Contribution of PKC to the Maintenance of 5-HT-Induced Short-Term Facilitation at Sensorimotor Synapses of *Aplysia*

Abbreviated Title: PKC Contribution to Maintenance of Short-Term Facilitation

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ABSTRACT

Aplysia sensorimotor synapses provide a useful model system for analyzing molecular processes that contribute to heterosynaptic plasticity. For example, previous studies demonstrated that multiple kinase cascades contribute to serotonin (5-HT)-induced short-term synaptic facilitation (STF), including protein kinase A (PKA) and protein kinase C (PKC). Moreover, the contribution of each kinase is believed to depend on the state of the synapse (e.g., depressed or nondepressed) and the time after application of 5-HT. Here, a previously unappreciated role for PKC-dependent processes was revealed to underlie the maintenance of STF at relatively nondepressed synapses. This PKC dependence was revealed when the synapse was stimulated repeatedly after application of 5-HT. The contributions of the PKA and PKC pathways were examined by blocking adenylyl cyclase-coupled 5-HT receptors with methiothepin and by blocking PKC with chelerythrine. STF was assessed 20 s after 5-HT application. The effects of PKC were consistent with enhanced mobilization of transmitter, as assessed by application of hypertonic sucrose solutions to measure the readily-releasable-pool (RRP) of vesicles and recovery of the RRP after depletion. A computational model of transmitter release demonstrated that a PKC-dependent mobilization process was sufficient to explain the maintenance of STF at nondepressed synapses and the facilitation of depressed synapses.

KEYWORDS

Sensorimotor Synapses, Synaptic Plasticity, Homosynaptic Depression, Heterosynaptic Facilitation, Transmitter Mobilization, Computational Model
INTRODUCTION

Short-term sensitization and dishabituation of withdrawal reflexes in *Aplysia* are mediated, in part, by serotonin (5-HT)-induced short-term facilitation (STF) of sensorimotor (SN-MN) synapses (Antonov et al. 2010; for review see Byrne and Kandel 1996). Serotonin activates multiple second-messenger cascades in the pre- and postsynaptic cells (Bernier et al. 1982; Braha et al. 1990; Dumitriu et al. 2006; Jin et al. 2011; Müller and Carew 1998; Ocorr et al. 1986; Sugita et al. 1992; Villareal et al. 2009). The relative contribution of each cascade depends on the state (depressed vs. nondepressed) of the synapse and the time at which STF is assessed after 5-HT treatment (for review see Byrne and Kandel 1996). For example, protein kinase A (PKA) contributes preferentially to STF of nondepressed synapses, whereas protein kinase C (PKC) contributes to STF at depressed synapses (Antonov et al. 2010; Braha et al. 1990; Byrne and Kandel 1996; Ghirardi et al. 1992; Goldsmith and Abrams 1991; Jin et al. 2011; Manseau et al. 2001; Sugita et al. 1997a, b). The present study used cultured SNs and SN-MN co-cultures to examine in greater detail the state-dependent contributions of PKA and PKC to STF.

Although the mechanisms underlying state-dependent STF are not fully understood, PKA is believed to facilitate nondepressed synapses by enhancing presynaptic Ca\(^{2+}\) influx and synaptic release (Antonov et al. 2010; Klein 1994; Klein and Kandel 1980; Leal and Klein 2009), whereas PKC is hypothesized to enhance vesicle mobilization (Houeland et al. 2007; Khoutorsky and Spira 2005). An important prediction of this hypothesis is that PKC-mediated mobilization should help maintain STF during repetitive stimulation following 5-HT treatment. This prediction has yet to be
tested, and thus, the goal of the present study was to examine the maintenance of STF during repeated stimulation, which can also be viewed as slowing of depression after 5-HT-induced facilitation. The studies reported here focused on STF at \( \leq 2.5 \) min after 5-HT treatment, when the plasticity is presynaptic (Antonov et al. 2010; Jin et al. 2011). Facilitation was measured in SN-MN co-cultures. After a pre-test measurement, SNs were stimulated once every 20 s (0.05 Hz), and 5-HT was applied 20 s prior to the 0.05 Hz stimulation. The relative contributions of each kinase were assessed by blocking 5-HT activation of PKA with methiothepin and PKC activity with chelerythrine. The contributions of each kinase to the maintenance of STF were assessed by measuring facilitation throughout the 0.05 Hz stimulation. In addition, the contribution of PKC to mobilization of transmitter was measured by examining the recovery of release following depletion of transmitter induced by hypertonic solution. Finally, the data were used to extend a previously developed computational model of presynaptic release. Simulations suggested that PKC-dependent mobilization helps to maintain STF during repeated stimulation.
MATERIALS AND METHODS

