Changes in the Readily Releasable Pool of Transmitter and in Efficacy of Release Induced by High-Frequency Firing at Aplysia Sensorimotor Synapses in Culture

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INTRODUCTION

Synaptic transmission can be dramatically altered by short trains of high-frequency stimulation (HFS), with depression immediately after a train (Zucker 1999; Zucker and Regehr 2002) being replaced at some synapses by long-term facilitation lasting hours or days (Bliss and Collingridge 1993). Many synapses in both vertebrates and invertebrates exhibit an increase in transmission lasting for seconds to minutes after HFS. This increase, known as posttetanic potentiation (PTP), results from an increase in transmitter release from the presynaptic terminals and depends on presynaptic calcium entry (Zucker 1999; Zucker and Regehr 2002). PTP occurs at Aplysia sensorimotor synapses as well (Zhao et al. 1997; Walters and Byrne 1984). The induction of PTP at these synapses depends on postsynaptic depolarization and calcium entry also (Bao et al. 1997; Lin and Glanzman 1994; Schaffhausen et al. 2001), but the postsynaptic contribution is limited to connections of initially low synaptic strength (Schaffhausen et al. 2001). In this report, we examine the underlying mechanisms of PTP at the sensorimotor synapses of Aplysia.

Zhao, Yali and Marc Klein. Changes in the readily releasable pool of transmitter and in efficacy of release induced by high-frequency firing at Aplysia sensorimotor synapses in culture. J Neurophysiol 91: 1500–1509, 2004. First published November 26, 2003; 10.1152/jn.01019.2003. Synaptic transmission at the sensory neuron-motor neuron synapses of Aplysia, like transmission at many synapses of both vertebrates and invertebrates, is increased after a short burst of high-frequency stimulation (HFS), a phenomenon known as posttetanic potentiation (PTP). PTP is generally attributable to an increase in transmitter release from presynaptic neurons. We investigated whether changes in the readily releasable pool of transmitter (RRP) contribute to the potentiation that follows HFS. We compared the changes in excitatory postsynaptic potentials (EPSPs) evoked with action potentials to changes in the RRP as estimated from the asynchronous transmitter release elicited by a hypertonic solution. The changes in the EPSP were correlated with changes in the RRP, but the changes matched quantitatively only at connections whose initial synaptic strength was greater than the median for all experiments. At weaker connections, the increase in the RRP was insufficient to account for PTP. Weaker connections initially released a smaller fraction of the RRP with each EPSP than stronger ones, and this fraction increased at weaker connections after HFS. Moreover, the initial transmitter release in response to the hypertonic solution was accelerated after HFS, indicating that the increase in the efficacy of release was not restricted to excitation-secretion coupling. Modulation of the RRP and of the efficacy of release thus both contribute to the enhancement of transmitter release by HFS.

Synaptic transmission is determined by the amount of neurotransmitter released from the presynaptic neuron and by the transduction of the chemical signal into an electrical response by the target cell. Transmitter release, in turn, depends on the size of the readily releasable pool (RRP) of transmitter (Gillis et al. 1996; Rosenmund and Stevens 1996), thought to represent the release-ready synaptic vesicles docked at the active zone (Schikorski and Stevens 2001), and on the efficacy of the release process.

The RRP and the efficacy of release can be modulated by electrical activity and by signaling molecules recruited by neurotransmitters. For example, the RRP is decreased in long-term depression (Goda and Stevens 1998) and is increased through the action of protein kinase C (Berglund et al. 2002; Stevens and Sullivan 1998; Waters and Smith 2000). Efficacy of release is also modulated in short-term synaptic plasticity at the sensorimotor synapses of Aplysia (Zhao and Klein 2002).

In a study on Aplysia sensorimotor synapses (Zhao and Klein 2002), we examined the underlying basis of homosynaptic depression, caused by low-frequency stimulation, and of the facilitation of depressed synapses by serotonin, an endogenous facilitatory transmitter. We found that both the size of the RRP and the efficacy of release were modulated and that the changes in the efficacy of release were limited to excitation-secretion coupling. We now present a similar analysis of the potentiation of synaptic transmission that follows HFS.

Changes in the EPSP at initially strong connections are matched quantitatively by changes in the RRP. At initially weak connections, there is, in addition, an increase in the efficacy of release. This increase can be detected even when release is elicited without action potentials, implicating a process that is independent of excitation-secretion coupling. Taken together with previous findings, our results suggest that an increase in the RRP contributes to PTP at all connections, and in addition, a retrograde signal from the motor neuron may contribute to the increase in the efficacy of transmitter release at weaker connections.

METHODS

Preparation of cultures

Adult Aplysia californica (75–150 g; Marine Specimens Unlimited, Pacific Palisades, CA, and Alacrity Marine Biological Services, Redondo Beach, CA) were anesthetized by injection of 50–100 ml of

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385 mM (isotonic) MgCl$_2$. Tail sensory neurons (Walters et al. 1983) and siphon motor neurons (LFS neurons) (Frost and Kandel 1995) were isolated and maintained as previously described (Coulson and Klein 1997; Klein 1994; Schacher and Proshansky 1983; Zhao and Klein 2002). Both conventional sensory neuron-motor neuron co-cultures (Schacher and Proshansky 1983) and soma-to-soma co-cultures (Coulson and Klein 1997; Klein 1994; Zhao and Klein 2002) were used for the experiments with no differences detected between them in any of the parameters we examined.

