photobleaching trace for 20 Hz stimulation and twice as fast for 50 Hz stimulation. Fluorescent intensity was normalized to maximum. Curves are fitted with a single exponential. Final background after destaining has been subtracted.
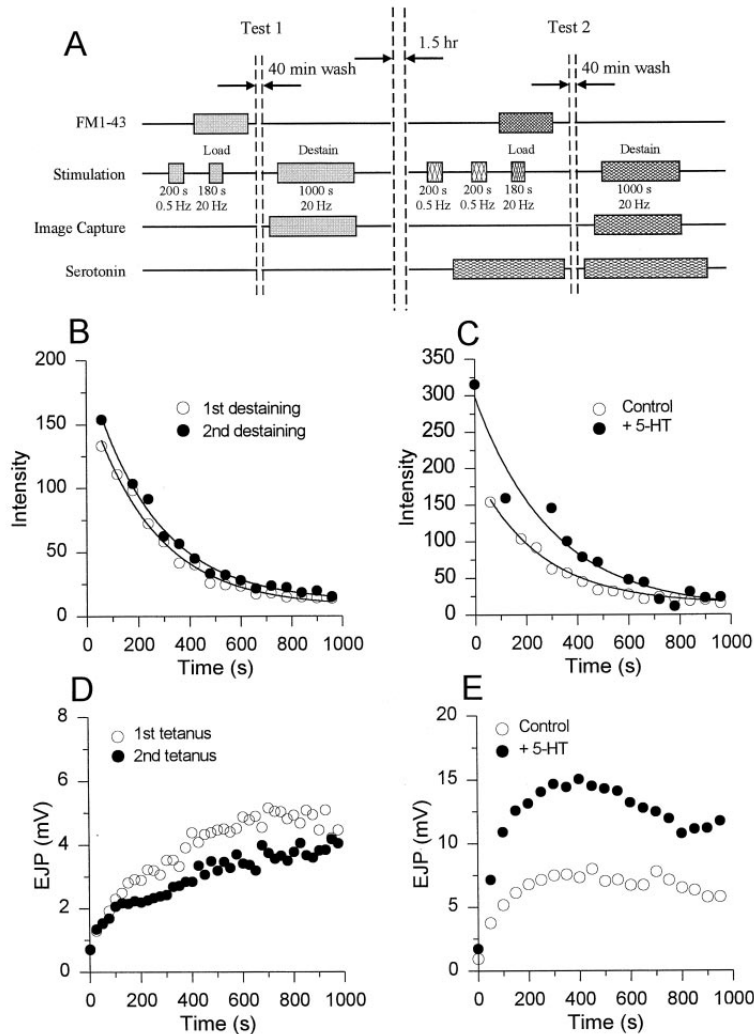
Figure 3. Effect of Serotonin on Staining and Destaining of FM1-43

(A) The protocols used for FM1-43 staining and destaining—control on the left, experimental on the right.

(B) Control: FM1-43 was loaded by a tetanus of 3600 pulses at 20 Hz and destained by 20,000 pulses at 20 Hz. The time constant was 253 s for the first destaining and 251 s for the second destaining. The sequence was repeated after a 90 min rest.

(C) Same protocol in a different experiment in which serotonin was present during the second stain/destain sequence. Curves are fitted exponentials. The time constant was 258 s for the control and 282 s for serotonin.

(D) The corresponding EJP amplitudes during destaining in (B).

(E) The corresponding EJP amplitudes during destaining in (C).

the destaining time course represents the utilization of the initial vesicle pool available for release (Ryan and Smith, 1995). At the crayfish neuromuscular junction, 95% of the initial synaptic vesicle pool was utilized in about 1000 s when stimulated at 20 Hz. Since the inverse of the time constant divided by stimulus frequency is the fraction of the vesicle pool released by each stimulation (see Appendix), each action potential released approximately 0.017% of the entire synaptic vesicle pool (Table 1).

As expected, at 50 Hz stimulation FM1-43 fluorescence decayed about 2.5 times faster, with a time constant of 95 ± 11 s ($n = 5$). This further confirms that the FM1-43 puncta are clusters of labeled synaptic vesicles. In contrast to the destaining process, photobleaching of the FM1-43 staining has a much slower time course. Therefore, the destaining process we measured was affected little by photobleaching.

The number of vesicles available in synaptic transmission (total vesicle pool) was calculated by the equation

$$T = \frac{m}{F},$$ (4)

where T is the total number of vesicles available for transmitter release, m is the number of vesicles released by a single action potential or the quantal content of an EJP, and F is the fraction of the total vesicle pool released by a single action potential (see Appendix). During stimulation, EJP size stabilized rapidly to a high level due to facilitation and augmentation. At room temperature and 20 Hz stimulation, a small amount of potentiation or LTF developed slowly during the tetanus (Figures 3D and 3E). We used a value of m derived from averaged EJPs during the tetanus.

Based on this method, we calculated the initial pool size and found it was regulated by serotonin, as suggested by depression experiments. The staining and destaining protocol is outlined in Figure 3A. In control solution the pool size was $490,000 \pm 20,000$ vesicles ($n = 5$; Table 1). After a 1.5 hr rest and if there was no LTF, 5 μM serotonin was added to the bath and the pool size was measured again. EJPs were greatly increased (Figure 3E and Table 1), but the destaining rate was almost unchanged (Figure 3C); the pool size increased to $720,000 \pm 80,000$ vesicles, while the fraction of pool released per action potential showed only a statistically

insignificant tendency (t test or nonparametric tests) to increase slightly.

We performed control experiments to test if these effects were due to the presence of serotonin (Figures 3B and 3D). The procedure was the same as above except no serotonin was added in the second loading and destaining (Figure 3A). In the first measurement, the pool size was 570,000 ± 100,000 vesicles (n = 4). After 1.5 hr rest, the pool size was almost unchanged (Table 1). The fraction of the pool released by an action potential also remained constant at 0.016 %. On average the pool size increased 47% in serotonin and increased only 10% in controls; the difference was significant (p < 0.05).

In these experiments, we often recorded EJPs from central fibers or distal fibers near the central fibers that were imaged to measure FM1-43 destaining. We tested whether it makes any difference which type of muscle fiber was used to record EJP. At low frequency, EJP amplitudes recorded from distal fibers are larger then those recorded from central fibers. But since the EJPs in central fibers facilitate more, they reach levels similar to those in central fibers at higher frequency (Atwood and Wojtowicz, 1986). We found that the average EJP amplitude at 20 Hz recorded from distal fibers (4.6 ± 0.4 mV, n = 8) was about the same as that from central fibers (4.2 ± 0.5 mV, n = 6).

Does Serotonin Activate Silent Synapses?

The changes in pool size that we have measured may be explained in two ways: either the number of vesicles participating in release at each release site increases, or the number of participating release sites increases. The second mechanism would imply an activation of presynaptically silent synapses, by which we mean release sites that did not previously secrete transmitter. We evaluated both possibilities.