Electrophysiology. Sensorimotor (SN-MN) co-cultures were prepared as described previously (Angers et al. 2002; Liu et al. 2008). Recordings were performed in saline consisting of 50% isotonic L15 and 50% artificial seawater (L15-ASW). An EPSP was elicited in a motor neuron by delivering a brief (1 ms) extracellular electrical stimulus to a presynaptic sensory neuron using a blunt patch electrode filled with L15-ASW. EPSPs were recorded with 12–15 MΩ sharp electrodes filled with 3 M potassium acetate. In each of the cell co-cultures, a pre-test was performed to verify the existence of a synaptic connection (termed EPSP0). Once the synaptic connection was confirmed, the preparation was allowed to rest for 5-15 min. Two criteria were used for data exclusion. First, as in our previous studies (e.g., Phares and Byrne 2005), only synapses with pre-test EPSP amplitudes ≥ 3 mV were used. Seven preparations were excluded based on this a priori criteria. Second, the extracellular stimulus to a SN typically elicited a unitary EPSP in the MN, indicative of a response produced by a single spike in the SN. However, in some cases, overlapped multiple component EPSPs in the MN were observed, which was indicative of the triggering by the extracellular stimulus of multiple presynaptic spikes in the SN. Because a burst of multiple spikes in SNs can affect the subsequent depression of EPSPs at the SN-MN synapse (Wan et al. 2011), any EPSPs recorded after multicomponent EPSPs were eliminated from the analysis. Fifteen preparations were excluded based on this post hoc criteria. Motor neurons were hyperpolarized to –90 mV to prevent the EPSPs from triggering spikes. In general, each culture was used only once per experimental manipulation. The only exceptions were preparations that were used to examine the
effects of different interstimulus intervals (ISIs) on paired-pulse ratios (PPRs, i.e., EPSP₂/EPSP₁). PPRs at four different ISIs were measured during one of the following conditions: control cultures treated with vehicle, 5-HT-treated cultures, 5-HT-treated cultures in the presence of a PKC inhibitor (20 μM chelerythrine, Manseau et al. 1998; Sossin 2007; Yanow et al. 1998), and 5-HT-treated cultures in the presence of methiothepin (100 μM), which blocks adenylyl cyclase-coupled 5-HT receptors (Cohen et al. 2003; Dumitriu et al. 2006). Previous studies indicated that these two inhibitors did not affect the basal amplitude of SN-MN EPSPs. Cai et al. (2011) showed that chelerythrine (Che) did not affect the SN-MN EPSP, although a somewhat lower concentration (5-10 uM) was used than that in the present study (20 uM). Similarly, methiothepin did not affect basal EPSPs at the same concentration used in this study (Dumitriu et al. 2006). Moreover, in the present study no significant difference was observed in the amplitude of the pre-5-HT treatment EPSPs among the four experimental groups (see Results). Methiothepin or chelerythrine was applied 15 min before the first paired pulse and remained in the chamber for the duration of the experiment. Each test of an ISI (ISI = 1, 5, 10, 20 s) was separated by washing the chamber with 5-HT-free medium containing methiothepin or chelerythrine, followed by a 10 min rest period before 5-HT or vehicle was reapplied. The orders of ISIs for any one condition were chosen pseudo-randomly from preparation to preparation. Data acquisition was performed using pClamp version 9 (Molecular Devices, Union City, CA). All experiments were performed at room temperature (22–25°C).

In experiments examining asynchronous release, the hypertonic stimulating solution consisted of L15-ASW (50% isotonic L15 and 50% artificial seawater) with 1 M
sucrose. Asynchronous release was measured following the methods of Zhao and Klein (2002, 2004). The number of mEPSPs was counted within the first 1 min after addition of the hypertonic solution, and this total number was taken as the RRP size (Zhao and Klein 2002, 2004). Between the first and second application of sucrose (Fig. 5), the chamber was perfused with either vehicle or chelerythrine for 2.5 min to wash away the residual sucrose. Estimated perfusion volume was about four times the bath volume.

Data analysis and statistics. In each experiment, a Shapiro-Wilk test for normality indicated that data were normally distributed. Thus, data were analyzed using parametric statistical tests, such as Student’s t test (two groups) or analyses of variance (ANOVA, three or more groups) followed by post hoc analysis with Student–Newman–Keuls (SNK) tests. Statistical tests were performed using Sigmaplot 11 (Jandel Scientific, San Jose, CA). All data are expressed as means ± standard error of the mean (SEM). Differences between means were considered significant with a P value < 0.05.

Drugs. Methiothepin was used to block 5-HT-induced activation of PKA cascades in sensory neurons of Aplysia (Cohen et al. 2003; Dumitriu et al. 2006; Lee et al. 2009; Nagakura et al. 2008). Chelerythrine was selected for use as an inhibitor of PKC because of its effectiveness in blocking dishabituation and the associated facilitation of depressed SN-MN synapses (Antonov et al. 2010; Villareal et al. 2009). Solutions of 5-HT and methiothepin (Sigma-Aldrich, St. Louis, MO) were prepared on the day of each experiment by dissolving the drug in recording saline (L15-ASW). Chelerythrine (EMD Biosciences-Calbiochem, Billerica, MA) was prepared as a 1 mM
stock solution in distilled water and stored at -20°C. On the day of an experiment, an aliquot of the stock solution was diluted in L15-ASW immediately before perfusion of the cells. During the experiment, a bolus of 5-HT (40 μl at 5 mM) was applied in the vicinity of the MN to achieve a final bath concentration of 50 μM (Liu et al. 2008). Chelerythrine and/or methiothepin were perfused into the experimental chamber with a peristaltic pump (Gilson Minipulse 3). Preparations were pretreated with methiothepin (100 μM, final concentration in L15-ASW) or chelerythrine (20 μM) for 15 min before testing synaptic connections. The treatment groups continued to receive methiothepin or chelerythrine until the end of the experiment.