**Electrophysiology**

An Axoclamp 2A or 2B amplifier (Axon Instruments) and borosilicate glass micropipettes (tip resistance: 10–20 MΩ) filled with 2 M potassium acetate (pH 7.5) were used for intracellular recordings from the LFS neuron. Recordings were carried out in artificial seawater [ASW; composition (in mM): 460 NaCl, 10 KCl, 11 CaCl$_2$, 55 MgCl$_2$, and 10 HEPES, pH 7.5]. Neuron type was confirmed by the characteristic notch on the rising phase of the voltage in LFS cells after release from hyperpolarizing current (Eliot et al. 1994). Throughout the experiments, the LFS motor neuron was hyperpolarized to −80 mV in current-clamp mode to minimize spiking. To elicit an EPSP in the motor neuron, an extracellular patch pipette filled with ASW was put into contact with the sensory neuron membrane, and a single action potential was evoked in the sensory neuron with a 1- to 5-ms pulse of current.

Voltage recording was done rather than voltage clamp because of the large difference in the amplitudes of EPSPs and of miniature EPSPs: voltage clamp that would be appropriate for the large synaptic currents (>10 nA) would be too noisy to resolve the miniatures (~20 pA), while measurement of the large currents would be inaccurate when clamping to examine the miniature synaptic currents. We did not switch between voltage recording for the large responses and voltage clamp for the miniatures, or vice versa, because we wanted to use the amplitudes of the miniatures to estimate the quantal content (or mini content; see following text) of the electrically evoked responses, which required using the same recording method for both.

To examine PTP, the sensory neuron was fired at 20 Hz for 1 s, and a single EPSP was elicited 30 s after the train. PTP is expressed as the ratio of the EPSP following the train to the first EPSP of the train (Fig. 1).

We estimated the size of the RRP from the number of miniature synaptic potentials (minis) triggered by application of a hypertonic solution. The hypertonic solution used to elicit this asynchronous release consisted of ASW with sucrose added to the desired final concentration. Either 0.5 or 1 M sucrose was added to the ASW. Hypertonic solution was applied either with a 5- to 10-s puff from a microperfusion system (tip diameter: ~50–100 μm; Warner Instruments) or by direct addition of 50 μl of the solution from a hand-held Pipetman onto the neurons.

The number of minis in the RRP was estimated on the basis of the dose-response relationship shown in Zhao and Klein (2002). Specifically, because 0.5 M sucrose releases ~50% of the RRP and 1 M releases ~80%, the RRP was estimated to contain twice the number of minis elicited with the 0.5 M solution and 1.25 times the number elicited with the 1 M solution.

Miniature synaptic potentials elicited by the hypertonic sucrose were detected and analyzed as described in Zhao and Klein (2002). Each mini was counted as a single event in spite of the likelihood that some of the largest minis probably reflected multiquantal release events. Our conclusions are not affected by counting all minis as single events because after HFS the average mini amplitude either did not change (weak connections) or decreased slightly (strong connections; see Fig. 5) with the result that the estimated relation between the RRP and the EPSP was affected minimally, if at all.

Because the mini frequency declined to very low levels within 1 min after sucrose application (Zhao and Klein 2002), all minis detected during the first 55 s after application of the hypertonic solution were counted. All applications of hypertonic solution within an experiment were done in the same way, i.e., either with a puff or with an aliquot; the results were not affected by the manner of application of the hypertonic solution. The preparation was washed with ASW for 10 min between tests.

The quantal content—or the “mini content,” because the average mini amplitude is not equal to the quantal amplitude—of EPSPs was estimated by dividing the EPSP amplitude by the average mini amplitude for EPSPs that were ≤10 mV in amplitude. For larger EPSPs, the peak EPSP amplitudes for the whole experiment were first plotted against the maximal slope of the EPSPs (corresponding to the peak synaptic current). We measured the maximal slope over 1 ms on the rising limb of the EPSP; where the EPSP triggered a spike or a local active response, the measurement was taken before the inflection point marking the active response. A partial correction for nonlinearity of summation was then done as follows: the linear relation between amplitude and maximal slope for the smaller EPSPs was extrapolated to the larger-amplitude EPSPs. The maximal slope of the larger EPSPs was then multiplied by a factor that made it fall on the extrapolated line, and the corresponding amplitude was divided by the average mini amplitude to estimate the mini content. The idea behind this correction is that the maximal slope of the EPSP is less affected by nonlinearity summation than the peak because the voltage at which the maximal slope occurs is much less depolarized than the EPSP peak. Because the maximal slope is still affected by nonlinear summation, albeit to a lesser extent, the mini content of larger EPSPs will still be somewhat

![FIG. 1. Posttetanic potentiation (PTP) varies with initial synaptic strength.](http://jn.physiology.org/)

