IntroducTion

Neuromodulation can cause dynamic, state-dependent changes in cellular and synaptic properties that reflect patterns of internal signal transduction within the modulated neuron rather than patterns of synaptic input. For example, at Aplysia sensory-motor synapses, serotonin induces at least three distinct biochemical cascades with different time courses, producing a complex temporal pattern of short-, intermediate, and long-term heterosynaptic potentiation (Ghirardi et al. 1995; Mauelshagen et al. 1996). However, previous work on intracellular signaling related to neuromodulation has tended to focus on responses to application of exogenous neuromodulatory substances that occur over minutes or hours; less is known about the mechanisms and dynamics of more transient forms of heterosynaptic modulation caused by activation of neuromodulatory neurons.

We previously described a transient but complex form of neuromodulation in the swim central pattern generator (CPG) of the mollusk, Tritonia diomedea; spike timing-dependent neuromodulation (STDN) depends on the relative timing of the spiking activities of the serotonergic dorsal swim interneuron (DSI, http://NeuronBank.org/Tri0001043) and another swim CPG neuron, ventral swim interneuron-B (VSI, http://NeuronBank.org/Tri0002436) (Fig. 1A) (Sakurai and Katz 2003). Immediately after DSI activation, there is a transient potentiation of VSI synaptic strength that is rapidly superseded by a longer-lasting depression of VSI synaptic strength.

The potentiation and depression phases of STDN are pharmacologically separable (Sakurai et al. 2006), indicating that they depend on distinct intracellular signals that operate over different time scales. The depression phase of STDN appears to be caused by a serotonergic enhancement of a transient voltage-gated potassium current known as the A-current, which contributes to spike narrowing and thus depression of VSI synaptic strength (Sakurai et al. 2006). Blockade of the A-current by 4-aminopyridine (4-AP) broadens the VSI action potential and eliminates the depression phase of DSI-induced STDN (Fig. 1), enabling the short-term potentiation phase of STDN to be studied in isolation.

In this study, we investigated the mechanisms underlying the potentiation phase of STDN. We applied a rapid depletion protocol to VSI to estimate changes in the size of the readily releasable pool of neurotransmitter, the fraction of release, and the replenishment rate (Millar et al. 2002; Sakaba et al. 2002; Schneggenburger et al. 2002; von Gersdorff and Borst 2002). These results were incorporated into a phenomenological simulation of VSI, enabling us to predict heterosynaptic potentiation dynamics and test these predictions physiologically. Finally, we examined a physiological mechanism underlying the potentiation by depleting Ca\(^{2+}\) stores with cyclopiazonic acid (CPA). The results indicate that the potentiation phase of STDN is mediated by a transient increase in the fraction of release at VSI synapses and that this increase requires mobilization of internal Ca\(^{2+}\) stores. Furthermore, the results show that the effect of serotonergic neuromodulation is dependent on the state of...
the synapse. Thus the dynamics of intracellular signaling play an important role in determining the dynamics of short-term heterosynaptic neuromodulation.

Portions of this work have been presented in abstract form (Sakurai et al. 2005).
Depletion protocol

We characterized a VSI synaptic dynamics with a rapid depletion protocol. Specifically, a VSI spike train was evoked at 5 Hz for 10–15 s (see Fig. 2B). This relatively modest VSI activation produced rapid depletion because of our use of 4-AP to block the depression phase of STDN. In addition to eliminating STDN depression (Sakurai et al. 2006), 4-AP broadens VSI spikes, thereby enhancing synaptic release and speeding synaptic depletion. Prior to each depletion protocol, three to six baseline EPSCs were evoked at 0.2 or 0.5 Hz. Average baseline measures were used to normalize EPSC amplitudes during the depletion train.

Rapid depletion protocols allow the estimation of important synaptic parameters, including the size of the readily releasable pool (RRP), the fraction of the RRP released per spike, and the rate at which the RRP is refilled (Iwasaki and Takahashi 2001; Sakaba et al. 2002; Schneggenburger et al. 1999, 2002). These parameters were extracted by plotting cumulative EPSCs (see Fig. 3B) and fitting a regression line through the linear portion of the plot that occurs at the end of the spike train (last 5 spikes in the train; Eq. 1). The linear portion of the plot represents the steady-state level of release where neurotransmitter depletion and replenishment are balanced. Thus the slope of the regression line provides an indicator of the rate of transmitter replenishment. Tracing the line back to the beginning of the spike train factors out the total replenishment across the train. For this reason, the y intercept of the regression line is an indicator of the size of the RRP at the start of the train. Finally, dividing the initial EPSC by the estimated RRP size provides an indicator of the fraction of release at the synapse. This method of estimating synaptic parameters is well established (Iwasaki and Takahashi 2001; Sakaba et al. 2002; Schneggenburger et al. 1999, 2002) and has been previously used to probe the mechanisms of synaptic plasticity (von Gersdorff and Borst 2002).

To determine if the potentiation phase of STDN involves modification of these parameters, we applied a depletion protocol to VSI before, during, and after stimulation of DSI. In some experiments, STDN was directly induced by a 10-Hz, 5-s activation of a serotonergic DSI. However, the potentiation phase of STDN is transient, lasting <15 s. Therefore we also utilized bath application of 5-HT as a stand-in for the potentiation phase of STDN. In 4-AP, bath application of 5-HT produces a stable and persistent potentiation of VSI synapses (Sakurai et al. 2006). The effect of 5-HT on transmitter release was determined by comparing the cumulative EPSC plots made before and during bath application of 5-HT.