**Computational model and simulations.** The model (Fig. 2A) was modified from the Gingrich-Byrne (G-B) lumped parameter model of the SN-MN synapse of *Aplysia* (Gingrich and Byrne 1985). The modifications accomplished three goals: 1) adapting the model to simulate the characteristics of homosynaptic depression of SN-MN synapses in culture versus the characteristics of depression in ganglia; 2) improving simulations of the effects of cAMP on heterosynaptic plasticity; and 3) incorporating a novel role for PKC in heterosynaptic plasticity, which is the subject of the present study.

To guide our modifications, the present study used a parameter optimization algorithm known as particle swarm optimization (PSO) (Schwaab et al. 2008; Zhang et al., 2012) (see below).

Briefly, the original G-B model consisted of two pools of vesicles, a readily releasable pool (RRP) and a reserve pool (RP). A set of ordinary differential equations was used to describe the dynamics of the pools of vesicles, Ca^{2+} influx, regulation of
intracellular Ca\(^{2+}\) concentration, synaptic transmission and vesicle mobilization from RP to RRP.

In the G-B model, vesicle mobilization was described by three fluxes: \(I_S\), \(I_F\), \(I_{VD}\). \(I_S\) and \(I_F\) represented the slow and fast fluxes from RP to RRP, respectively, and these two fluxes were regulated by the concentration of intracellular Ca\(^{2+}\). \(I_{VD}\) was a flux driven by concentration differences between vesicles in the RRP and RP.

For the present study, a new term for PKC-dependent vesicle mobilization (\(I_{PKC}\)) was added. In addition, the capacity of the RRP was modified to include an upper bound. Thus, all vesicle mobilization fluxes from RP to RRP were limited by the difference between the current concentration of vesicles in the RRP (\(C_{RRP}\)) and its maximum value (\(C_{RRPMax}\)). Depletion of vesicles in the RP was replenished by a relatively slow flux \(I_{IN}\) (see Eq. 15 of Gingrich and Byrne 1985).

With these modifications, the total flux of vesicle mobilization from RP to RRP became:

\[
I_{Replenish} = I_{S1} + I_{F1} + I_{VD1} + I_{PKC}
\]

[1]

where \(I_{S1}\), \(I_{F1}\) and \(I_{VD1}\) were the modified fluxes \(I_S\), \(I_F\) and \(I_{VD}\) and were described by:

\[I_{S1} = \alpha \times I_S \times (C_{RRPMax} - C_{RRP}),\]

\[I_{F1} = \alpha \times I_F \times (C_{RRPMax} - C_{RRP}),\] and

\[I_{VD1} = \alpha \times I_{VD} \times (C_{RRPMax} - C_{RRP}).\]
α is a constant scale factor with value 0.01; $C_{RRPMax}$ is equal to 100 (dimensionless units); and $I_S$, $I_F$ and $I_{VD}$ were the same as the original G-B description. The flux of PKC-dependent vesicle mobilization was described by:

$$I_{PKC} = \beta_{PKC} \times (C_{RRPMax} - C_{RRP})$$  \[2\]

where $\beta_{PKC}$ was the contribution of the PKC-dependent process to vesicle mobilization.

Note at nondepressed synapses, $C_{RRP}$ was at its maximum value $C_{RRPMax}$ (i.e., $C_{RRPMax} - C_{RRP} = 0$), and thus, $I_{PKC}$ would not contribute to mobilization even if $\beta_{PKC}$ was fully activated. This saturation effect was chosen to be consistent with the previous observations that the contribution of PKC to the facilitation of depressed synapses becomes progressively more important as synaptic transmission becomes more depressed (Ghirardi et al. 1992). Published data and the present results (Figs. 1 and 3) suggest that the PKC-dependent contribution has both activation and inactivation components (Dumitriu et al. 2006; Manseau et al. 2001). Thus, $\beta_{PKC}$ was described by:

$$\beta_{PKC} = F_{PKC} \times m \times h$$  \[3\]

where $F_{PKC}$ is the maximum contribution of the PKC-dependent process to vesicle mobilization and was estimated to have a value of 14.2. The activation and inactivation components were represented by $m$ and $h$, respectively. Activation, $m$, varied from 0 to 1 and was described by $m = 1 - e^{-t/\tau_1}$, where $t$ is set to 0 with the application of 5-HT. The activation time constant, $\tau_1$, was estimated to be 0.4 s, based on obtaining a good fit between model and data in Figs. 2B and 3. The inactivation component, $h$, varied from 1 to 0 and was described by: $h = e^{-t/\tau_2}$, where $t$ is set to 0 with the application of
5-HT. The inactivation time constant $\tau_2$ was estimated to be 110 s, based on obtaining a good fit between model (Fig. 2) and data (Fig. 1) (see also below).