**A:** experimental protocol. A train of excitatory postsynaptic potentials (EPSPs) in a motor neuron elicited by extracellular stimulation of a sensory neuron at 20 Hz was followed after 30 s by a single stimulus to measure PTP (top). Potentiation of the EPSP is expressed as the ratio of the maximal slope of the EPSP following the train (post-HFS) to that of the 1st EPSP of the train (pre-HFS). **B:** post- and pre-HFS EPSPs are shown on an expanded scale. Calibrations: top, 10 mV and 100 ms; bottom, 20 mV and 20 ms. **B:** PTP decreases as the initial synaptic strength increases. Each point represents 1 experiment; dotted line shows PTP = 1 or no potentiation. **C:** control experiment showing that the inverse relationship shown in B cannot be attributed to a statistical artifact. Two EPSPs were elicited at a 1-min interval without intervening HFS; there is no correlation between the magnitude of the initial EPSP and the ratio of the 2nd EPSP to the 1st EPSP (see Results for further explanation). **D:** connections were divided into weak (n = 14) and strong (n = 11) at the median of the initial maximal slope (14 mV/ms), and PTP at weak and strong connections were averaged separately. The means are significantly different (P < 0.02).

J Neurophysiol • VOL 91 • APRIL 2004 • www.jn.org
PTP is greater at initially weak connections than at initially strong connections

HFS of the sensory neuron at sensorimotor synapses of *Aplysia* is followed by an increase in synaptic transmission (Walters and Byrne 1984) similar to the PTP observed at many other synapses (Zucker 1999; Zucker and Regehr 2002). The magnitude of PTP at *Aplysia* synapses in intact ganglia varies inversely with the initial synaptic strength of the connection, and the mechanisms of PTP appear to differ at connections of different synaptic strength (Schaffhausen et al. 2001). As the first step in our analysis, we examined the relationship between the magnitude of PTP and the initial synaptic strength at sensorimotor connections in cell culture.

A 1-s train of presynaptic action potentials at 20 Hz (Fig. 1A) was followed by PTP that varied inversely with the initial synaptic strength of the connection (Fig. 1B). When the connections were divided into strong and weak at the approximate median initial synaptic strength (maximal slope of 14 mV/ms; see METHODS), the EPSP was potentiated about fourfold at the initially weak connections \((P = 0.0003\) compared with the first of the train) and about twofold at the initially strong connections \((P = 0.012\); Fig. 1C), almost exactly matching the PTP measured in intact ganglia (Schaffhausen et al. 2001).

Before proceeding with the analysis, however, it was first necessary to deal with the possibility that the inverse relationship of Fig. 1B (and Fig. 3A), as well as that reported in Schaffhausen et al. (2001), might be due to a statistical artifact (see for example Kim and Alger 2001). If fluctuating synaptic responses are sorted by the amplitude of the initial response and the amplitude of a second response is compared with the amplitude of the first response, there will often be a negative correlation between the amplitude of the first response and the ratio of the second to the first response. This negative correlation can be a statistical artifact that has no physiological significance.

An artifactual negative correlation can arise from the fact that a response that is near the upper end of the amplitude distribution is more likely to be followed by a smaller response, whereas a small response, which is near the bottom of the distribution, is more likely to be followed by a larger response. If the response can vary between a very small and an arbitrarily large value, the ratio of the second to the first response can vary up to infinity if the first response is small, whereas the ratio can vary only up to 1 if the first response is at the upper end of the amplitude distribution. Plotting the ratio of the second to the first response against the amplitude of the first response will result in a negative correlation similar to that shown in Fig. 1B.

This artifact can occur if the synaptic responses belong to a single population with a given mean and variance. In practice, this situation arises if the response of a single synaptic connection is sampled repeatedly or if the comparison is done on a population of synaptic connections that vary around more or less the same mean and have similar variances. If different connections have different means and variances, or, in the most extreme case, if the different connections do not overlap at all in their amplitude distributions, there should be no correlation between the magnitude of the first response and the ratio of the second response to the first. Thus for example, a large response from a connection whose average response is large may or may not be near the upper end of the distribution for that connection, and it is therefore equally likely that a second response will be greater than the first as that it will be smaller. A similar argument holds for a connection whose average response is small: a small response at such a connection is equally likely to be followed by a smaller response as by a greater response. We used this test to examine the possibility that the inverse relationship between the initial synaptic response and the magnitude of PTP might be a statistical artifact.

If the inverse relationship of Fig. 1B were the result of the artifact described in the preceding text, then sampling two successive synaptic responses without intervening HFS should also result in a negative relationship between the magnitude of the first response and the ratio of the second response to the first response. We therefore examined two successive responses separated by a 1-min interval in the absence of the HFS. In 17 experiments looking at initial synaptic responses in the same range as those of Fig. 1B, there was no correlation between the magnitude of the initial response and the ratio of the second response to the first \((F = 0.394, P = 0.049, P = 0.394, n = 17)\). This finding indicates that the negative correlation between the extent of PTP and the magnitude of the initial synaptic response cannot be attributed to a statistical artifact but rather that it results from a physiologically meaningful difference between potentiation at initially weak connections and that at initially strong connections.