To test for an activation of previously silent synapses, we used an FM1-43 and FM4-64 dual loading protocol (Henkel and Betz, 1995). FM4-64 is a fluorescent dye similar to FM1-43, but it has a different emission wavelength. We loaded FM1-43 first in the absence of serotonin. Then in the presence of serotonin we loaded FM4-64 with a short tetanus (see Figure 4A and Experimental Procedures). The length of the second tetanus was adjusted so that FM1-43 was destained to about 50% and was therefore still bright enough to be measured. If presynaptically silent synapses were activated in the presence of serotonin, they would be labeled by FM4-64 but by not FM1-43.

Figure 4B shows a control experiment in which no serotonin was added during FM4-64 loading. FM4-64 staining is shown in Figure 4B1 and FM1-43 staining in Figure 4B2; the staining patterns are almost identical. If we use red to represent FM4-64 staining and green to represent FM1-43 staining and merge the two images, then yellow puncta would represent sites labeled by both dyes. If there were activation of previously silent synapses in the second tetanus, the synapses would be labeled only by FM4-64 and, therefore, would be red. As shown in Figure 4B3, there was little evidence of red staining.

To investigate this more closely, we calculated the ratio of FM4-64 intensity to FM1-43 intensity at individual puncta and plotted the frequency distribution of the logarithm of the ratios. If there were presynaptically silent synapses, the distribution of the histogram would be skewed to the right. As shown in Figure 4B4, the histogram was symmetrical. In four experiments consisting of 73–144 puncta, the average skewness was 0.3 ± 0.4.

In the presence of serotonin during FM4-64 loading, we did not detect any change in the staining pattern. As shown in Figures 4C1 and 4C2, all the puncta labeled by FM4-64 were also labeled by FM1-43. The distribution of intensity ratios was again symmetrical (Figures 4C3 and 4C4). In six experiments consisting of 70–151 puncta, the average skewness was 0.3 ± 0.2.

A clear difference between control and serotonin experiments was that the ratios of FM4-64 to FM1-43 intensities were larger in serotonin. In the six serotonin experiments, the log ratios varied between 0.112 and 0.498 (representing an approximate doubling of average intensity), while in the four control experiments, the log ratios were between −0.008 and 0.067. The difference is statistically significant (p < 0.01). Thus, serotonin increased the number of labeled vesicles at individual puncta.

An increase in vesicle pool size was also inferred from the results of the single labeling experiments. As shown in Table 1, FM1-43 staining was brighter in the presence of serotonin (p < 0.05). Since in both depletion and FM1-43 destaining experiments the fraction of the vesicle pool recycled by a single pulse did not change significantly in control experiments and in serotonin tests (p > 0.3), similar fractions of the pool were labeled in each case. The brighter labeling in serotonin therefore suggests that the number of vesicles available for release was increased in serotonin.

A final approach to this question was to load FM1-43 in serotonin and destain in serotonin-free medium (Figure 5). This should result in some previously active synapses becoming "silent," in which previously recruited vesicles cannot be accessed, and destaining will be incomplete. We found that 23% ± 5% (n = 4) of dye could not be destained 90 min after washing out serotonin, compared to 4.2% ± 1.7% in controls (p < 0.05; see Figure 3B and control traces of Figures 3C and 6B). Destaining rates (τ = 383 ± 41 s) were unchanged from those in normal solution (329 ± 36 s, p > 0.2) or in serotonin (235 ± 31 s, p > 0.05) throughout the experiment. If we calculated the pool size increase in serotonin and divided it by the pool size in serotonin based on the FM1-43 destaining experiments, we would expect a 34.2% ± 6% increase. This is similar to the percentage of dye that could not be destained.

Calcium Changes Pool Size in a Different Way

To further test our methods and also to determine whether the regulation of the pool size was a unique feature of serotonin, we studied the effect of external calcium concentration ([Ca^{2+}]$_e$) on pool size. We decreased [Ca^{2+}]$_e$ to test for effects on pool size and release and recovery rates. In principle, if low [Ca^{2+}]$_e$ reduces the fraction of the pool of transmitter released by action potentials without altering the pool size, then it should take a longer time to deplete the pool.
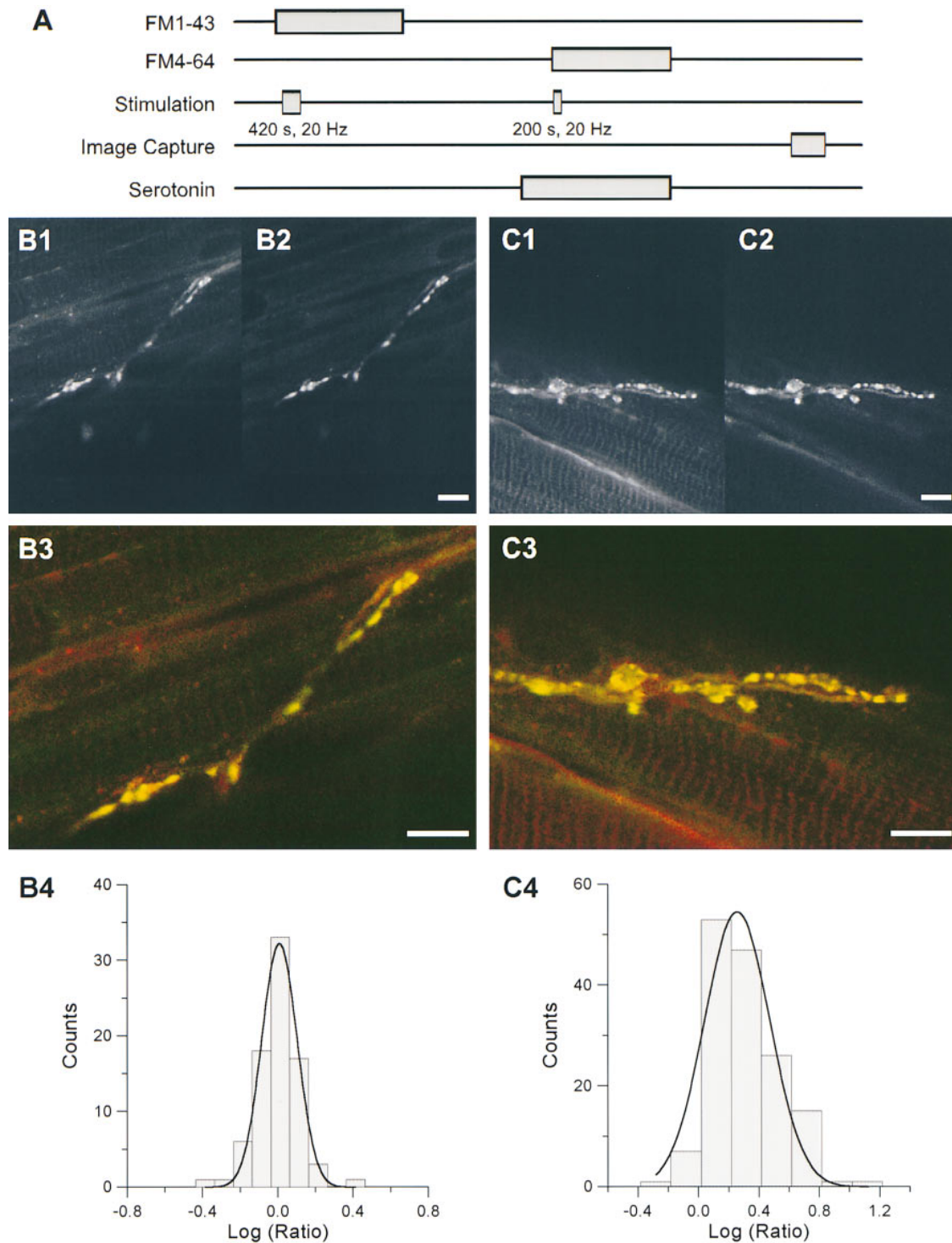
Figure 4. FM1-43 and FM4-64 Dual Labeling