VSI synapse model

We explored the mechanisms of 5-HT-induced potentiation with a phenomenological model of the VSI-to-VFN synapse (Tsodyks et al. 1998; Tsodyks and Markram 1997). In this modeling scheme, the synapse has a static quantity of neurotransmitter (R_total) partitioned into three pools: releasable (R), active (A), and used (U). Each presynaptic spike (at time t_sp) causes a fraction (F_sp) of the releasable pool (R) to be released into the active pool (A, Eq. 1; see Fig. 4A). Resources in A inactivate with time constant τ_inact into U (Eq. 2), and resources in U replenish the releasable pool with time constant τ_R (Eq. 1). The resources in U can be calculated simply as the portion of total resources that are not releasable and not active (Eq. 3). At rest, all resources accumulate into R (i.e., R = R_total). Thus R represents the current level of the readily releasable pool and R_total represents its size or maximum capacity. Accordingly

\[
\frac{dR}{dt} = \frac{U}{\tau_{inact}} - F_{sp} \cdot R \cdot \delta(t - t_{sp}) \\
\frac{dA}{dt} = -\frac{A}{\tau_{inact}} + F_{sp} \cdot R \cdot \delta(t - t_{sp}) \\
U = R_{total} - R - A
\]

The net postsynaptic current (I) is set by the level of active resources, a static weight (G) and the driving force on the synapse (V_post - E_rev)

\[
I = G \cdot A \cdot (V_{post} - E_{rev})
\]

where V_post is the membrane potential of the postsynaptic VFN and E_rev is the reversal potential of the VSI-evoked EPSC.

This model incorporates synaptic depression through depletion of the resources in the releasable pool. Facilitation is incorporated by calculating an activity-dependent fraction of release (F_sp). Each spike causes an increase in fraction of release that is proportional to a baseline level of release (F_base) and scaled by the degree of release saturation (Eq. 5). In the absence of activity, the fraction of release decays with time constant τ_facil

\[
\frac{dF_{sp}}{dt} = -F_{sp} \cdot \tau_{facil} + F_{base} \cdot (1 - F_{sp}) \delta(t - t_{sp})
\]

This model provides an abstract representation of synaptic transmission with only a few free parameters. Nevertheless, it can reproduce postsynaptic responses to both regular and irregular spike trains with a high level of fidelity (Tsodyks et al. 1998; Tsodyks and Markram 1997). In this description, we have slightly altered the labels from Tsodyks and Markram (1997) for clarity. The model was implemented both in NEURON (Hines and Carnevale 1997, 2001) and within a Microsoft Excel spreadsheet (Redmond, WA). A NEURON implementation of Tsodyks et al. (1998) is also available on ModelDB (http://senselab.med.yale.edu/senselab/modeldb).

The model was fit to EPSCs elicited in VFN by VSI spike trains (5 Hz, 10 s). Parameters were fit using a descending-gradient search algorithm. Fractional error was measured for each EPSC in the train [absolute value of [(empirical – model)/empirical]], and the mean fractional error across the train was used as the measure of fit (Varela et al. 1997). V_post was fixed to -50 mV to reflect our voltage-clamp protocol, and E_rev was fixed to 0 mV, the hypothetical reversal

FIG. 2. The rapid depletion protocol. VSI was stimulated to fire at 5 Hz using repeated intracellular current pulses (7 nA, 20 ms) in control Hi-Di saline (A) and in the presence of 1 mM 4-AP (B). C: peak amplitudes of EPSCs evoked in control saline (●) and in 1 mM 4-AP saline (gray triangles) were plotted. 4-AP increased the amplitude of the initial EPSC and shortened the time for the EPSCs to reach maximum facilitation and then maximum homosynaptic depression. In the presence of 4-AP, the steady-state EPSC amplitude was less than the initial EPSC amplitude.
potential for this synapse. Based on the decay rate of a single VSI-evoked EPSC, $\tau_{un}$ was set to 30 ms.

For each experimental preparation, we fit the model to EPSCs elicited before and during bath-application of 5-HT. For control trains, the size of the RRP ($R_{total}$) was set to one (an arbitrary value). Synaptic weight ($G$) was set at each iteration to scale the first model EPSC to match the first empirical EPSC. Control fits were then determined for three free parameters: $F_{base}$, $\tau_{facilt}$ and $\tau_{rep}$. For 5-HT trains, the search algorithm was seeded with the best-fit control parameters. For this phase, $G$ was fixed at its control level, and $R_{total}$ was freed. The fitting algorithm thus selected four free parameters: $R_{total}$, $F_{base}$, $\tau_{facilt}$ and $\tau_{rep}$. This procedure enabled us to estimate changes in synaptic parameters attributable to 5-HT.

Simulating the dynamics of STDN

After estimating the synaptic changes caused by a steady-state concentration of 5-HT, we developed a model of the dynamic potentiation phase of STDN evoked by stimulation of the serotonergic DSI. This was modeled as a dynamic enhancement of the basal fraction of release, $F_{base}$. Similar to frequency-facilitation, we assumed that each DSI spike produces a percent increase, $F_{mod}$, in the fraction of release, but that this modulation decays back to an unmodulated level of release, $F_{unmod}$, with an exponential time constant $\tau_{mod}$. Thus $F_{base}$ became a dynamic variable in the model calculated as

$$\frac{dF_{base}}{dt} = -\frac{F_{base} - F_{unmod}}{\tau_{mod}} + F_{mod} \cdot F_{base} \cdot \delta(t - t_{stim})$$

This scheme is similar to popular models of short-term plasticity such as frequency facilitation (e.g., Magleby and Zengel 1975, 1976). Note, however, that unlike frequency-facilitation this enhancement in the fraction of release of the VSI synapse is indexed to DSI spikes, representing a heterosynaptic potentiation.