HFS increases the RRP at both initially strong and initially weak synapses but increases the efficacy of release only at initially weak synapses

We next looked for changes in the size of the RRP and in the efficacy of release that might underlie this potentiation. We estimated the size of the RRP from the asynchronous release caused by application of a hypertonic sucrose solution (see METHODS) and compared EPSPs and responses to hypertonic solution after HFS to the test values before HFS (Fig. 2). A test EPSP was elicited by stimulating the sensory neuron with one action potential, and the hypertonic bathing solution was then applied. After 10 min of wash and rest, a 1-s train of action potentials at 20 Hz was evoked in the sensory neuron to induce PTP. Thirty seconds after the end of the train, the EPSP and the response to hypertonic solution were tested again and were compared with the test responses at the beginning of the

**RESULTS**

**PTP is greater at initially weak connections than at initially strong connections**

The protocol used to examine EPSPs and asynchronous release consisted of a single EPSP elicited by electrical stimulation of the sensory neuron followed within 1 min by application of the hypertonic solution (see Fig. 2). In some of the experiments, homosynaptic depression of the EPSP was first induced by stimulating the sensory neuron five times at 30-s intervals before HFS.

Experiments were recorded on a Power MacIntosh computer running Axograph 4 (Axon Instruments), and EPSPs and minis were analyzed with that program.

Data files were transferred to Microsoft Excel (version 7) and GraphPad Prism (version 2) for statistical analysis and plotting. All data are expressed as means ± SE. Two-tailed t-test (paired or unpaired, as appropriate) were used for statistical comparisons, and differences were considered significant if the P value was <0.05.

**METHODS**

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experiment. Note that in this comparison, unlike that of Fig. 1B, the EPSP measured in the first run of the experiment, before a 10-min wash, was compared with the EPSP elicited after HFS in the second run of the experiment, after the wash. These are the same experiments as those shown in Fig. 1B, but to compare the effects of HFS on the EPSP and on the RRP, it was necessary to measure both parameters before and after an intervening wash to remove the hypertonic solution.

Eliciting 20 action potentials at these synapses at an inter-stimulus interval of 30–60 s results in depression of \(75\%\) in the EPSP and of \(50\%\) in the RRP compared with the initial test values (Zhao and Klein 2002). In the present experiments, the first EPSP of the high-frequency train was depressed compared with the initial test value as a result of the prior electrical stimulation and hypertonic stimulus (to \(0.644 \pm 0.087\) of the initial value, \(n = 25\), \(P = 0.0002\)). After HFS, the EPSP and the RRP averaged \(1.711 \pm 0.272\) and \(1.127 \pm 0.075\) of the initial test value, respectively (\(n = 29\)), neither of which was significantly different from the test value (\(P = 0.182\) for the EPSP and \(P = 0.357\) for the RRP). HFS thus reversed homosynaptic depression and led to an increase in the EPSP and in the RRP over the initial values in many instances (Fig. 3, A1 and B1).

As was true for PTP, the change in the EPSP was negatively correlated with the maximal slope of the initial EPSP (Fig. 3A1), and, in addition, the change in the RRP was also negatively correlated with the initial synaptic strength (Fig. 3B1).

**FIG. 2.** Protocol for comparing changes in the EPSP and in the readily releasable pool of transmitter (RRP) after HFS. A single EPSP was elicited with a stimulus to the sensory neuron, and asynchronous transmitter release in the form of miniature synaptic potentials was triggered by application of a hypertonic sucrose solution (test). After a 10-min wash, a train of stimuli at 20 Hz was given (HFS), and the EPSP and response to hypertonic solution were elicited again (post). The EPSP after HFS triggered an action potential, which is truncated in this record. Calibrations: 25 mV and 15 ms for the EPSPs and 3.5 mV and 1 s for the minis.

**FIG. 3.** Weak and strong connections respond differently to HFS. A: the ratio of the post-HFS EPSP to the test EPSP was negatively correlated with the initial synaptic strength (A1; \(r^2 = 0.298\), \(P = 0.002\)). Circles with asterisks, experiments in which the test EPSP had first been depressed by stimulating the sensory neuron 5 times (see METHODS and RESULTS). Inset: experiment controlling for statistical artifact (explained in RESULTS). The ratio of the 1st pre-HFS EPSP of the 2nd run of the experiment to the test EPSP is plotted against the maximal slope of the initial EPSP. Unlike the ratio of the post-HFS EPSP to the test EPSP (A1), there is no correlation between the maximal slope of the initial EPSP and the pre-HFS-to-test EPSP ratio. A2: after HFS, the EPSP showed a significant increase (\(P = 0.002\)) at initially weak connections (from 7.847 \(\pm\) 1.019 to 18.136 \(\pm\) 2.799 mV/ms, \(n = 14\)), and a significant decrease (\(P = 0.014\)) at initially strong connections (from 20.673 \(\pm\) 2.770 to 16.443 \(\pm\) 1.899 mV/ms, \(n = 15\)).