In the modified G-B model, $\beta_{PKC}$ also contributed to the vesicle mobilization that replenishes the RP. The modified flux $I_{IN1}$ replenishing the RP was described by:

$$I_{IN1} = I_{IN} * (1 + \beta_{PKC})$$

As in the original G-B model (Gingrich and Byrne 1985, 1987), the effects of PKA were simulated by increasing the duration of the presynaptic action potential. In the present study, the duration was increased by 147% (i.e., from 3 to 4.4 ms). This increase was estimated using PSO based on the fit between model (Fig. 2, 5-HT + Che) and data (Fig. 1, 5-HT + Che). The increase in this one variable was used to simulate the combined actions of 5-HT-induced enhancement of $Ca^{2+}$ influx associated with spike broadening (Baxter et al. 1999) and the direct enhancement of the $Ca^{2+}$ conductance (Leal and Klein 2009). The simulated EPSP is represented by the total amount of vesicle released during a stimulus (Eqs. 9 and 10 of Gingrich and Byrne 1985).

Parameter optimization. The original G-B model was designed to simulate the dynamics of the SN-MN synapse of the abdominal ganglion (Byrne 1982), which differ somewhat from that of the SN-MN synapses in culture. For example, stimuli with interspike intervals (ISIs) of 1 s and 20 s led to similar rates of depression in cultures (see Fig. 7B, pre 5-HT), but not in ganglia (Gingrich and Byrne 1985). In addition, the original G-B model did not include a description of the PKC-dependent vesicle mobilization process.
To facilitate the selection of parameter values, PSO was used to determine the best fit between the empirical data and the output of the model (Schwaab et al. 2008; Zhang et al. 2012). Data from Figs. 1 (Control), and 7B (pre 5-HT) were used to estimate a subset of five parameters of the model related to homosynaptic depression (HSD). All other parameters remained unchanged from the original model of Gingrich and Byrne (1985). These five parameters were: $K_R$, the synaptic release probability; $V_R$ and $V_F$, the volumes of the releasable and reserve pools respectively; $K_F$ and $N_F$, the rate constant and Hill coefficient of the fast flux of vesicle mobilization respectively. The original values (from the original G-B model) and modified values of these five parameters are presented in Table 1.

For this optimization, fitness error was represented by:

$$
fitness = \sqrt{\sum_{j=1}^{m} (\text{EPSP}(\text{sim}, \text{Fig. 1}, j) - \text{EPSP}(\text{exp}, \text{Fig. 1}, j))^2 / m + 0.5 * } \\
\left( \sqrt{\sum_{j=1}^{n} (\text{EPSP}(\text{sim}, \text{Fig. 7}(\text{ISI} = 1s), j) - \text{EPSP}(\text{exp}, \text{Fig. 7}(\text{ISI} = 1s), j))^2 / n + } \\
\sqrt{\sum_{j=1}^{n} (\text{EPSP}(\text{sim}, \text{Fig. 7}(\text{ISI} = 20s), j) - \text{EPSP}(\text{exp}, \text{Fig. 7}(\text{ISI} = 20s), j))^2 / n) [5]}
$$

where $j$ represented stimulus number, $\text{sim}$ represented simulated EPSP, and $\text{exp}$ represented experimental EPSP. The PSO-generated values for the above five parameters improved the fitness error to 0.021, as compared to 0.067 using the parameters in the original G-B model.
After estimating the control parameter values for homosynaptic depression in the absence of 5-HT, the PSO analysis was repeated to estimate parameters related to 5-HT-induced heterosynaptic facilitation. Data from Fig. 1 (5-HT + Che) were used in conjunction with the first term of Eq. 5 to estimate a single parameter, the spike duration (SpikeWidth), related to the PKA-induced spike broadening. Data from Fig. 1 (5-HT + Met) were then used in conjunction with the first term of Eq. 5 to estimate parameters related to the PKC-dependent processes (i.e., activation constant ($\tau_1$), inactivation constant ($\tau_2$) and maximum contribution of the PKC-dependent process to vesicle mobilization ($F_{PKC}$)).

Values of parameters are provided in Table 1. Unless stated otherwise, no further parameter adjustments were made when the model was used to simulate the results in Figs. 1 (5-HT), 3, 5, 6, and 7 (post 5-HT). Integrations were performed using the forward-Euler method and the integration stepsize was 0.1 ms. Further reductions in step size did not alter the results. Simulations were performed in Visual C++. The source code is available at ModelDB website (Hines et al. 2004).
RESULTS

Maintenance of 5-HT-induced STF. The primary focus of the present study was the maintenance of STF, which was measured by repeatedly stimulating the sensorimotor connection following application of 5-HT. The methods used to evaluate the maintenance of STF are illustrated in Fig. 1. After the initial pre-test EPSP was measured (Fig. 1, EPSP₀), cultures were rested for 5 min. Then, STF and its maintenance were examined via a train of 8 stimuli at 0.05 Hz (Fig. 1, EPSP₁₋₈) in the continued presence of 5-HT, which was applied 20 s prior to EPSP₁ (Fig. 1A). Control cultures were treated with vehicle alone. Here, maintenance refers to the reduced rate of depression during a train of test stimuli following 5-HT application, as compared to the rate of depression in control preparations treated with vehicle. It can also be viewed as slowing of depression after 5-HT-induced facilitation.