The new parameters required to model this dynamic potentiation were derived directly from available data. We set $F_{unmod}$ to the fraction of release estimated in control, unmodulated trains. To determine the time constant of decay, $\tau_{mod}$, we analyzed new and archival records (Sakurai et al. 2006) of the decay of the potentiation phase in 4-AP (Fig. 1B) and fit these data to a single-exponential decay function. Finally, the increase in the fraction of release per DSI spike, $F_{mod}$, was set such that a 5-s, 10-Hz DSI stimulation would produce the same 64% increase in fraction of release observed during bath application of 5-HT. Thus all additional model parameters were well-constrained by empirical data.

**Paired-pulse protocol**

In most cases, the VSI synapse showed initial frequency facilitation in response to repetitive stimulation in either Hi-Di saline or Hi-Di with 1 mM 4-AP saline. The magnitude of the frequency facilitation was strongly affected by changes in the strength of the synapse. Increasing the EPSC size by raising $[Ca^{2+}]_o$, reduced the magnitude of the frequency facilitation, whereas reducing EPSC size by lowering $[Ca^{2+}]_o$, enhanced it. This is because increased synaptic output causes synaptic depression concurrently with the frequency facilitation. 2×$[Ca^{2+}]_o$ saline enhanced synaptic depression suitably for a depletion protocol, but it also made voltage-clamp of the postsynaptic cell difficult. We used 1×$[Ca^{2+}]_o$ saline with 4-AP for the depression protocol. On the other hand, we used low-$Ca^{2+}$ saline to minimize contamination of synaptic depression for the analysis of frequency facilitation (see following text).

A paired-pulse protocol was used to measure the decay of homosynaptic facilitation at VSI synapses. To avoid contamination from transmitter depletion, we used a low-$Ca^{2+}$ saline, containing 25% $[Ca^{2+}]_o$ of normal saline (Augustine and Charlton 1986; Rosenmund and Stevens 1996; Zucker 1989). The time constant governing the decay of facilitation was extracted by fitting the paired-pulse data to a single-exponential curve. Fits were conducted using TableCurve2D (Systat Software, San Jose, CA). We also simulated the same protocol using our phenomenological model of the VSI synapse.

**RESULTS**

4-AP isolated the enhancement phase of STDN and sped depletion

These experiments were designed to identify the mechanisms underlying the potentiation phase of the biphasic STDN produced at VSI synapses by stimulation of the serotonergic DSI (Fig. 1) (see also Sakurai and Katz 2003). To isolate the potentiation phase of this neuromodulation, we utilized bath application of the potassium channel blocker 4-AP. It was shown previously that 4-AP (1 mM) blocks the depression phase of STDN (Sakurai et al. 2006). Conveniently, 4-AP does not alter the amplitude of the potentiation phase of STDN (Fig. 1, B and C), providing an effective means for studying this process in isolation.

Bath application of 4-AP also causes VSI spike broadening and a concomitant increase in neurotransmitter release at VSI synapses (Sakurai et al. 2006); notice that the basal EPSC amplitude is larger in Fig. 1, B than in A. This speeds the depletion of neurotransmitter from VSI terminals during sustained activation. In Hi-Di saline (control), a train of VSI action potentials (15 s, 5 Hz) produced an initial facilitation of VSI-evoked EPSCs followed by a slight depression to a somewhat steady-state level (Fig. 2, A and C). Under these conditions, the EPSC amplitude at steady state was often greater than that at the start of the train, implying that this terminal contained reserved neurotransmitter that had not been depleted. With the addition of 4-AP (1 mM), however, initial release was much higher, facilitation was briefer, and the steady-state EPSC amplitude was well below that of the initial EPSC of the train (Fig. 2, B and C). This implies that the terminal had been depleted of readily releasable transmitter and was now releasing only what had been refilled between spikes. We used this *rapid depletion protocol* (a 15-s, 5-Hz train of VSI spikes in 4-AP) to probe the mechanisms of STDN potentiation.

**Cumulative EPSCs: 5-HT increased the fraction of release**

Monitoring synaptic output during a rapid depletion protocol enables the estimation of important synaptic parameters, including the size of the RRP, the fraction of the RRP released per spike, and the rate at which the RRP is refilled (Iwasaki and Takahashi 2001; Sakaba et al. 2002; Schneggenburger et al. 1999, 2002; von Gersdorff and Borst 2002). To determine if the potentiation phase of STDN involves modification of these parameters, we applied a depletion protocol to VSI before and during bath application of 5-HT. We utilized 5-HT as a stand-in for DSI-evoked STDN, which produces very transient synaptic changes (see Methods). Consistent with previous results, applying 5-HT in the presence of 4-AP produced a sustained and stable enhancement of VSI synapses when VSI was stimulated every 30 s (Fig. 3A, inset) (Sakurai et al. 2006). When VSI was stimulated using the depletion protocol, 5-HT increased the amplitude of the initial EPSCs of the train by an average of 60 ± 20% [mean ± SD, t(7) = 3.34, P < 0.05 comparing initial EPSCs in control and 5-HT trains]. However,
this enhancement was rapidly overwhelmed by the depletion of the synapse; there was no difference in the amplitude of steady-state EPSCs near the end of a 5 Hz train (Fig. 3A). When VSI was allowed to rest for 3 min, the initial EPSC amplitude and the degree of DSI-evoked potentiation recovered completely. Thus depletion of the VSI synapse overwhelms the potentiating effects of 5-HT on VSI synaptic strength.