(A) The dual labeling protocol. Concentration (in micromolars): 8 FM1-43, 30 FM4-64, and 5 serotonin. In the control experiment, no serotonin was added during FM4-64 loading.

(B) Control: (B1) is a fluorescent image of boutons labeled by FM4-64; (B2) is a simultaneously scanned fluorescent image of boutons labeled by FM1-43. (B3) is a merged image of (B1) and (B2). FM1-43 is represented by green and FM4-64 by red. If both FM1-43 and FM4-64 label a spot with similar intensities, the color becomes yellow. (B4), Frequency distribution of the logarithms of ratios of FM4-64 intensity to FM1-43 intensity of individual puncta. The histogram was fitted to a Gaussian distribution, with a mean of 0.0082 and SD of 0.094.

(C) Effect of serotonin: (C1–C4) are similar to (B1–B4), except that serotonin was added during FM4-64 loading. In (C4) the mean of the Gaussian distribution is 0.26 and SD is 0.22.
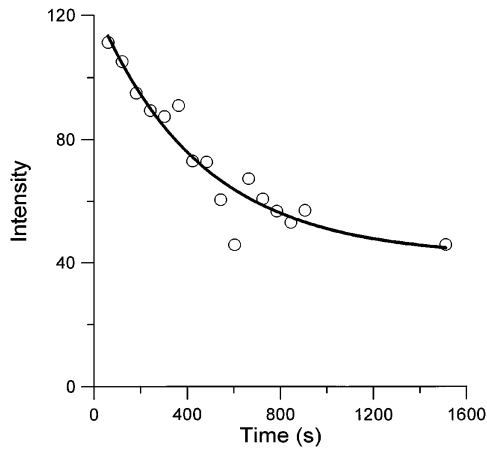
Figure 5. FM1-43 Loaded in Serotonin Does Not Fully Destain in Normal Medium

FM1-43 was loaded in 5 μM serotonin with a 3 min 20 Hz tetanus and destained after serotonin was washed out.

We attempted to test this idea by analysis of depression. In 7 mM, 3.5 mM, and 2.5 mM $[Ca^{2+}]_e$, the maximum EJP amplitude at 50 Hz reached the same level as in the normal $[Ca^{2+}]_e$. This is due to a saturation of transmission that has been reported previously at these synapses (Zucker, 1974). If we further decreased $[Ca^{2+}]_e$ to 2 mM, as shown in Figure 6A, the maximum EJP amplitude was reduced, but the time constant of the decay did not change much (Table 1). Perhaps ϕ was still saturated at the 50 Hz needed to observe depression, even in 2 mM $[Ca^{2+}]_e$. In any case, the results imply a decrease in pool size.

An important difference from the serotonin results was that in low $[Ca^{2+}]_e$ the recovery rate decreased substantially (p < 0.05). In fact, in some experiments the depressed EJP amplitude was so low that it was indistinguishable from noise level; this leads to a value of ρ = 0 (Equation 3). It may be that in low $[Ca^{2+}]_e$ we fail to activate a priming step in the vesicle cycle that makes vesicles available for release, thus inhibiting recovery and reducing the usable size of the releasable vesicle pool (see Discussion).

We also tested whether low $[Ca^{2+}]_e$ affected pool size using FM1-43 destaining. We tried to use 2 mM $[Ca^{2+}]_e$ in destaining, since it was the concentration used in the depression experiments. However, at this concentration there was very little destaining even after 20,000 stimuli (about 17 min) and it was impossible to fit the fluorescence decay to an exponential curve. We then used 3.5 mM $[Ca^{2+}]_e$; the results are shown in Figure 6B.

Unlike at 50 Hz stimulation (needed to produce depression) in 3.5 mM $[Ca^{2+}]_e$, at 20 Hz stimulation used for destaining EJP amplitudes did not reach the saturated maximum seen in higher $[Ca^{2+}]_e$. In 3.5 mM $[Ca^{2+}]_e$, FM1-43 destained more slowly than in normal $[Ca^{2+}]_e$ (time constant 401 ± 21 s in low $[Ca^{2+}]_e$ versus 289 ± 16 s in control). Since the average EJP amplitude decreased to 28% of its original value (Table 1 and Figure 6C) and the fraction of the pool released per action potential decreased by 22%, the pool size decreased to about
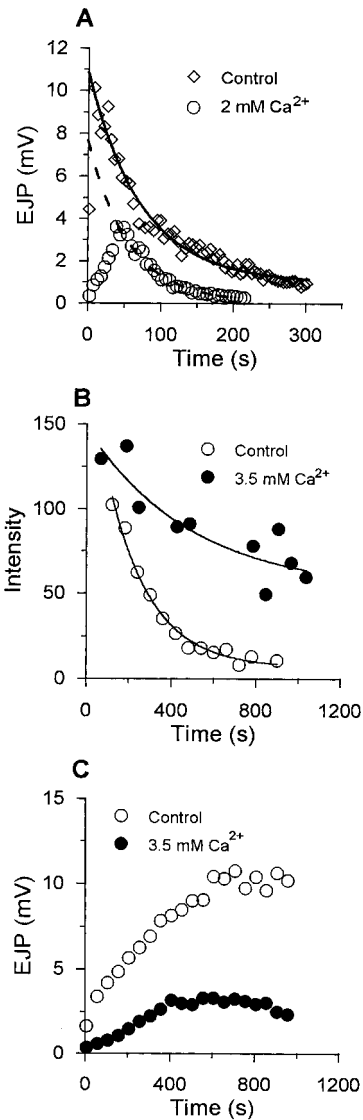

Figure 6. Effect of Reduced $[Ca^{2+}]_e$ on Depression and FM1-43 Destaining

(A) EJP depression in normal $[Ca^{2+}]_e$ (13.5 mM) and 2 mM $[Ca^{2+}]_e$. Experimental procedure was the same as in Figure 1, except low $[Ca^{2+}]_e$ replaced serotonin, and the early EJPs showing facilitation and augmentation have been included.
(B) FM1-43 destaining in 3.5 mM $[Ca^{2+}]_e$. Experimental procedure was the same as in Figure 3, except low $[Ca^{2+}]_e$ replaced serotonin. The time constant is 200 s for control and 555 s for 3.5 mM $[Ca^{2+}]_e$.
(C) The corresponding EJP amplitudes during destaining in (B).