B: the ratio of the number of minis in the response to hypertonic solution after HFS to the number of minis in the test response was also negatively correlated with the initial synaptic strength (B1; \(r^2 = 0.335\), \(P = 0.001\)); circles with asterisks, depressed EPSPs (as in A1). Only the response at weak connections changed significantly after HFS (B2). The number of minis in the RRP increased at initially weak connections (from 113.500 \(\pm\) 13.462 to 151.357 \(\pm\) 21.994, \(n = 14\), \(P = 0.019\)) and was unchanged at initially strong connections (124.267 \(\pm\) 12.986 before and 110.600 \(\pm\) 11.556 after HFS; \(n = 15\), \(P = 0.113\)).
As a control for the statistical artifact discussed earlier, the ratio between the first pre-HFS EPSP and the test EPSP was plotted against the maximal slope of the initial EPSP (Fig. 3A1, inset). There was no correlation between the two values ($r^2 = 0.001$, $P = 0.870$, $n = 27$), demonstrating the physiological significance of the inverse correlation between the amount of potentiation and the initial EPSP.

When the connections were divided into weak and strong groups, as in the preceding text, there was a significant increase after HFS in the EPSP and in the size of the RRP compared with the test values at the weak connections, whereas the strong connections showed no significant change in the RRP and a small decrease in the EPSP (Fig. 3, A2 and B2). Although the EPSP at the initially strong connections did not increase over its initial value, it was potentiated compared with the first EPSP of the train (Fig. 1B). The average potentiated EPSP at the weak connections (18.136 ± 2.704 mV/ms, $n = 15$) did not differ from the average potentiated EPSP at the strong connections (16.443 ± 1.966, $n = 14$; $P = 0.616$).

In some of the experiments, the high-frequency train was preceded by five action potentials at 30-s intervals in the sensory neuron to examine the influence of prior homosynaptic depression on the potentiation by HFS. Prior homosynaptic depression had no effect on the change in the EPSP or in the RRP following HFS, relative to the test value (Fig. 3, A1 and B1, circles with asterisks).

If the effect of the high-frequency train on the EPSP were the result of an increase in the size of the RRP, then the changes in the two parameters should be positively correlated. This was in fact the case. Plotting the EPSP ratio (post-HFS/test) against the corresponding RRP ratio (Fig. 4A) showed that the two measures were significantly correlated ($r^2 = 0.52$, $P < 0.0001$, $n = 28$, 1 outlier excluded). The fact that the changes in the EPSP and in the RRP were correlated with each other for the strong and the weak connections when examined separately ($r^2 = 0.432$, $P = 0.008$ and $r^2 = 0.436$, $P = 0.014$, respectively, with 1 weak outlier excluded) suggests that the changes in the RRP could be contributing to the changes in the EPSP at all connections. However, closer examination of the plot of Fig. 4A revealed that the changes in the EPSP and in the RRP were more nearly the same for the strong connections (●) than for the weak connections (○). On average, the changes in the EPSP and in the RRP were similar at strong connections (Fig. 4B; ratio of post-HFS to test 0.852 ± 0.060 and 0.938 ± 0.077, respectively, $n = 14$, $P = 0.151$), whereas the increase in the EPSP was significantly greater than the increase in the RRP at the weak connections (Fig. 4B: 2.633 ± 0.433 and 1.329 ± 0.107, respectively, $n = 15$, $P = 0.010$).

If the changes in the EPSP were due entirely to changes in the RRP, then the EPSP ratio should be equal to the RRP ratio in each experiment. It can be seen in Fig. 4A that these ratios are approximately equal for the initially strong connections (●), but not for the initially weak connections (○). Figure 4C shows a plot of the EPSP ratio divided by the RRP ratio as a function of initial synaptic strength. This quotient is ~1 for the initially stronger connections but significantly >1 for the weaker connections. We excluded the possibility of a statistical artifact (see beginning of RESULTS) by plotting this quotient for control preparations in which the HFS was omitted. The was no correlation between the quotient and the initial synaptic strength ($r^2 = 0.027$, $n = 22$, $P > 0.25$). These observations suggest that the effect of HFS on the EPSP might be fully explained by an effect on the size of the RRP at strong connections but that additional factors must be implicated at weak connections.

Potentiation of the EPSP is not accompanied by an increase in postsynaptic sensitivity

The differences between the effects of HFS on synaptic transmission at initially weak and initially strong connections support the idea that there is a qualitative difference between the two types of synaptic connection. Schaffhausen et al. (2001) showed that potentiation of synaptic transmission by HFS includes a postsynaptic contribution but only at initially weak connections. One possible postsynaptic mechanism that could contribute to an increase in the EPSP is an increase in the postsynaptic sensitivity to the neurotransmitter as expressed in an increase in the quantal amplitude. If this were to occur, it would be expected that the average amplitude of the miniature synaptic potentials would increase.

To test the hypothesis that an increase in postsynaptic sensitivity amplifies synaptic transmission after HFS, we compared the average mini amplitude before and after HFS. At the weak connections ($n = 14$), the average amplitude of the minis evoked by application of hypertonic solution after the HFS did not differ from the amplitude before HFS (Fig. 5A; 0.762 ± 0.096 and 0.801 ± 0.089 mV, respectively, $P = 0.448$).