Synaptic parameters were extracted by plotting cumulative EPSCs and fitting a regression line through the linear portion of the plot, corresponding to the steady-state EPSC amplitudes at the end of the spike train (Fig. 3B, see METHODS). The effect of 5-HT on the EPSCs evoked by a VSI spike train appeared as a positive shift in the steady-state portion of the plot with no change in slope (Fig. 3B). In this study, we used the y intercept of the regression line as an indicator of the size of the RRP at the start of the train, and the ratio of the initial EPSC to the estimated RRP size as an indicator of the initial fraction of release at the synapse. Thus in comparison to control trains, 5-HT caused an apparent increase in both the size of the RRP (Fig. 3Ci, P < 0.01, paired t-test, n = 8) and the fraction of the RRP released per spike (Fig. 3Cii, P < 0.02, paired t-test, n = 8) but did not change the refill rate (Fig. 3Ciii, P = 0.29, paired t-test, n = 8).

If changes in the size of the RRP and the fraction of release contribute to the potentiation phase of STDN, then the magnitude of these changes should correlate with the amplitude of 5-HT-induced potentiation across preparations. There was only a weak negative correlation between measures of RRP size and potentiation caused by 5-HT ($R^2 = 0.11$, $P < 0.42$; Fig. 3Di). However, the fraction of release exhibited a strong positive correlation with the change in initial EPSC size ($R^2 = 0.78$, $P < 0.01$; Fig. 3Dii). Note however, that in preparations exhibiting low levels of 5-HT induced potentiation, there was no measurable increase in the fraction of release. This inconsistency suggests some errors in estimating the fraction of release (see METHODS). Taken together, these results suggest that the potentiation phase of STDN is driven primarily by an increased fraction of the RRF released per spike. However, a change in RRP size could not be completely ruled out.

**Phenomenological model: 5-HT increased only the fraction of release**

Because the cumulative EPSC analysis was not definitive, we utilized a second approach to extract estimates of synaptic parameters from this data. Specifically, we fit each pair of control and 5-HT trains to a phenomenological model of
synaptic transmission (Tsodyks et al. 1998; Tsodyks and Markram 1997). This model involves only a few free parameters but is highly accurate at replicating complex synaptic dynamics (Fig. 4A). Like the cumulative EPSC plot, this computational approach provides indicators of the size of the RRP ($R_{\text{total}}$ in the model), the initial fraction of the RRP released per spike ($F_{\text{base}}$), and the refill rate ($\tau_{\text{rep}}$). In addition, this model provides an estimate of the decay time constant for frequency facilitation ($\tau_{\text{facil}}$). This model represents only a single pool of transmitter in the synaptic terminal; it assumes that stimulation rates and durations are insufficient to involve significant recruitment of transmitter from reserve pools.

We fit the model to each pair of EPSC trains measured in control 4-AP saline and the 4-AP saline containing 5-HT (Fig. 4B). Fits were produced using a descending gradient search algorithm. The quality of fit was calculated as the average of the error per spike across each train (see METHODS). This type of approach to estimating synaptic parameters is well established (e.g., Dobrunz 2002; Sun and Dobrunz 2006) and has been used previously to probe the mechanisms underlying synaptic modulation (Varela et al. 1997). The fit algorithm yielded parameter sets that precisely reproduced the dynamics of the measured EPSC amplitudes. Across eight pairs of trains, the average fractional error per spike was 6.1% and was never more than 8.9% across a single VSI train. Fits were equally good for both control and 5-HT trains with no significant differences in mean fractional error across conditions ($t(8) < 1$).

The mean best-fit parameters for both the control and the 5-HT conditions are listed in Table 1 and compared in Fig. 4C. Parameter values for both conditions were within the range of published values in other systems (Fisher et al. 1997). Paired comparisons between parameters from the control and 5-HT conditions indicated no significant differences in the decay of facilitation ($\tau_{\text{facil}}$; $t(8) < 1$), replenishment rate [($\tau_{\text{rep}}$; $t(8) < 1$), or size of the RRP ($R_{\text{total}}$; $t(8) < 1$). Baseline fraction of release ($F_{\text{base}}$), however, was significantly increased by an average of 64% in models of 5-HT trains ($t(8) = 5.25, P < 0.001$). Consistent with these results, the change in fraction of release was strongly and significantly correlated with the amplitude of 5-HT-induced potentiation across preparations (Fig. 4D; $R^2 = 0.60, n = 8, P < 0.05$). No other estimated parameter correlated with the effects of 5-HT ($\tau_{\text{facil}}$: $R^2 = 0.25, P < 0.2$; $\tau_{\text{rep}}$: $R^2 = 0.21, P < 0.25$; $R_{\text{total}}$: $R^2 = 0.01, P < 0.94$). Moreover, it was possible to accurately fit 5-HT trains by altering only the baseline fraction of release from the control parameter set. This yielded an average error of 11% per spike across the eight 5-HT trains, accounting for more than half of the improvement in fit over the control parameter set. No other single-parameter change could fit the 5-HT data as well. Taken together, these results suggest that the 5-HT-elicited potentiation involves an increase in the fraction of release without altering other aspects of synaptic function.