37% of its original size. After correcting for photobleaching, we found that 52% ± 12% of the FM1-43 intensity cannot be destained by stimulation (Figure 6B). This agrees with results from the depression experiments. The reduced pool size and inability to destain half the FM1-43 labeling suggest that some step in vesicle supply is inhibited by low $[Ca^{2+}]_e$ or that some synapses cease to release transmitter and recycle vesicles (see Discussion).

Since the depression experiments indicated a reduction in ρ in low $[Ca^{2+}]_e$, we tested whether endocytosis

was inhibited in this condition. We loaded FM1-43 in 2 mM $[Ca^{2+}]_e$ and found that FM1-43 staining reached normal levels (data not shown). Therefore, low $[Ca^{2+}]_e$ did not block vesicle endocytosis.

Discussion

In this paper, we used two independent methods, one based on analysis of synaptic depression and the other on FM1-43 destaining coupled with postsynaptic recording, to determine the number of vesicles available for release and rates of release from and recovery to that pool. We also tested effects of serotonin and low $[Ca^{2+}]_e$ on these parameters. We found that serotonin increases the apparent number of vesicles available for release. There was no significant increase in fraction of pool released per action potential, although our results suggest that there is also a small increase in release rate that remained below statistical significance. The apparent increase in pool size is due either to an activation of presynaptically silent synapses or to an increase in the number of the vesicles available for release at each release site. FM1-43/FM4-64 dual labeling could not detect activation of presynaptically silent synapses. Using FM1-43 labeling, we found that the increase occurs within each punctum. Low $[Ca^{2+}]_e$ reduced both the size of the vesicle pool available for release and the recovery of released vesicles into the releasable pool. It is appropriate to discuss several points that affect the reliability of our methods and the implications of our results.

What Synaptic Vesicle Pool Did We Measure?

The pool size measured by FM1-43 destaining is the total number of vesicles participating in recycling and available for release. However, for the measured pool size to correspond to the total vesicular pool, FM1-43-labeled vesicles must be well mixed with unlabeled vesicles so that labeled and unlabeled vesicles have an equal chance of being released. If this condition is true, destaining will occur at the same rate regardless of whether the synaptic pool is totally or only partially stained (Betz and Bewick, 1992; Ryan and Smith, 1995). If labeled vesicles did not mix well with preexisting unlabeled vesicles, destaining would be slower from a partially labeled pool. We found the destaining rate to be independent of level of loading (data not shown), confirming this assumption. Thompson and Atwood (1984) also found that horseradish peroxidase labeling of synaptic vesicles appeared uniformly throughout the presynaptic vesicle pool, which also supports the assumption of a well-mixed pool.

Synaptic depression has often been used to estimate pool size by assuming that it is caused by depletion of that pool (e.g., Elmqvist and Quastel, 1965). Most models, including the one used here, postulate that the fraction of the store released by an action potential remains constant. A similar assumption underlies the analysis of pool size by FM1-43 destaining. Due to effects of facilitation, augmentation, and potentiation (Zucker, 1974; Delaney et al., 1989; Delaney and Tank, 1994), this assumption seems unlikely to be true. However, facilitation

and augmentation reach steady state within 10–20 s (Zucker, 1974; Delaney and Tank, 1994). Potentiation should continue to grow during the 50 Hz tetani used to observe depression, but the uniexponential decays of EJP amplitude suggests that this is not occurring. It has previously been shown that EJP amplitude saturates to a maximum fixed level at 50 Hz stimulation in normal $[Ca^{2+}]_o$ (Zucker, 1974), apparently due to saturation of transmitter release. This tends to fix the release rate, ϕ, at a constant near-maximal level during 50 Hz stimulation. At 20 Hz and room temperature, we sometimes did observe some potentiation during the destaining tetanus (e.g., in Figure 3E but not Figure 3F). However, if F increased substantially during 20 Hz stimulation, staining should not decay according to a single exponential (Christensen and Martin, 1970). Thus, the assumption that release rate remains roughly constant throughout most of the tetanus is likely to be sufficiently accurate so that our estimates of destaining rate, release fraction, and pool size are not likely to be affected greatly.

In the case of depression, this simplification leads to an overestimation of pool size, because extrapolation of the decaying exponential back to time zero overestimates the number of quanta released at the beginning of the train. We can roughly correct for this error by estimating pool size at a time after the beginning of stimulation, when facilitation and augmentation have reached steady state, and by adding back the number of vesicles already released. This correction suggests that initial pool size was overestimated by at most 15% for control and serotonin experiments. For low calcium experiments, initial pool size was overestimated by 15%–30%. Therefore, the actual reduction in pool size in low calcium was probably slightly larger than indicated in the uncorrected results of the depression experiments shown in Table 1.

Depression at the crayfish neuromuscular junction occurs much more gradually than at most synapses used to study depression. Indeed, depression can be observed only with prolonged stimulation at frequencies in excess of 40 Hz. At many other synapses, PSP amplitudes drop rapidly with just a few low frequency stimuli, indicating the rapid depletion of a small store. This immediately releasable store is often much smaller than the total number of vesicles at nerve terminals (e.g., at hippocampal synapses; Dobrunz and Stevens, 1997). Depletion of the total pool of vesicles may be observed by more prolonged stimulation at moderate frequencies (e.g., compare Dobrunz and Stevens, 1997, to Liu and Tsien, 1995). Many other synapses show multiple phases of depression. The first rapid phase of depression is usually thought to correspond to a small readily releasable pool of docked or primed vesicles, while later phases are thought to reflect depletion of more distant pools and eventually the entire pool of presynaptic vesicles (reviewed in Zucker, 1989 and 1996). The absence of such a rapid phase of depression at crayfish opener neuromuscular junctions suggests that the immediately releasable pool is replenished with unusual rapidity after each action potential at these synapses.