Interestingly, there was a significant decrease in the average mini amplitude after HFS at the initially strong connections (Fig. 5A; from 0.628 ± 0.071 to 0.544 ± 0.042, $n = 15$, $P = 0.004$). To examine this change in the mini amplitude in more detail, we compared the distributions of the mini amplitudes before and after HFS at the initially strong connections. In
practically all experiments, the first peak of the amplitude frequency histogram (presumably the unitary quantal amplitude) did not shift its position, but there was a redistribution of the amplitudes toward smaller amplitudes after HFS (see examples in Fig. 5B). Because peaks at larger amplitudes often appeared to be integral multiples of the amplitude of the first peak, the larger minis may represent multiquantal release events (see also Zhao and Klein 2002). The decrease in the average mini amplitude might therefore reflect a decrease in the relative proportion of multiquantal events. Alternatively, the decrease in the mini amplitude could represent a change in vesicle filling among other possibilities. In any case, there was clearly no increase in the mini amplitude at either weak or strong connections that could have contributed to the increase in the EPSP.

Efficacy of release is lower at initially weak connections and increases after HFS

To see if there were other differences between initially weak and initially strong connections, we compared them with respect to several other parameters. There was no difference in the initial average mini amplitude between the two groups (Fig. 6A1) nor was there any correlation between the mini amplitude and the initial synaptic strength (Fig. 6A2). The initial pool size, although not significantly different between weak and strong connections (Fig. 6B1, but see DISCUSSION), was correlated with the initial synaptic strength (Fig. 6B2).

We next compared the fraction of the RRP that was released in the initial EPSP, a measure of the efficacy of release. To compute this value, we first divided the estimated amplitude of the EPSP by the average mini amplitude for each experiment (see METHODS for details). If the average mini amplitude was the same as the quantal amplitude, this calculation would yield an estimate of the quantum content of the EPSP. Because it is unlikely that the average mini amplitude and the quantal amplitude are the same in our experiments (see preceding text), this calculation gives an estimate of the number of minis comprising the EPSP. The number of minis in the EPSP divided by the total number of minis in the RRP gives the fraction of the RRP released with the EPSP.

Because the size of the RRP and the average mini amplitude were not significantly different between the two groups of connections, it is to be expected that the difference in synaptic strength was due to a difference in the fraction of the RRP released in the EPSP. In fact, there was a highly significant difference between the RRP fractions released at weak and at

![Fig. 5. Amplitudes of miniature synaptic potentials before and after HFS. A: at initially weak connections, there is no change in the mini amplitude after HFS, whereas at initially strong connections, there is a small decrease. B: 3 examples of amplitude frequency histograms of miniature synaptic potentials taken from experiments on initially strong connections. The positions of the peaks are similar before and after HFS in each experiment, but there is a decrease in the relative proportion of larger-amplitude events and a corresponding increase in the relative proportion of smaller-amplitude events.](image)
A correlation between RRP size and initial synaptic strength (Fig. 7A1), with a single EPSP at strong connections (Fig. 7A2), more than twice the fraction of the RRP compared with weak connections (Fig. 7B). There was no significant difference at weak and strong connections (Fig. 8A), whereas there is a correlation between mini amplitude and initial strength (Fig. 8B). There was no increase in the average number of minis at any time point at the initially strong connections (Fig. 8C).

Although there was no increase in the number of minis at the initially strong connections, there was a modest acceleration of the time course of release at the strong connections also. When the release at each time point was plotted as a fraction of the total number of minis in each experiment, rather than simply as the number of minis, there was an increase after HFS in the fraction released in the first 5 s at all connections (Fig. 7; weak connections: from 0.184 ± 0.021 to 0.290 ± 0.034, n = 13, P < 0.004; strong connections: from 0.190 ± 0.015 to 0.250 ± 0.028, n = 12, P < 0.05). If this change in the kinetics of the release at each time point is plotted as a fraction of the total number of minis in each experiment, rather than simply as the number of minis, there was an increase after HFS in the fraction released in the first 5 s at all connections (Fig. 7; weak connections: from 0.184 ± 0.021 to 0.290 ± 0.034, n = 13, P < 0.004; strong connections: from 0.190 ± 0.015 to 0.250 ± 0.028, n = 12, P < 0.05).
same number of action potentials at 20 Hz resulted in potentials at an interstimulus interval of 10 s. (Klein 2002). In contrast, we report here that triggering the release by hypertonic solution reveals an increase in the rate of release in the 1st 5 s of the application at both weak ($P < 0.004$) and strong ($P < 0.05$) connections.

asynchronous release reflects a change in the efficacy of release, then this change in efficacy is not due to modulation of excitation-secretion coupling per se because the release induced by hypertonic solution is dependent on neither presynaptic depolarization nor presynaptic calcium entry from the outside (Rosenmund and Stevens 1996; Zhao and Klein 2002).