5-HT did not alter paired-pulse facilitation

Together, our cumulative EPSC analysis and our phenomenological model suggest that 5-HT has limited effects on

Fig. 4. Phenomenological model VSI showed that an increase in the fraction of release can account for the effects of 5-HT. A: modeling scheme: The synapse has a static quantity of resources partitioned into 3 pools: releasable (R), active (A), and used (U). Each presynaptic spike (at time $t_p$) causes a fraction ($F_{\text{rep}}$) of R to be released into A. Resources in A inactivate to U with a time constant $\tau_{\text{inf}}$, and resources in U replenish R with a time constant $\tau_{\text{rep}}$. Synaptic depression is incorporated through depletion of R. Facilitation is incorporated by having each presynaptic spike increase the fraction of release which then decays back to a baseline level, $F_{\text{base}}$, at a time constant $\tau_{\text{facil}}$. See METHODS for details. B: fitting the model to the peak amplitudes of the EPSCs evoked in the control saline (1 mM 4-AP in Hi-Di saline, top trace) and in the presence of 5-HT (0.1 mM 5-HT, 1 mM 4-AP in Hi-Di saline, bottom trace). $\bullet$, peak amplitudes of the model EPSCs fit to these data. C: paired comparisons between parameters from the control and 5-HT conditions indicate no significant differences in the decay of facilitation ($\tau_{\text{facil}}$), replenishment rate ($\tau_{\text{rep}}$), or total size of the RRP ($R_{\text{total}}$); Baseline fraction of release ($F_{\text{base}}$), however, was significantly increased by an average of 64% in models of 5-HT trains. D: values in individual preparations of the changes in the fraction of release plotted against the magnitude of 5-HT-induced potentiation in that preparation. The change in the fraction of release had a positive correlation with 5-HT-induced potentiation (see text).
In contrast, activation of the serotonergic DSIs produces a tonic increase in VSI synaptic strength. Expression of STDN depends on the level of synaptic depletion, we directly measured the effects of 5-HT on the decay of facilitation using a paired-pulse protocol. This also provided an opportunity to further validate our phenomenological model of the VSI synapse. The parameter sets that we derived from spike trains suggest that facilitation decays with a time constant of ~250 ms (Table 1) with no substantial difference between control and 5-HT conditions. As shown in Fig. 5A, the model exhibits identical rates of decay in a paired-pulse protocol across a wide range of values for fraction of release. Thus the model predicts that 5-HT would not affect the extent or dynamics of facilitation, which we could measure independently using a paired-pulse protocol.

Paired-pulse stimuli were given to VSI to evoke EPSCs at various intervals (Fig. 5B). After a control protocol, 5-HT was applied and the protocol was repeated. Results were analyzed with a two-way repeated-measures ANOVA (condition × interval). In both control and 5-HT conditions, frequency facilitation was greatest at shorter intervals (0.1 s) and decayed completely at intervals >0.8 s. This was evident as a significant main effect of inter-pulse interval \( [F(4) = 14.73, P < 0.001] \). As predicted by our model, 5-HT had no effect on the frequency facilitation ratio in this new data set. Specifically, the main effect of condition (control vs. 5-HT) was not significant \( [F(1) < 1] \) nor was the interaction between condition and interval \( [F(4) < 1] \) nor any comparison between conditions at specific inter-pulse intervals.

We next examined the time constant for the decay of facilitation. Specifically, we fit an exponential function through the control paired-pulse data set. The resulting curve was highly accurate \( (R^2 = 0.97) \). The estimated decay rate of facilitation was 220 ms. This is a close match \( (z = 1.03, P < 0.29) \) to the value that we independently derived from fitting the train data \( (254 ± 33 \text{ ms in control trains, Table 1}) \), showing that our phenomenological model of the VSI synapse has good predictive power even with an independent data set not used during parameter selection. Similar fits were obtained through the 5-HT data \( (R^2 = 0.96) \). These results further support the hypothesis that the change in the fraction of release is sufficient to explain the effect of 5-HT.

Expression of STDN depends on the level of synaptic depletion

When VSI is stimulated at low rates in 4-AP, bath application of 5-HT produces a tonic increase in VSI synaptic strength (Fig. 3A, inset). In contrast, activation of the serotonergic DSIs

### Table 1. Synaptic parameter estimates for control and 5-HT trains

<table>
<thead>
<tr>
<th>Synaptic Parameter</th>
<th>Control</th>
<th>5-HT</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of readily releasable pool: ( R_{\text{total}} )</td>
<td>1</td>
<td>0.98 ± 0.05</td>
<td>−2.0%</td>
</tr>
<tr>
<td>Initial fraction of release: ( F_{\text{base}} )</td>
<td>3.9 ± 0.8%</td>
<td>6.4 ± 0.6%</td>
<td>64.1%*</td>
</tr>
<tr>
<td>Refill rate: ( \tau_{\text{rec}} )</td>
<td>8.1 ± 1.3 s</td>
<td>8.1 ± 0.7 s</td>
<td>0.0%</td>
</tr>
<tr>
<td>Decay of facilitation: ( \tau_{\text{facil}} )</td>
<td>254 ± 33 ms</td>
<td>247 ± 74 ms</td>
<td>−2.8%</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Indicates significantly different between control and 5-HT conditions \( (P < 0.01) \).

FIG. 5. No effect of 5-HT on the paired-pulse facilitation ratio. A: plots of frequency facilitation based on the output of the phenomenological model. Facilitation index (FF index) was calculated as \( (2\text{nd EPSC} - 1\text{st EPSC})/1\text{st EPSC} \). Changes in fraction of release do not strongly influence the magnitude or decay of paired-pulse facilitation in the phenomenological model. Shown are plots of FF index with \( F_{\text{base}} \) set to control levels (empty circle), 150% of control (black triangle), and 50% of control (gray square). Each condition produced similar paired-pulse facilitation. B: plots of FF index based on electrophysiological measurements. 5-HT had little effect on the FF index. Inset: pairs of EPSCs evoked at intervals of 0.1, 0.2, 0.4, and 0.8 s in reduced Ca\(^{2+}\) saline. Data obtained in the control saline (black traces) and in the presence of 100 \( \mu \text{M} \) 5-HT (red traces) are shown overlaid (top traces). Traces are depicted superimposed after normalization to the 1st EPSC (bottom traces). Average measures from 7 preparations are plotted together. There is no significant difference between control and 5-HT measurements at any interval. The curves represent the exponential fit for both control and 5-HT.
produces the highly dynamic potentiation phase of STDN (Fig. 1B). Nevertheless, it seems likely that both processes operate via similar mechanisms, through an elevation of the fraction of release. To test this hypothesis, we examined how the level of VSI depletion influences the expression of DSI-evoked potentiation. Transmitter depletion should mask changes in the fraction of release (which requires available transmitter for expression) but not changes in vesicle recycling or pool size (which can transiently overcome depletion).