There are two reasons to believe that the pool we measure from depression represents the total number

of releasable vesicles. First, our estimates of pool size from depression and FM1-43 destaining are similar (approximately 850,000 versus 550,000). The estimates are not significantly different (compare pool sizes under various control conditions from the two methods in Table 1), and the difference is partly explained by the overestimation of pool size from depression discussed above. Second, our estimates of pool size are similar to those derived from ultrastructural observations. Wojtowicz et al. (1994) estimated that the number of vesicles in one active zone at the crayfish neuromuscular junction is 600. If there are about 50 active zones per bouton or varicosity (Cooper et al., 1995) and approximately 50 boutons or extracellularly isolatable recording sites per muscle fiber (Bittner and Kennedy, 1970), then there are a total of roughly 1,500,000 vesicles in all the synapses associated with one muscle fiber. This should correspond to the maximum size of the releasable pool that we observe under any conditions (approximately 1,150,000 vesicles; Table 1). Given the roughness of the factors used to calculate the total number of vesicles from ultrastructural data, as well as estimates of quantal size used in our calculations, the agreement with our measurements is very good.

In our experiments, serotonin increased transmission more modestly (often only doubling EPSP amplitude; Table 1) than in earlier studies (Glusman and Kravitz, 1982). It appears that the tetanic enhancement of synaptic transmission occludes somewhat the action of serotonin. This would be expected if part of the tetanic enhancement of transmission involves the recruitment of some of the same silent synapses activated by serotonin. Recruitment of silent synapses has previously been implicated in activity-induced synaptic enhancement (Wojtowicz et al., 1994), and interactions between serotonin action and augmentation and potentiation phases of activity-induced synaptic enhancement have recently been observed (Qian and Delaney, 1997).

The Effect of Low $[Ca^{2+}]_e$ on Pool Size and Recovery Rate

The results in low $[Ca^{2+}]_e$ were unexpected. We needed to reduce $[Ca^{2+}]_e$ to 2 mM in order to desaturate transmission at 50 Hz. Then we calculated from the depletion model of depression a reduction in pool size (N_a) and recovery rate (ρ), with little change in release fraction, ϕ. Perhaps release fraction was still saturated at 50 Hz. The reduction in ρ was apparently not due to an inability to take up released vesicle membrane, since FM1-43 staining proceeded normally in low $[Ca^{2+}]_o$. At 20 Hz and 3.5 mM $[Ca^{2+}]_e$ used for destaining measurements, we observed the expected reduction in release fraction, F, but the calculated pool size was again reduced. Even more remarkable was our finding that part of the previously labeled pool was unreleasable. This again suggests a reduction in recyclable pool size. At 2 mM $[Ca^{2+}]_e$, so little of the previously labeled pool was recyclable that we could not accurately estimate destaining rate.

These results suggest that in low $[Ca^{2+}]_e$, vesicles become trapped at some intermediate step in the vesicle cycle. They are not all readily available for release. This accounts for the reduction in releasable pool size inferred from both depression and FM1-43 destaining, the reduction in average recovery rate of released vesicles to a releasable pool, and the inability to destain fully the previously labeled vesicle pool. Perhaps the low levels of average $[Ca^{2+}]_i$ reached during stimulation in low $[Ca^{2+}]_e$ are unable to sustain a step normally needed to mobilize vesicles to release sites or otherwise prime them or make them release competent or available for release.

An alternative possibility is that active zones are nonuniform, and that at 3.5 mM $[Ca^{2+}]_e$ and 20 Hz stimulation some active zones essentially stopped releasing transmitter and became silent. These synapses would cease to recycle vesicles, and they would not destain. Since they do not transmit, the EJP would be reduced and this would also lead to the calculation of a reduced pool size. However, since both initial and steady state EJP amplitudes should be reduced in depression experiments, ρ should not be reduced. The selective "silencing" of some synapses cannot easily account for the apparent effect on recovery rate seen at 2 mM $[Ca^{2+}]_e$ and 50 Hz stimulation, but it certainly may be all or part of the reason why the apparent pool size is decreased in low $[Ca^{2+}]_e$ solution.

What Does Each Punctum Represent?

We have shown in this paper that in serotonin the number of vesicles released and recycled within each punctum was increased. What does each punctum represent structurally? In our experiments, we used an objective with a numerical aperture of 0.75. This means that the resolution of our optical system was about 0.5 μm in the XY plane and about 1.2 μm in the Z direction. Since many synapses are less than 0.5 μm apart and most puncta are about 1–2 μm across, most of the puncta labeled with FM1-43 probably include several active zones.

Based on this, the serotonin-induced increase in the number of vesicles contained within each punctum may be explained in at least three ways: the actual number of vesicles at individual synaptic contacts or active zones may be increased, a previously noncycling subpool of synaptic vesicles at each release site may be recruited, or there may be activation of previously silent synapses. At our optical resolution, we cannot distinguish these alternatives, and the dual labeling results remain ambiguous. The ability of serotonin washout to trap some prestained vesicles suggests one of the latter two alternatives. If presynaptically silent synapses are activated by serotonin, they have to be close to the synapses that are already active. From statistical analysis of transmitter release, Wojtowicz et al. (1994) proposed that LTF induces activation of previously silent synapses. Like serotonin, LTF expression involves cAMP (Dixon and Atwood, 1989b, 1989c). Perhaps similar effects on vesicle pools are involved in LTF induction and serotonin action.

Crayfish (*Procambarus clarkii*, 2–2.5 in) were obtained either from Atchafalaya Biological Supplies (Raceland, LA) or Niles Biological (Sacramento, CA). During experiments, autotomized legs were pinned in saline containing (in millimolars) 195 NaCl, 13.5 CaCl$_2$, 5.4 KCl, 2.6 MgCl$_2$, and 10 Na-HEPES (pH 7.4) at 14°C–17°C. A 5 mM solution of serotonin (Sigma, St. Louis, MO) was diluted to 5 μM in crayfish ringer before experiments. FM1-43 and FM4-64 (Molecular Probes, Eugene, OR) stock solutions were made to 10 mM and 20 mM, respectively, and then diluted to the desired concentration before experiments.

Electrophysiology

Intracellular electrodes were back-filled with 3 M KCl and had resistances of 5–17 MΩ. EJPs were recorded from central muscle fibers in depression experiments and from central or distal fibers adjacent to central fibers used in FM1-43 destaining experiments. The electrical signal was amplified (Neuroprobe 1600 Amplifier, A-M Systems, Everett, WA), filtered with a 2 kHz low-pass filter, digitized at 3.33 kHz (DigiData 1200A, Axon Instruments, Foster City, CA), and every fifth or tenth EJP saved to a pentium-based computer (software: pClamp 6.0, Axon Instrument). EJP amplitudes obtained during a tetanus were measured offline by subtracting peak amplitude from the extrapolated falling phase of the previous EJP and corrected for the nonlinear relationship between postsynaptic potential and transmitter release (Martin, 1955), assuming an EJP reversal potential of 0 mV (Taraskevich, 1971).

In the experiments measuring pool size, we first stimulated the nerve and measured the number of vesicles and the rates in the absence of serotonin and then waited for at least 1.5 hr to allow the vesicles to recycle and LTF to run down. If by this time the single EJP amplitude was identical to that before the tetanus, 5 μM serotonin was added to the perfusion solution, and we waited 10 min to allow its effect to reach steady state.