After 20 s of 5-HT, EPSP₁ was facilitated by 187 ± 14% (n = 6) relative to the pre-test (Fig. 1B₁). Thereafter, STF was relatively stable (i.e., maintained) in the presence of continued testing, particularly when compared to the rapid decrement of the control EPSPs. The maintenance of STF is more clearly illustrated in Fig. 1B₂ where EPSP₁₋₈ were re-normalized to EPSP₁ for the 5-HT-treated and control groups. For example, the control EPSP₄ (i.e., the midpoint in the post-5-HT stimulus train) decremented to 40 ± 3% of its initial level, whereas the 5-HT EPSP₄ decremented to only 76 ± 4% (Fig. 1B₂).

PKC contributes to the maintenance of STF. To examine the contribution of PKA- and PKC-dependent processes to the maintenance of STF, 5-HT was applied in the presence of 20 μM chelerythrine (Antonov et al. 2010) or 100 μM methiothepin (Dumitriu et al. 2006) (Fig. 1A, 1B₁, 5-HT + Met; 5-HT + Che). No significant difference
was observed in the amplitude of the pre-5-HT treatment EPSPs among the four experimental groups (Control, 5-HT, 5-HT + Che and 5-HT + Met, $F_{(3,19)} = 0.66, P = 0.59$). However, pretreatment with either methiothepin or chelerythrine reduced the initial 5-HT-induced facilitation of EPSP₁ to 110% and 122% of EPSP₀, respectively. EPSP₁ in the non-treated group was 79% of EPSP₀ (EPSP₁ / EPSP₀ ± SEM: Control, 79 ± 6%, n = 6; 5-HT, 187 ± 14%, n = 6; 5-HT + Met, 110 ± 6%, n = 5; 5-HT + Che, 122 ± 8%, n = 6). A one-way ANOVA revealed a significant difference among the groups ($F_{(3,19)} = 24.4, P < 0.001$). Post hoc pair-wise comparisons (SNK) indicated that 5-HT significantly increased EPSP₁ as compared to all other groups (5-HT vs. Control, $q_4 = 11.8, P < 0.001$; 5-HT vs. 5-HT + Met, $q_3 = 8.0, P < 0.001$; 5-HT vs. 5-HT + Che, $q_2 = 7.1, P < 0.001$). Although both methiothepin or chelerythrine reduced the 5-HT induced facilitation, the facilitation was nevertheless significantly greater than the non-treated control group (Control vs 5-HT + Met, $q_2 = 3.2, P < 0.05$; Control vs. 5-HT + Che, $q_3 = 4.6, P < 0.05$), and there was no significant difference between 5-HT + Met and 5-HT + Che groups (5-HT + Met vs. 5-HT + Che, $q_2 = 1.2, P = 0.4$). These results suggest both PKA- and PKC-dependent processes contributed to the initial 5-HT-induced facilitation. Previous studies showed that PKA-dependent processes mainly contribute to STF of nondepressed synapse, whereas PKC-dependent processes mainly contribute to STF of depressed synapse, with the extent of the contribution of PKC to STF directly related to the extent of prior depression (Ghirardi et al. 1992; Manseau et al. 2001). Because the control synapses were slightly depressed (i.e., EPSP₁ = 79% of EPSP₀), we expected that the 5-HT-induced facilitation of EPSP₁ would be dependent on both PKA and PKC and therefore attenuated by both inhibitors (Byrne and Kandel 1996).
In contrast, the two drugs had very different effects on the maintenance of STF. Maintenance was unaffected by methiothepin, but was abolished in the presence of chelerythrine (Fig. 1B2). A statistical analysis of EPSP₄ indicated a significant overall difference among the four groups (EPSP₄ / EPSP₁ ± SE: Control, 40 ± 3%, n = 6; 5-HT, 76 ± 4%, n = 6; 5-HT + Met, 71 ± 10%, n = 5; 5-HT + Che, 39 ± 4%, n = 6; $F(3,19) = 12.7$, $P < 0.001$). Subsequent pair-wise comparisons revealed that EPSPs after treatment with 5-HT were significantly larger than the vehicle-treated control PSPs (Control vs. 5-HT: $q₃ = 6.4$, $P < 0.001$) as were the EPSPs after treatment with 5-HT in the presence of methiothepin (Control vs 5-HT + Met, $q₄ = 5.3$, $P < 0.01$). However, the EPSPs after treatment with 5-HT in the presence of chelerythrine were not larger than control PSPs (Control vs. 5-HT + Che, $q₂ = 0.42$, $P = 0.77$). Moreover, addition of chelerythrine reduced the amplitude of EPSP₄ (5-HT vs. 5-HT + Che, $q₄ = 6.8$, $P < 0.01$), whereas the addition of methiothepin did not (5-HT vs 5-HT + Met, $q₂ = 0.76$, $P = 0.6$). These results indicated that PKC-dependent processes contributed to the maintenance of STF, whereas PKA-dependent processes did not.