DSI-evoked potentiation (5-s, 10-Hz DSI activation) was induced before and during a VSI depletion protocol (15-s, 5-Hz VSI activation), representing both nondepleted and depleted states of the synapse, respectively. Stimulating DSI before depletion produced a transient potentiation (Fig. 6Ai), observable as a 29.1% ± 3.2 (SE) increase in synaptic efficacy 2.5 s after induction \([n = 4, r(3) = 9.1, P < 0.01; \text{Fig. 6Aii}i]\). This potentiation quickly dissipated, however, back to control levels, likely due to both the decay of heterosynaptic potentiation and to the depletion of transmitter. In contrast, the expression of DSI-evoked potentiation was almost completely abolished when induced in a depleted VSI synapse (Fig. 6Aiiii). Specifically, there was no enhancement observable 2.5 s after induction \([5.6 \pm 9.9\%, \text{mean} \pm \text{SE}, n = 4, r(3) = 0.56, P = 0.61; \text{Fig. 6Aiii}i]\). Thus DSI-evoked potentiation is not expressed in a depleted synapse, suggesting that it involves a selective modification in the fraction of release at VSI synapses.

We next explored if our phenomenological model of the VSI synapse could reproduce the influence of VSI synaptic state on the expression of DSI-evoked potentiation. To do this, we extended the model to incorporate a dynamic heterosynaptic regulation of the fraction of release. Specifically, we coupled the baseline fraction of release, \(F_{\text{base}}\), at the VSI synapse to the level of DSI activity so that the fraction of release would increase with each DSI spike and decay back to an unmodulated level in the absence of DSI activity (see METHODS).

The three new parameters added to the model were well-constrained by prior experiments and archival data. The decay time constant, \(\tau_{\text{mod}}\), was set to 6.95 s based on fits to archival records of DSI-evoked potentiation in 4-AP (e.g., Fig. 1B). We set the unmodulated level of release, \(F_{\text{unmod}}\), to 0.04, the average fraction of release in control trains in previous experiments (Table 1). Finally, the fraction of release per DSI spike, \(F_{\text{mod}}\), was set to 1.33\%, so that a 5-s, 10-Hz DSI stimulation would produce the same 64% increase in fraction of release as observed during bath application of 5-HT (Table 1). Note that these constraints do not utilize the data exploring the influence of VSI depletion on the expression of DSI-evoked potentiation (Fig. 6A). We thus used this data as an independent validation of the dynamic model. Note also that we did not attempt to model the depression phase of STDN but that the influence of this...
process should be relatively small over the course of the short protocols used to validate the model.

We tested the ability of the dynamic model to replicate the expression of DSI-evoked potentiation (5-s, 10-Hz DSI activation) in 4-AP before and during a VSI depletion protocol (15 s, 5 Hz). Stimulating DSI prior to the depletion protocol produced a transient elevation of VSI synaptic efficacy (Fig. 6Bi). This was observable as a 36% increase in EPSC size 2.5 s after the end of induction (Fig. 6Bi). This was similar to the 29.1 ± 3.2% increase observed in the parallel physiology experiment (Fig. 6Aiii) although statistically higher (z = 2.15; P < 0.03). In addition, the model correctly showed a masking of DSI-evoked potentiation during VSI depletion (Fig. 6Bii). Specifically, there was only a 3% increase in EPSC size 2.5 s after induction during a VSI spike train (Fig. 6Biii), similar to the lack of potentiation observed in the parallel physiology experiment (compare with 5.6 ± 9.9% increase; z = 0.26; P < 0.79; Fig. 6Aiii).

Reduction of synaptic depletion restores the expression of potentiation

If the level of depletion can mask changes in the fraction of release, then decreasing depletion should unmask DSI-evoked potentiation, increasing both the magnitude and the time course of expression. We thus repeated the same protocols in control Hi-Di saline without 4-AP. Under these conditions, VSI release is reduced and the synapse is not fully depleted (Fig. 2, A and C). Furthermore, under these conditions, both the potentiation and depression phases of STDN are expressed (Fig. 1A). However, the depression phase of STDN has a longer time course than the potentiation phase, so it should not have a strong influence during these short protocols.

Recording in the absence of 4-AP reduced baseline VSI release and transformed output during a VSI spike train from depressing to facilitating (control plots in Fig. 7A, compare with Fig. 6A). In addition, the expression of DSI-evoked potentiation was transformed. Consistent with our predictions, stimulating DSI prior to the VSI train produced a stronger initial enhancement (Fig. 7Aii), observable as a 47.7 ± 9.2% increase in synaptic efficacy 2.5 s after the end of the simulated DSI train (iii). ○ control EPSCs without DSI stimulation; ▲ EPSCs during runs with DSI stimulation.
pattern of expression suggests that DSI-evoked potentiation selectively modulates the fraction of release at VSI synapses.