Optical Measurements and Microscopy

A Bio-Rad (Pinole, CA) MRC 600 laser scanning unit coupled with a Nikon Optiphot microscope with a 40× 0.75 n.a. Olympus water immersion objective was used in collecting images. To minimize photobleaching, a 1% neutral density filter was used in FM1-43 destaining experiments and a 3% filter was used in dual labeling experiments. Fluorescence was excited using the 488 nm line of an argon laser, and FM1-43 emission was detected with a 515 nm long-pass filter. In FM1-43 and FM4-64 dual labeling experiments, FM1-43 emission was detected with a 540 ± 15 nm filter and FM4-64 emissions with a 600 nm long-pass filter. Plotted fluorescence intensities are average levels in regions completely encompassed by 3–5 puncta. No significant differences in the behavior of individual puncta were observed. Where necessary, effects of photobleaching were corrected by dividing intensities by the reduction expected from the number of images that had been taken, as determined from Figure 2D.

FM1-43 Destaining Protocol

To stain terminals, 10 μM FM1-43 was added to the bath, and a short tetanus, either 50 Hz for 14 s (2 experiments) or 20 Hz for 3 min (all other experiments), was used. The preparation was then washed in dye-free saline for at least 30 min. The nerve was then destained with a 20 Hz, 20,000 pulse tetanus. Images were saved to record the time course of destaining and EJPs were recorded simultaneously. Muscle movements sometimes required refocusing, leading to gaps in records of staining versus time.

One and an half hours later, if there were no LTF, 5 μM serotonin was added to the perfusion solution, and 10 min later, 10 μM FM1-43 and 5 μM serotonin were added to the bath for loading. After loading, FM1-43 was thoroughly rinsed out using ordinary ringer, and then 5 μM serotonin was returned to the bath during destaining.

FM1-43 and FM4-64 Dual Labeling

To stain with FM1-43, 8 μM of this dye was added to the bath and a 20 Hz, 8400 pulse tetanus was applied. FM1-43 was left in the bath for 30 min before being washed out to allow all released vesicles to be endocytosed. In serotonin experiments, 5 μM was added to the bath for 10 min before FM4-64 loading. To stain with FM4-64,

30 μM of the dye with or without 5 μM serotonin, depending on the experiment, was added to the bath, and a 20 Hz, 4,000 pulse tetanus was applied. Again FM4-64 was kept in the bath for about 30 min to allow for vesicle recycling. Since there was no FM4-64 bleedthrough, FM1-43 destaining was monitored during FM4-64 loading to ensure that FM1-43 was not excessively destained. FM1-43 and FM4-64 images were collected at different gains so that a full range of gray values could be used. The difference in the intensity caused by the difference in gain was corrected in the analysis of ratio calculation, which was determined separately for each punctum analyzed.

Statistics

The two-tailed Student t test was used in testing for significant differences. Since distributions of pool size changes are likely to be skewed instead of normal, we tried different ways to transform the data (e.g., logarithmic). We found that the p values were little affected by these manipulations.

Appendix

We derive here the equations used in the FM1-43 destaining experiments to estimate the pool size. The destaining process can be described as:

$$A \xrightarrow{F} B \qquad (A1)$$

where A represents presynaptic labeled vesicles and B represents labeled vesicles that are released. F is the fraction of labeled vesicles released by a single action potential. Then

$$\Delta A = -FfA\Delta t, \qquad (A2)$$

where f is the frequency of the tetanus in Hz.

$$\frac{dA}{A} = -Ffdt$$

$$A = A_0 e^{-Fft}$$

By fitting the FM1-43 destaining time course to an exponential, we are able to estimate F. Staining will decay with a time constant τ in seconds, given by

$$\tau = \frac{1}{Ff} \qquad (A3)$$

If labeled vesicles mix well with unlabeled vesicles, then the fraction of total vesicles in the pool (T) that are released will be the same as the fraction of labeled vesicles released. The number of quanta released in a single EJP, m, will be given by

$$m = FT \qquad (A4)$$

EJP amplitude (V) is the product of number of quanta released and quantal size (q),

$$V = mq \qquad (A5)$$

and from (A4) to (A6),

$$T = \frac{m}{F} = \frac{Vf\tau}{q}. \qquad (A6)$$

References

Atwood, H.L., and Wojtowicz, J.M. (1986). Short-term and long-term plasticity and physiological differentiation of crustacean motor synapses. Intl. Rev. Neurobiol. *28*, 275–362.

Augustine, G.J., Charlton, M.P., and Smith, S.J. (1987). Calcium

action in synaptic transmitter release. Annu. Rev. Neurosci. *10*, 633–693.

Baxter, D.A., and Byrne, J.H. (1989). Serotonergic modulation of two potassium currents in the pleural sensory neurons of *Aplysia*. J. Neurophysiol. *62*, 665–679.

Belardetti, F., Kandel, E.R., and Siegelbaum, S.A. (1987). Neuronal inhibition by the peptide FMRFamide involves opening of S K$^+$ channels. Nature *325*, 153–156.

Betz, W.J., and Bewick, G.S. (1992). Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. Science *255*, 200–203.

Betz, W.J., and Bewick, G.S. (1993). Optical monitoring of transmitter release and synaptic vesicle recycling at the frog neuromuscular junction. J. Physiol. *460*, 287–309.

Betz, W.J., Bewick, G.S., and Ridge, R.M. (1992a). Intracellular movements of fluorescently labeled synaptic vesicles in frog motor nerve terminals during nerve stimulation. Neuron *9*, 805–813.

Betz, W.J., Mao, F., and Bewick, G.S. (1992b). Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. J. Neurosci. *12*, 363–375.

Bittner, G.D., and Kennedy, D. (1970). Quantitative aspects of transmitter release. J. Cell Biol. *47*, 585–592.

Capogna, M., Gähwiler, B.H., and Thompson, S.M. (1995). Presynaptic enhancement of inhibitory synaptic transmission by protein kinases A and C in the rat hippocampus in vitro. J. Neurosci. *15*, 1249–1260.

Capogna, M., Gähwiler, B.H., and Thompson, S.M. (1996). Presynaptic inhibition of calcium-dependent and -independent release elicited with ionomycin, gadolinium, and α-latrotoxin in the hippocampus. J. Neurophysiol. *75*, 2017–2028.

Christensen, B.N., and Martin, A.R. (1970). Estimates of probability of transmitter release at the mammalian neuromuscular junction. J. Physiol. *210*, 933–945.

Cooper, R.L., Marin, L., and Atwood, H.L. (1995). Synaptic differentiation of a single motor neuron: conjoint definition of transmitter release, presynaptic calcium signals, and ultrastructure. J. Neurosci. *15*, 4209–4222.

Critz, S.D., Baxter, D.A., and Byrne, J.H. (1991). Modulatory effects of serotonin, FMRFamide, and myomodulin on the duration of action potentials, excitability, and membrane currents in tail sensory neurons of *Aplysia*. J. Neurophysiol. *66*, 1912–1926.