**Discussion**

**Potentiation by HFS**

In a previous study, we reported that evoking 15–20 action potentials at an interstimulus interval of 10–30 s depressed the EPSP by $\sim 75\%$ and reduced the RRP by $\sim 50\%$ (Zhao and Klein 2002). In contrast, we report here that triggering the same number of action potentials at 20 Hz resulted in potentiation of the EPSP relative to the first EPSP of the high-frequency train (Fig. 1B). To examine the relationship between changes in the EPSP and in the RRP resulting from HFS, we had to use a protocol in which the EPSP and the RRP after HFS were compared with the values at the beginning of the experiment (Fig. 2), which yielded a smaller measured potentiation of the EPSP (Fig. 3A1). Using this protocol, there was either no change in the RRP or a small increase (Fig. 3B2). HFS thus prevented homosynaptic depression of both the EPSP and the RRP. It is also noteworthy that the effects of HFS on the EPSP and on the RRP were the same whether or not homosynaptic depression was first induced, indicating that the depression is reversed by the HFS (Fig. 3, A1 and B1, circles with asterisks).

At a mammalian synapse, the accumulation of calcium resulting from HFS increases the rate of recovery of the RRP from depletion (Wang and Kaczmarek 1998). Although it is not clear that homosynaptic depression at *Aplysia* sensorimotor synapses results from depletion (Gover et al. 2002; Royer et al. 2000), HFS may act in a similar way to prevent and to reverse depression.

**Changes in vesicle recruitment or in the efficacy of the hypertonic stimulus as alternative explanations**

We have considered the changes in the number of minis to be changes in the size of the RRP. However, there are other possible explanations for the effects of HFS on the response to the hypertonic stimulus. First, it is possible that rather than there being a change in the size of the RRP, the HFS leads to increased recruitment of vesicles during the hypertonic stimulus.

Increased recruitment is unlikely to be the explanation for the increased number of minis, for several reasons. First, our previous study (Zhao and Klein 2002) suggested that there is little or no recruitment during the hypertonic challenge because cross-depletion experiments using electrical and hypertonic stimulation showed a good quantitative agreement between the RRP that is available for each of these types of stimulation. In addition, the fact that the asynchronous release ceases almost completely after about a minute (see Figs. 8 and 9) suggests that there is little if any rapid replenishment of the RRP during the hypertonic challenge. Finally, if the increase in the number of minis were due to increased replenishment of the RRP during the period of exposure to the hypertonic solution, it would be necessary to maintain that the close match between the changes in the number of minis in the EPSP (at least for strong connections—see Fig. 4) is the result of a highly unlikely coincidence.

A different possible explanation for the change in the number of minis released in response to the hypertonic sucrose after HFS is that there might be an increase in the effectiveness of the sucrose, such that the maximal releasable number of minis (the RRP) remains the same, but that there occurs a shift in the dose-response relationship between the sucrose concentration and the asynchronous release. If this were true, we would then have to reinterpret the change in the number of minis after HFS as a change in the efficacy of release rather than a change in the size of the RRP. Here too, the fact that the changes in the number of minis and in the EPSP are in such close agreement argues that whatever is responsible for the increased sensitivity to sucrose is also responsible for the increased efficacy of an action potential in triggering release. We believe that this possibility is not very likely because the number of minis after HFS returns to close to the pre-HFS values (as opposed to the decrease that occurs with low-frequency stimulation), and if the restoration of the response to hypertonic solution were due to a shift in the dose-response relationship, there is no reason why the pre- and post-HFS values should be so close to each other (see Fig. 3). In any event, this hypothesis does not address the differences between the responses of initially weak and initially strong connections to HFS.

**Distinguishing between weak and strong connections**

We and others report quantitative and qualitative differences between plasticity at initially weak and initially strong sensorimotor connections (Jiang and Abrams 1998; Schaffhausen et al. 2001; Sugita et al. 1992). How do we decide whether to classify a given connection as strong or weak? All of these studies adopted the rough criterion of designating connections stronger than the mean or the median for all experiments as “strong,” and those weaker as “weak.” Because there is no reason a priori why the dividing line between the two types of connections should fall exactly at the mean or the median, it is clear that some of the connections designated as strong might actually be weak, and vice versa, especially in the region of assumed overlap around the mean or median value. In Fig. 3A1, for example, the points in the plot fall into two distinct groups, with a PSP ratio of 2.5–3 for connections with initial maximal slopes less than about 11 mV/ms, and a PSP ratio of slightly less than one for connections with initial maximal slopes $>$12–13 mV/ms. Connections in the intermediate region
of \sim 11–13 \text{ mV/ms} are distributed between the two groups. There may be a similar clustering of points into two groups in Fig. 7A2, although more experiments would be needed to show this definitively.

The absence of a clear criterion for distinguishing initially weak from initially strong connections will lead to an underestimate of the differences between the two putative groups of connections. For example, we reported that there was no significant difference in the size of the RRP between weak and strong connections (Fig. 6B1). However, if connections with initial maximal slopes \(<11\text{ mV/ms}\) are compared with those with initial maximal slopes \(>13.5\text{ mV/ms}\) (Fig. 6B2), there is a significant difference in the average RRP size \((P = 0.022)\). Thus the lack of a statistically significant difference between the RRP sizes of strong and weak connections using the median value as the dividing line is probably not meaningful, and the size of the RRP is likely to be an additional parameter in which strong and weak connections differ.