We also simulated the pattern of DSI expression under conditions of lower release, similar to our physiological experiments in control Hi-Di saline without 4-AP. For these simulations, the unmodulated fraction of release, \( F_{\text{unmod}} \), was decreased to 17% of the value we had established in 4-AP (Table 1). This is representative of the level of VSI release in normal saline, which is 17% of the level observed in 4-AP (Fig. 2) (Sakurai et al. 2006). As in our physiological experiments, lowering the unmodulated fraction of release in the model transformed output during a VSI spike train from depressing to facilitating (control plot in Fig. 7Bi; compare with the control plot in Ai). In addition, the expression of DSI-evoked potentiation was transformed. Consistent with the physiological data, stimulating DSI prior to VSI activation, produced a stronger, longer-lasting synaptic enhancement (Fig. 7Bi). This was expressed as a 41% increase in EPSC size 2.5 s after induction (Fig. 7Bii), comparable to the 47.7 ± 9.2% increase (\( z = 0.72, P < 0.047 \)) observed in the parallel physiological experiment (Fig. 7Aii). Stimulating DSI during VSI activation (Fig. 7Bii) also produced a persistent potentiation, evident as a 26% increase 2.5 s after induction. In this case, however, the model significantly underestimated the 61.2 ± 6.5% increase in EPSC size observed in our physiological experiments (\( z = 5.41, P < 0.001 \)). This could have been due to an overestimation of the unmodulated fraction of release in normal saline and/or too rapid decay of the modulated fraction of release. Overall, however, the dynamic model was reasonably accurate at capturing both the magnitude and time course of plasticity under these novel conditions, reinforcing the hypothesis that the potentiation phase of STDN is due to a dynamic regulation of the fraction of release.

Depleting the internal calcium store blocked 5-HT-induced potentiation

Taken together, our computational and physiological data indicate that the potentiation phase of STDN is caused by a transient enhancement of the fraction of release at VSI synapses due to the release of 5-HT from the DSIs. The fraction of release is dependent on the presynaptic Ca\(^{2+}\) level (Augustine and Charlton 1986; Bardo et al. 2002; Sakaba and Neher 2001; Zucker 1999). To determine whether Ca\(^{2+}\) released from internal stores plays a role in the 5-HT-evoked potentiation, we depleted the calcium store with CPA, which blocks the refilling of calcium stores by sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pumps. We then tested the effect of bath-applied 5-HT in the presence of CPA.

Before application of CPA, 5-HT caused a sustained increase in VSI-evoked EPSC amplitude (Fig. 8A and Ci). Bath application of CPA reduced the overall amplitude of VSI-evoked EPSCs from 4.1 ± 1.2 to 1.3 ± 0.5 (SE) nA (Fig. 8, B and E) and eliminated the 5-HT-induced potentiation of the EPSCs (from 102.8 ± 33.5 to 8.1 ± 12.7%; Fig. 8, Cii and F). When the EPSCs were evoked by a train of stimuli (50 pulses at 5 Hz), CPA blocked the 5-HT-induced increase in the amplitude of the initial portion of the EPSC train (cf., Fig. 3A) but preserved other characteristics such as the frequency facilitation (Fig. 8, D and G). The continued presence of frequency facilitation in CPA strongly indicates that CPA does not act by
depleting the releasable transmitter nor does it decrease the sensitivity to changes in residual calcium level. Rather it indicates that CPA specifically blocks 5-HT-induced changes in the release process that require stored calcium. Thus we conclude here that calcium release from internal stores is necessary for 5-HT-induced potentiation of VSI synaptic strength.

**Discussion**

In the *Tritonia* swim CPG system, activation of a serotonergic DSI produces STDN (Sakurai and Katz 2003), a dynamic neuromodulation of VSI-VFN synaptic strength consisting of two distinct phases: an early, short-lasting synaptic potentiation that is insensitive to 4-AP and a delayed, longer-lasting synaptic depression that is blocked by 4-AP (Sakurai et al. 2006). Because this is one of the few systems where neuromodulation of central synapses has been directly induced by activation of an aminergic neuron as opposed to bath application (see, however: Katz and Harris-Warrick 1990; Mackey et al. 1989), it provides a unique opportunity to understand the short-term dynamics of neuromodulation, which may have important consequences for neural circuit functions such as attention, working memory, and conditioning (Clayton et al. 2004; Roitman et al. 2004; Schultz and Dickenson 2000; Stuber et al. 2005; Thivierge et al. 2007; Williams and Goldman-Rakic 1995).

It has previously been shown that the depression phase of STDN may be produced by a transient decrease in VSI spike duration (Sakurai et al. 2006). In this study, we explored the mechanisms underlying the short-term potentiation phase of STDN by blocking the depression phase with 4-AP. We utilized both a depletion protocol and a phenomenological model to track changes in VSI synaptic dynamics. Our results indicate that both 5-HT and direct DSI activation produce a dynamic increase in the fraction of release at VSI synapses without altering the size of the RRP, the replenishment rate, or the magnitude and the time course of facilitation. Furthermore, the potentiating effects of 5-HT are blocked by depleting the intracellular stores of Ca$^{2+}$. This suggests that intracellular Ca$^{2+}$ stores play a role in determining the fraction of release and that their interaction with the synaptic release process is modifiable by serotonergic neuromodulatory input.

**Potentiation phase of STDN is caused by an increase in the fraction of release**

Both the cumulative EPSC analysis and the phenomenological model suggest that a transient increase in the fraction of release is sufficient to account for the potentiation phase of STDN. This is broadly consistent with findings in other central and peripheral synapses where 5-HT produces synaptic facilitation, including crab neuromuscular junction (Jorge-Rivera et al. 1998). In sensory-motor synapses of cultured *Aplysia* neurons, the effects of 5-HT are less selective, producing an increase in RRP size as well as fraction of release (Zhao and Klein 2002).