Dale, N., and Kandel, E.R. (1990). Facilitatory and inhibitory transmitters modulate spontaneous transmitter release at cultured *Aplysia* sensorimotor synapses. J. Physiol. *421*, 203–222.

Delaney, K.R., and Tank, D.W. (1994). A quantitative measurement of the dependence of short-term synaptic enhancement on presynaptic residual calcium. J. Neurosci. *14*, 5885–5902.

Delaney, K.R., Zucker, R.S., and Tank, D.W. (1989). Calcium in motor nerve terminals associated with posttetanic potentiation. J. Neurosci. *9*, 3558–3567.

Delaney, K., Tank, D.W., and Zucker, R.S. (1991). Presynaptic calcium and serotonin-mediated enhancement of transmitter release at crayfish neuromuscular junction. J. Neurosci. *11*, 2631–2643.

Dittman, J.S., and Regehr, W.G. (1996). Contributions of calcium-dependent and calcium-independent mechanisms to presynaptic inhibition at a cerebellar synapse. J. Neurosci. *16*, 1623–1633.

Dixon, D., and Atwood, H.L. (1989a). Phosphatidylinositol system's role in serotonin-induced facilitation at the crayfish neuromuscular junction. J. Neurophysiol. *62*, 239–246.

Dixon, D., and Atwood, H.L. (1989b). Conjoint action of phosphatidylinositol and adenylate cyclase systems in serotonin-induced facilitation at the crayfish neuromuscular junction. J. Neurophysiol. *62*, 1251–1259.

Dixon, D., and Atwood, H.L. (1989c). Adenylate cyclase system is essential for long-term facilitation at the crayfish neuromuscular junction. J. Neurosci. *9*, 4146–4252.

Dobrunz, L.E., and Stevens, C.F. (1997). Heterogeneity of release probability, facilitation, and depletion at central synapses. Neuron *18*, 995–1008.

Edmonds, B., Klein, M., Dale, N., and Kandel, E.R. (1990). Contributions of two types of calcium channels to synaptic transmission and plasticity. Science *250*, 1142–1147.

Elmqvist, D., and Quastel, D.M.J. (1965). A quantitative study of endplate potentials in isolated human muscle. J. Physiol. *178*, 505–529.

Fossier, P., Baux, G., and Tauc, L. (1994). N- and P-type Ca^{2+} channels are involved in acetylcholine release at a neuroneuronal synapse: only the N-type channel is the target of neuromodulators. Proc. Natl. Acad. Sci. USA *91*, 4771–4775.

Fu, W.-M., Liou, J.-C., Lee, Y.-H., and Liou, H.-C. (1995). Potentiation of neurotransmitter release by activation of presynaptic glutamate receptors at developing neuromuscular synapses of *Xenopus*. J. Physiol. *489*, 813–823.

Gillis, K.D., Möβner, R., and Neher, E. (1996). Protein kinase C enhances exocytosis from chromaffin cells by increasing the size of the readily releasable pool of secretory granules. Neuron *16*, 1209–1220.

Glusman, S., and Kravitz, E.A. (1982). The action of serotonin on excitatory nerve stimulation in lobster in nerve–muscle preparations. J. Physiol. *325*, 223–241.

Goldsmith, B.A., and Abrams, T.W. (1992). cAMP modulates multiple K$^+$ currents, increasing spike duration and excitability in *Aplysia* sensory neurons. Proc. Natl. Acad. Sci. USA *89*, 11481–11485.

Gray, R., Rajan, A.S. Radcliffe, K.A., Yakehiro, M., and Dani, J.A. (1996). Hippocampal synaptic transmission enhanced by low concentrations of nicotine. Nature *383*, 713–716.

Heidelberger, R., and Matthews, G. (1991). Inhibition of calcium influx and calcium current by γ-aminobutyric acid in single synaptic terminals. Proc. Natl. Acad. Sci. USA *88*, 7135–7139.

Henkel, A.W., and Betz, W.J. (1995). Staurosporine blocks evoked release of FM1-43 but not acetylcholine from frog motor nerve terminals. J. Neurosci. *15*, 8246–8258.

Hochner, B., Klein, M., Schacher, S., and Kandel, E.R. (1986). Additional component in the cellular mechanism of presynaptic facilitation contributes to behavioral dishabituation in *Aplysia*. Proc. Natl. Acad. Sci. USA *83*, 8794–8798.

Holz, G.G., Kream, R.M., Spiegel, A., and Dunlap, K. (1989). G proteins couple α-adrenergic and GABA$_B$ receptors to inhibition of peptide secretion from peripheral sensory neurons. J. Neurosci. *9*, 657–666.

Hori, Y., Endo, K., and Takahashi, T. (1996). Long-lasting synaptic facilitation induced by serotonin in superficial dorsal horn neurones of the rat spinal cord. J. Physiol. *492*, 867–876.

Huang, C.-C., Hsu, K.-S., and Gean, P.-W. (1996). Isoproterenol potentiates synaptic transmission primarily by enhancing presynaptic calcium influx via P- and/or Q-type calcium channels in the rat amygdala. J. Neurosci. *16*, 1026–1033.

Huston, E., Cullen, G.P., Burley, J.R., and Dolphin, A.C. (1995). The involvement of multiple calcium channel sub-types in glutamate release from cerebellar granule cells and its modulation by GABA$_B$ receptor activation. Neuroscience *68*, 465–478.

Isaacson, J.S., and Hille, B. (1997). GABA$_B$-mediated presynaptic inhibition of excitatory transmission and synaptic vesicle dynamics in cultured hippocampal neurons. Neuron *18*, 143–152.

Kamiya, H., and Zucker, R.S. (1994). Residual Ca^{2+} and short-term synaptic plasticity. Nature *371*, 603–606.

Kato, M., Chapman, C., and Bicknell, R.J. (1992). Activation of κ-opioid receptors inhibits depolarisation-evoked exocytosis but not the rise in intracellular Ca^{2+} in secretory nerve terminals of the neurohypophysis. Brain Res. *574*, 138–146.

Klein, M. (1993). Differential cyclic AMP dependence of facilitation at *Aplysia* sensorimotor synapses as a function of prior stimulation: augmentation versus restoration of transmitter release. J. Neurosci. *13*, 3793–3801.

Klein, M., Camardo, J., and Kandel, E.R. (1982). Serotonin modulates a specific potassium current in the sensory neurons that show presynaptic facilitation in *Aplysia*. Proc. Natl. Acad. Sci. USA *79*, 5713–5717.

Lagnado, L., Gomis, A., and Job, C. (1996). Continuous vesicle cycling in the synaptic terminal of retinal bipolar cells. Neuron *17*, 957–967.

Landò, L., and Zucker, R.S. (1994). Ca^{2+} cooperativity in neurosecretion measured using photolabile Ca^{2+} chelators. J. Neurophysiol. 72, 825–830.