**Computational simulations of PKC- and PKA- dependent processes.** The empirical data suggested that a PKC-dependent process was responsible for the maintenance of STF of nondepressed synapses (Fig. 1). Because previous studies suggest that PKC enhances vesicle mobilization from a reserve pool (RP) to a readily releasable pool (RRP) (Byrne and Kandel 1996; Hochner et al. 1986; Houeland et al. 2007; Khoutorsky and Spira 2005; Zhao and Klein 2002, 2004), the G-B model was extended by incorporating a PKC-dependent vesicle mobilization process (Fig. 2A, see Material and Methods).
In the modified G-B model, the contribution of a PKC-dependent process to vesicle mobilization ($\beta_{PKC}$) consists of three components: the maximum value of its contribution ($F_{PKC}$), an activation component ($m$), and an inactivation component ($h$). The value of $F_{PKC}$ was constrained by the extent to which the PKC-dependent process maintains STF. The parameters of $m$ and $h$ were constrained by transient effects of the PKC-dependent process on the maintenance of STF. PKC-dependent processes appeared to make a substantial contribution to the maintenance of STF at a relatively nondepressed synapse when 5-HT was applied 20 s prior to testing STF in the presence of a PKA cascade inhibitor (Figs. 1B1, 1B2 (5-HT + Met)). These data were used to estimate the parameters of activation components ($m$ and $F_{PKC}$) of PKC-dependent vesicle mobilization. In addition, to account for the slow decay of STF in the continued presence of 5-HT (Fig. 1B2), we assumed that the PKC-dependent vesicle mobilization process was not persistent and introduced the inactivation variable $h$. Additional support for this assumption is the rapid re-emergence of depression after the initial STF of depressed synapses in the continuous presence of 5-HT (Dumitriu et al. 2006; Manseau et al. 2001; see also Fig. 3).

The contribution of the PKA-dependent process to STF was simulated by increasing the duration of the action potential (Gingrich and Byrne 1985, 1987, also see Material and Methods). In SNs, modulation of membrane currents by PKA leads to spike broadening and increased Ca$^{2+}$ influx (Baxter et al. 1999; Klein et al. 1982; Leal and Klein 2009), which results in enhanced transmitter release and Ca$^{2+}$-dependent vesicle mobilization (see Eq. 14 of Gingrich and Byrne 1985). In the model, this enhanced transmitter release produced greater depletion of the RRP, but this depletion
was partially offset by Ca\textsuperscript{2+}-dependent fluxes from the RP to the RRP (I_F, I_S). Data from Fig. 1B2 (5-HT + Che) were used to constrain the parameters related to the PKA-dependent process.

Figure 2B illustrates a simulation of STF and its maintenance using the same stimulus protocol in Fig. 1. STF induced by 5-HT in the presence of methiothepin was simulated by the activation of PKC alone; STF induced by 5-HT in the presence of chelerythrine was simulated by activation of PKA alone; and overall effects of 5-HT on STF were simulated by combined activation of PKA and PKC. Similar to empirical data, STF was assessed by the ratio EPSP_n / EPSP_0 (Fig. 2B1), whereas maintenance of STF was assessed by the ratio of EPSP_n / EPSP_1 (Fig. 2B2). Initial facilitation in the simulations is illustrated in Fig. 2B1 (EPSP_1 / EPSP_0: Control, 73%; 5-HT, 144%; 5-HT + Met, 100%; 5-HT + Che, 105%). The maintenance of STF at EPSP_4 for the four groups (Fig. 2B2, EPSP_4 / EPSP_1: Control, 42%; 5-HT, 85%; 5-HT + Met, 87%; 5-HT + Che, 49%) indicated that initial STF in 5-HT was maintained at a level much greater than that of the control and this maintenance was due to the PKC-dependent mobilization process, but not the PKA-dependent process.

**PKC-dependent vesicle mobilization process could also account for the 5-HT-induced STF of depressed synapses.** Because PKC underlies the 5-HT-induced STF of depressed synapses (Ghirardi et al. 1992; Manseau et al. 2001), we next examined the extent to which the PKC-dependent vesicle mobilization process that was incorporated into the model to simulate the data of Fig. 1 could account for the facilitation of a highly depressed synapse.
We first performed experiments in which synapses were depressed by stimulating sensory neurons with a 0.05 Hz train of 8 stimuli in the presence of 100 μM methiothepin (Fig. 3). The EPSPs depressed significantly during the train with the amplitude of EPSP₄ reaching 33 ± 4%, of EPSP₁. Serotonin was applied immediately after EPSP₈ and 20 s later another 0.05 Hz train of 8 stimuli were elicited to examine the STF of EPSP₉ – EPSP₁₆ (n = 5, Fig. 3A). Application of 5-HT led to facilitation within 20 s, which peaked at EPSP₁₀ and then exhibited a re-emerging slow depression (EPSP₁₁ – EPSP₁₆). The filled circles in Fig. 3B illustrate results from a simulation using the same stimulus protocol and with parameters of the model identical to those used for the simulations in Fig. 2B. The fit between data and model (Fig. 3B) suggested that the same fundamental PKC-dependent process, β_{PKC}, and its kinetics, can account for the dynamics of 5-HT induced short-term facilitation of both nondepressed and depressed synapses.