Changes in RRP size also figure prominently in the potentiating effects of 5-HT at inhibitory and excitatory neuromuscular junctions in crayfish (Lin and Fu 2005; Logsdon et al. 2006; Sparks and Cooper 2004; Wang and Zucker 1998). However, the small magnitude of RRP change (25–120% during 5-HT) (Wang and Zucker 1998) does not correlate well with the large increase in synaptic efficacy produced by 5-HT (400–440% during 5-HT) (Dixon and Atwood 1989). Moreover, accelerated release kinetics have been suggested to supplement changes in the RRP at inhibitory junctions (Lin and Fu 2005). Direct measurement of fraction of release via a depletion protocol suggests that these other mechanisms do not include changes in fraction of release at the excitatory junction (Wang and Zucker 1998). However, 5-HT has been repeatedly shown to accelerate release at these junctions (Lin and Fu 2005; Southard et al. 2000; Vyshedskiy et al. 1998), which strongly suggests a net increase in the fraction of release per spike. One problem with comparing the effects of 5-HT across species and synapses is that the receptor subtypes are not known, and it is thus unclear how much consistency should be expected.

**Role of internal Ca$^{2+}$ stores in heterosynaptic potentiation**

Pharmacological experiments suggest that 5-HT may increase the fraction of release via an enhancement of Ca$^{2+}$ release from intracellular stores. Depletion of internal Ca$^{2+}$ stores by CPA reduced the baseline synaptic strength and completely abolished the 5-HT-evoked potentiation. These results further suggest that Ca$^{2+}$ release from intracellular stores plays a role in the unmodulated synaptic release process. There is increasing evidence that calcium-induced calcium release (CICR) from internal stores plays a role in synaptic release (Bouchard et al. 2003; Carter et al. 2002; Collin et al. 2005; Narita et al. 2000). CICR has been shown to contribute to somatic release in leech neurons (Trueta et al. 2004). Intracellular Ca$^{2+}$ stores help control neurotransmitter release in *Aplysia* synapses (Chameau et al. 2001). Ryanodine receptors play a role in controlling the quantal release size in *Lymnaea* synapses (Dunn and Syed 2006) and also control synaptic strength in mammalian basket cell to Purkinje cell synapses (Galante and Marty 2003).

It is less clear how internal calcium stores contribute to the synaptic effects of 5-HT. The potentiating effects of 5-HT have been associated with intracellular cascades that could alter internal calcium dynamics, including IP3, PLC, and ryanodine receptors at the crayfish neuromuscular junction (Dixon and Atwood 1989; Dropic et al. 2005) or that could be affected by intracellular stores, such as PKC in *Aplysia* sensory-motor synapses (Braha et al. 1990; Ghirardi et al. 1992). Imaging data from these systems, however, do not show an effect of 5-HT on internal calcium dynamics: in crayfish, there are no changes in Ca$^{2+}$ dynamics in synaptic terminals (Delaney et al. 1991; Vyshedskiy et al. 1998; Zhong et al. 2004); in *Aplysia*, there is an increase in spike-mediated Ca$^{2+}$ that is independent of internal store dynamics (Blumenfeld et al. 1990). The situation may be different in *Tritonia*, where activation of the serotonergic DSIIs produces increased spike-evoked Ca$^{2+}$ at other synapses (Hill and Katz 2006), which were previously shown to be modulated (Katz et al. 1994; Katz and Frost 1995). More research is needed to determine if this serotonergic modulation of Ca$^{2+}$ involves internal stores.
Expression of STDN neuromodulation interacts with ongoing synaptic state

We found that expression of the potentiation phase of STDN is influenced by the state of the synapse, particularly the level of available transmitter. When VSI is in a depleted state, DSI stimulation produces a modest synaptic enhancement that is rapidly overwhelmed by depression. In a non-depleted state, DSI stimulation produces a stronger, longer-lasting enhancement of VSI synaptic strength. This physiological results are remarkably consistent with our phenomenological model of the synapse. The depression phase of DSI-evoked STDN is also contingent on the firing history of VSI (see Fig. 10 in Sakurai and Katz 2003). Thus the overall profile of STDN expression will be shaped by the time courses of the potentiation and depression phases as well as the ongoing activity of VSI.

The functional consequences of this complexity remain to be illuminated. However, it is worth noting that DSI and VSI are both components of a central pattern generator and fire in alternating bursts to generate a rhythmic motor program. Furthermore, the modulatory actions of DSI is essential to this oscillation (McClellan et al. 1994). Thus the interactions of temporal VSI firing patterns and heterosynaptic neuromodulation may play an important role in initiating and regulating rhythmic motor pattern generation.

There are indications that neuromodulatory actions in other systems can be state dependent. For example, the intracellular signaling cascade that mediates 5-HT potentiation of sensory neurons in Aplysia is dependent on the depression state of those synapses (Byrne and Kandel 1996). State dependence in some systems could be determined by changes in the association of subcellular components or in the background level in other signaling pathways. The sensitivity of Aplysia bag cell neurons to protein kinase C is related to their previous firing history, which in turn affects the association of the kinase with particular ion channels (Magoski and Kaczmarek 2005). Similarly, the effect of 5-HT4 receptors on GABAergic signaling in the prefrontal cortex is dependent on the basal level of protein kinase A activity in the modulated neurons (Cai et al. 2002).

Spike timing-dependent neuromodulation

In summary, this study and previous studies (Sakurai and Katz 2003; Sakurai et al. 2006) suggest that the two opposite effects caused by DSI on the VSI synaptic strength are produced by independent intracellular signaling pathways. The dynamics of the neuromodulatory actions leading to STDN are dependent on the intracellular signaling that occurs after serotonin receptors are activated by DSI activity. This intracellular signaling produces the time and state dependence of the neuromodulation. Although difficult to observe in other systems because of experimental constraints, dynamic, short-term heterosynaptic plasticity, such as this, is likely to be generally important for neuronal processing.

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REFERENCES


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