**Changes in the efficacy of release at initially weak connections**

We found two ways in which the efficacy of release increased after HFS at initially weak connections: first, there was an increase in the fraction of the RRP released with a single EPSP (Fig. 7B). If the EPSP increases without a quantitatively similar increase in the size of the RRP and with no increase in the mini amplitude, then it must necessarily follow that the fraction of the RRP released in an EPSP increases. Although this would be true in any case, we observed that there was a greater than twofold difference between initially weak and initially strong connections in the fraction of the RRP released with an EPSP (Fig. 7A1). These observations suggest that weak connections are not simply scaled-down versions of strong connections but rather that the limiting steps in the release process differ at initially weak and initially strong connections. Thus at initially strong connections, where the fraction of the RRP released is already high, potentiation is mediated through an increase in the size of the RRP. At initially weak connections, by contrast, both parameters are upmodulated by HFS.

The second indication of an increase in efficacy of release at initially weak connections is the large increase after HFS in the frequency of miniature synaptic potentials in the first 10 s after application of hypertonic solution (Fig. 8B). Because the asynchronous release triggered by hypertonic solution depends on neither presynaptic depolarization nor presynaptic calcium entry from the outside (Rosenmund and Stevens 1996; Zhao and Klein 2002), this increase does not reflect a change in excitation-secretion coupling per se. By contrast, in our study of homosynaptic depression and its reversal by serotonin, we found that these forms of plasticity were accompanied by changes in the efficacy of release but that these changes could be detected only when release was triggered by action potentials, thereby implying modulation of excitation-secretion coupling (Zhao and Klein 2002).

**Postsynaptic factors in PTP**

PTP at the sensorimotor synapses of *Aplysia* comprises a postsynaptic component in addition to the presynaptic process that it shares with the PTP described elsewhere but only at initially weak connections (Schaffhausen et al. 2001). Our finding that the amplitude of the miniature synaptic potentials did not increase after HFS (Fig. 5A) implies that this postsynaptic contribution is not expressed as an increase in postsynaptic sensitivity. On the other hand, we show that a change in the size of the RRP can account fully for the change in the EPSP only at initially strong connections, suggesting that additional factors must play a role in the potentiation of the EPSP at weak connections (Fig. 4, A–C). We therefore suggest that modulation of the size of the RRP is mediated by an exclusively presynaptic process at all connections, whereas the changes in the efficacy of release at initially weak connections may be caused by a retrograde signal from the postsynaptic neuron.

**Differences between strong and weak connections**

What could account for the differences between initially weak and initially strong connections? One possibility is that the populations of sensory and/or motor neurons are not homogeneous but that different subclasses of these cells form connections with different properties as has been found for cricket synapses for example (Davis and Murphey 1993). Large differences in the fraction of the RRP released to a single action potential have been reported at tonic and phasic motor neurons of the crayfish, and, similarly to our findings, the two types of motor neuron show marked differences in their plastic properties (Milar et al. 2002). A second possibility is that the differences in the synaptic properties might be the result of differences in maturational state of the synapses as has been reported in several other systems. In mammals, LTP is more easily induced at immature synapses (Bolshakov and Siegelbaum 1995; Liao and Malinow 1996), and there appears to be a switch in the biochemical signaling pathways mediating LTP as synapses mature (Yasuda et al. 2003). Insertion of postsynaptic receptors in *Xenopus* neurons decreases as synapses mature (Wu et al. 1996), and, like PTP at the sensorimotor connections of *Aplysia*, the potentiation of weaker connections at developing neuromuscular synapses of *Xenopus* is dependent on elevation of postsynaptic calcium (Wan and Poo 1999). In *Aplysia*, the processes mediating synaptic facilitation by serotonin at the sensorimotor synapses change as the animal matures (Marcus and Carew 1998), and a similar change takes place as these synapses mature in culture (Sun and Schacher 1996). Taken together, these observations suggest that the initially weak sensorimotor synapses of *Aplysia* might be immature synapses, which are susceptible to forms of modulation that do not play as large a role in the plasticity of strong, or mature, synapses.

Whatever the basis for the differences in plasticity at connections of different initial synaptic strength, our study raises questions that bear on fundamental aspects of synaptic communication. It would be of great interest to test the idea that different steps in the release process are limiting at different subclasses of connections of the same type, whether as a consequence of maturational stage, of prior activity, or of the identity of the postsynaptic target cell, to take a few examples. These differences could reflect differences in the individual protein components of the synaptic apparatus (for example, differences in isofrom, in subunit composition or in phosphorylation state), in their relative abundance, or in their localiza-
tion at the synapse. Modulation of synaptic transmission, whether by intrinsic activity or by exogenous modulators, can thus take different forms at different synapses depending on the relative contributions of the various steps in the release process.

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