The re-emergence of depression (EPSP₁₁ – EPSP₁₆) in the simulation was described by the inactivation time constant (~ 68 s) of the variable β_{PKC}, which suggested that β_{PKC} is largely inactivated in minutes. To test the decay of PKC-dependent processes, we examined the facilitatory effects of applying 5-HT starting 150 s after the application of 5-HT rather than at 20 s after treatment as we had done in the experiments of Fig. 1. This delayed testing procedure produced facilitation of the first EPSP (i.e., EPSP₁) elicited after the treatment, which was similar to that produced by eliciting EPSP₁ 20 s after treatment. However, the 5-HT-induced facilitation was not maintained indicating that the PKC-dependent component of the facilitation had inactivated. Moreover, in this case neither Met nor Che altered the decay of the 5-HT-
induced facilitation during EPSP<sub>1-8</sub>. Statistical analyses revealed no significant differences among the four groups at EPSP<sub>4</sub> (EPSP<sub>4</sub> / EPSP<sub>1</sub> ± SEM: Control, 47 ± 4%, n = 4; 5-HT<sub>150</sub>, 52 ± 6%, n = 3; 5-HT<sub>150</sub> + Met, 55 ± 5%, n = 6; 5-HT<sub>150</sub> + Che, 53 ± 4%, n = 5; one-way ANOVA, F<sub>(3,17)</sub> = 0.60, p = 0.62). These data provide additional support that the 5-HT-induced activation of PKC decreases with time and has a time constant of decay < 100 s.

Dynamics of PKC- and PKA-dependent processes in the model. The simulated dynamics of PKC- and PKA-dependent processes were examined (Fig. 4) in the following four conditions: 1) no 5-HT (Fig. 4A1, Control); 2) 5-HT application prior to the first stimulus (Fig. 4A2, 5-HT); 3) 5-HT application prior to the first stimulus with the PKA cascade disabled (Fig. 4A3, 5-HT + Met); and 4) 5-HT application prior to the first stimulus with the PKC cascade disabled (Fig. 4A4, 5-HT + Che). Each stimulus of a train of 8 stimuli at 0.05 Hz triggered a transient Ca<sup>2+</sup> current influx (Fig. 4A1, upward deflections in the I<sub>Ca</sub> trace; also see Eqs. 1-4, Gingrich and Byrne 1985). Transmitter released during each stimulus produced a rapid depletion of vesicles in the RRP (Fig. 4A, downward deflections in the C<sub>RRP</sub> traces). As C<sub>RRP</sub> decreased, mobilization was initiated, in part, because of the difference: (C<sub>RRPmax</sub> - C<sub>RRP</sub>) (see Eq. 2). The sum of the four mobilization fluxes was represented by I<sub>Replenish</sub>. The activation of PKC-dependent processes was illustrated by the β<sub>PKC</sub> traces. The activation of PKA-dependent processes was simulated by increasing the duration of the simulated action potential from 3 ms to 4.4 ms, which led to a greater Ca<sup>2+</sup> influx (Fig. 4A, insets). The effect of 5-HT in the absence of inhibitors was simulated by activating both PKA- and PKC-dependent processes (Fig. 4A2). In Fig. 4, the time courses of I<sub>Replenish</sub> exhibit abrupt,
brief spikes. These spikes are due to the rapid kinetics of the $\text{Ca}^{2+}$-activated mobilization flux $I_{F1}$ (Eq. 1 and Gingrich and Byrne 1985). The PKC-activated flux, $I_{PKC}$ (Eq. 2), is of much lower amplitude but is sustained throughout the duration of PKC activity. Because the $I_{F1}$ spikes are brief, $I_{PKC}$ actually accounts for a greater proportion of the total, time-integrated mobilization.