Liu, G., and Tsien, R.W. (1995). Properties of synaptic transmission at single hippocampal synaptic boutons. Nature 375, 404–408.

Man-Son-Hing, H., Zoran, M.J., Lukowiak, K., and Haydon, P.G. (1989). A neuromodulator of synaptic transmission acts on the secretory apparatus as well as on ion channels. Nature 341, 237–239.

Martin, A.R. (1955). A further study of the statistical composition of the end-plate potential. J. Physiol. 130, 114–122.

McGehee, D.S., Heath, M.J.S., Gelber, S., Devay, P., and Role, L.W. (1995). Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. Science 269, 1692–1696.

Mudge, A.W., Leeman, S.E., and Fischbach, G.D. (1979). Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. Proc. Natl. Acad. Sci. USA 76, 526–530.

Mulkey, R.M., and Zucker, R.S. (1991). Action potentials must admit calcium to evoke transmitter release. Nature 350, 153–155.

Mulkey, R.M., and Zucker, R.S. (1993). Calcium released from DM-nitrophen photolysis triggers transmitter release at the crayfish neuromuscular junction. J. Physiol. 462, 243–260.

Neher, E., and Zucker, R.S. (1993). Multiple calcium-dependent processes related to secretion in bovine chromaffin cells. Neuron 10, 21–30.

Qian, S.-M., and Delaney, K.R. (1997). Neuromodulation of activity-dependent synaptic enhancement at crayfish neuromuscular junction. Brain Res. 771, 259–270.

Ramaswami, M., Krishnan, K.S., and Kelly, R.B. (1994). Intermediates in synaptic vesicle recycling revealed by optical imaging of Drosophila neuromuscular junctions. Neuron 13, 363–375.

Reuter, H. (1995). Measurements of exocytosis from single presynaptic nerve terminals reveal heterogeneous inhibition by Ca^{2+} channel blockers. Neuron 14, 773–779.

Ryan, T.A., and Smith, S.J. (1995). Vesicle pool mobilization during action potential firing at hippocampal synapses. Neuron 14, 983–989.

Ryan, T.A., Ziv, N.E., and Smith, S.J. (1996). Potentiation of evoked vesicle turnover at individually resolved synaptic boutons. Neuron 17, 125–134.

Scholz, K.P., and Miller, R.J. (1991a). Analysis of adenosine actions on Ca^{2+} currents and synaptic transmission in cultured rat hippocampal pyramidal neurones. J. Physiol. 435, 373–393.

Scholz, K.P., and Miller, R.J. (1991b). GABA$_B$ receptor–mediated inhibition of Ca^{2+} currents and synaptic transmission in cultured rat hippocampal neurones. J. Physiol. 444, 669–686.

Scholz, K.P., and Miller, R.J. (1992). Inhibition of quantal transmitter release in the absence of calcium influx by a G protein–linked adenosine receptor at hippocampal synapses. Neuron 8, 1139–1150.

Shapiro, E., Castellucci, V.F, and Kandel, E.R. (1980). Presynaptic inhibition in Aplysia involves a decrease in the Ca^{2+} current of the presynaptic neuron. Proc. Natl. Acad. Sci. USA 77, 185–189.

Silinsky, E.M., and Solsona, C.S. (1992). Calcium currents at motor nerve endings: absence of effects of adenosine receptor agonists in the frog. J. Physiol. 457, 315–328.

Singer, J.H., Bellingham, M.C., and Berger, A.J. (1996). Presynaptic inhibition of glutamatergic synaptic transmission to rat motoneurons by serotonin. J. Neurophysiol. 76, 799–807.

Smith, C.B., and Betz, W.J. (1996). Simultaneous independent measurement of endocytosis and exocytosis. Nature 380, 531–534.

Takeuchi, A., and Takeuchi, N. (1966). On the permeability of the presynaptic terminal of the crayfish neuromuscular junction during synaptic inhibition and the action of γ-aminobutyric acid. J. Physiol. 183, 433–449.

Taraskevich, P.S. (1971). Reversal potentials of L-glutamate and the excitatory transmitter at the neuromuscular junction of the crayfish. Biochem. Biophys. Acta 241, 700–703.

Thompson, C.S., and Atwood, H.L. (1984). Synaptic strength and horseradish peroxidase uptake in crayfish nerve terminals. J. Neurocytol. 13, 267–280.

Thomas, P., Lee, A.K., and Almers, W. (1994). A triggered mechanism retrieves membrane in seconds after calcium-stimulated exocytosis in pituitary cells. J. Cell Biol. 124, 667–675.

Toth, P.T., Bindokas, V.P., Bleakman, D., Colmers, W.F., and Miller, R.J. (1993). Mechanism of presynaptic inhibition by neuropeptide Y at sympathetic nerve terminals. Nature 364, 635–639.

Umemiya, M., and Berger, A.J. (1994). Activation of adenosine A$_1$ and A$_2$ receptors differentially modulates calcium channels and glycinergic synaptic transmission in rat brainstem. Neuron 13, 1439–1446.

von Gersdorff, H., and Matthews, G. (1994). Inhibition of endocytosis by elevated internal calcium in a synaptic terminal. Nature 370, 652–655.

von Rüden, L., and Neher, E. (1993). A Ca-dependent early step in the release of catecholamines from adrenal chromaffin cells. Science 262, 1061–1065.

Wojtowicz, J.M., Marin, L., and Atwood, H.L. (1994). Activity-induced changes in synaptic release sites at the crayfish neuromuscular junction. J. Neurosci. 14, 3688–3703.

Wu, L.G., and Saggau, P. (1994). Adenosine inhibits evoked synaptic transmission primarily by reducing presynaptic calcium influx in area CA1 of hippocampus. Neuron 12, 1139–1148.

Wu, L.-G., and Saggau, P. (1995). GABA$_B$ receptor-mediated presynaptic inhibition in guinea-pig hippocampus is caused by reduction of presynaptic Ca^{2+} influx. J. Physiol. 485, 649–657.

Yawo, H., and Chuhma, N. (1993). Preferential inhibition of ω-conotoxin-sensitive presynaptic Ca^{2+} channels by adenosine autoreceptors. Nature 365, 256–258.

Zucker, R.S. (1974). Characteristics of crayfish neuromuscular facilitation and their calcium dependence. J. Physiol. 241, 91–110.

Zucker, R.S. (1989). Short-term synaptic plasticity. Annu. Rev. Neurosci. 12, 13–31.

Zucker, R.S. (1996). Exocytosis: a molecular and physiological perspective. Neuron 17, 1049–1055.

Zucker, R.S., and Lara-Estrella, L.O. (1983). Post-tetanic decay of evoked and spontaneous transmitter release and a residual calcium model of synaptic facilitation at crayfish neuromuscular junctions. J. Gen. Physiol. 81, 355–372.