To compare the synaptic release across the four simulated conditions, the number of vesicles released by the first stimulus (initial release) was normalized to the upper bound of the RRP (Fig. 4B, blue bars). For the first stimulus, the normalized values were: Control, 0.37; 5-HT, 0.54; 5-HT + Met, 0.37; and 5-HT + Che, 0.54. Note that PKC-dependent vesicle mobilization (Fig. 4A3, 5-HT + Met) did not contribute to the initial synaptic release of nondepressed synapses compared with control simulation (Fig. 4A1, Control) (initial release = 0.37 in both cases) because the first stimulus (the equivalent of EPSP0 in Figs. 1 and 2) occurred at a time when the model was in its initial state with the RRP fully occupied (Fig. 4A1, 4A3, $C_{RRP}$). In contrast, PKA-dependent processes substantially increased the initial release because of the greater $\text{Ca}^{2+}$ influx (Fig. 4A2, 4A4, insets) (initial releases were increased to 0.54). This feature of the model is consistent with the observation that PKC-dependent processes have little effect on the STF of initial synaptic release of nondepressed synapses, whereas PKA-dependent processes play a dominant role in the STF of initial synaptic release of nondepressed synapses (Ghirardi et al. 1992; Jin et al. 2011; Khoutorsky and Spira 2005). To compare the initial recovery after the first release event across the four simulated conditions, the accumulated number of vesicles replenished between the first and second stimulus was normalized to the upper bound of the RRP (Fig. 4B, red bars).
The values for the normalized initial recovery were: Control, 0.03; 5-HT, 0.48; 5-HT + Met, 0.31; and 5-HT + Che, 0.11. After synaptic release in the control simulation (Fig. 4A1), the recovery of RRP was relatively slow (initial recovery = 0.03; Fig. 4B, Control). In contrast, the recovery of RRP was faster when $\beta_{PKC}$ was activated (Fig. 4A3, 5-HT + Met, initial recovery = 0.31; Fig. 4B, 5-HT + Met). Therefore, PKC-dependent mobilization contributed to the maintenance of STF of the second and subsequent EPSPs, as mobilization partially compensated for depletion of the RRP (see also Fig. 1B2, 2B2, green traces). Note that PKA-dependent processes also enhanced the vesicle mobilization (Fig. 4A4, 5-HT + Che, initial recovery = 0.11; Fig. 4B, 5-HT + Che). However, the PKA-dependent processes have, overall, little effect on the maintenance of STF because PKA activation also leads to greater synaptic release, which tends to cancel out the enhanced mobilization (see also Fig. 1B2, 2B2, blue traces).

**PKC enhanced the refilling of the RRP.** The model assumes that PKC enhances vesicle mobilization from the RP to the RRP. To test this assumption, we examined whether PKC enhanced the refilling of the RRP following its depletion by a hypertonic sucrose solution. Zhao and Klein (2002) found that the application of 1 M sucrose to SN-MN co-cultures led to an asynchronous release, which depleted the RRP within 1 min. After a 10-min wash, a second application of hypertonic solution indicated that the size of the RRP, which was measured by the accumulated asynchronous release over 1 min, was restored to ~80% of its initial level. Zhao and Klein (2002) also found that 5-HT increases asynchronous release from depressed synapses and speeds the refilling of the RRP. We hypothesized that this refilling process would be PKC dependent.
To examine the refilling process, we applied sucrose with a shorter interval (5 min, Fig. 5A1) so that the RRP would still be substantially depleted in control conditions. Sample responses are illustrated in Fig. 5A2. The percentage of recovery was measured by the ratio of the size of the RRP after the second sucrose application relative to the size after the first application. Control experiments in the absence of 5-HT showed that the percentage of recovery was 30 ± 4% (Fig. 5B, C1), whereas when 5-HT was applied 1 min before the second application of sucrose, the percentage of recovery was significantly increased to 95 ± 9% (Fig. 5B, C1; overall effects: \( F_{(2,12)} = 22.2, P < 0.001; \) Control vs. 5-HT; \( q_3 = 8.41, P < 0.001 \)). Similar to Zhao and Klein (2002, 2004), 5-HT did not increase the asynchronous response above its pre-depression level. Moreover, in the presence of 20 μM chelerythrine, the effect of 5-HT on the enhancement of the recovery of RRP was blocked (Fig. 5B, C1, blue bar) (Control vs 5-HT + Che, \( q_2 = 0.508, P = 0.91; \) 5-HT vs 5-HT + Che, \( q_2 = 7.90, P < 0.001 \)). These results suggested that PKC-dependent processes increased the rate of refilling of RRP depleted by sucrose.

To simulate the depletion of the RRP by the first application of sucrose, the size of the RRP (\( C_{RRP} \)) after the first application of sucrose was set to 0. No other parameters were changed from their values in Fig. 2. The percentage of recovery in the model was tracked by the ratio of \( C_{RRP} \) 5 min after the first application of sucrose to its maximum value (\( C_{RRP,Max} \)). The percentage of recovery of \( C_{RRP} \) in the control condition was 22%. When \( \beta_{PKC} \) was activated 1 min prior to the second application of sucrose, the percentage of recovery was increased to 99%. The good agreement between empirical results and the simulations of both synchronous (Figs. 2 and 3) and asynchronous
release (Fig. 5) indicated that the magnitude of PKC-dependent vesicle mobilization required to simulate the effects of 5-HT was in the physiological range.

*Contribution of PKC-dependent processes to maintenance of STF was dependent on stimulus frequency.* As illustrated in Fig. 1, PKC-dependent processes contributed to maintenance of STF of relatively nondepressed synapses when synapses were challenged with stimuli at 0.05 Hz (ISI = 20 s). We next examined whether the contribution of PKC-dependent processes to the maintenance of STF was dependent on stimulus frequency. We hypothesized that the contribution of PKC-dependent processes should increase as the testing frequency decreases because greater intervals between stimuli allow accumulation of more vesicles in the RRP. To examine this issue, EPSPs were evoked by paired-pulse stimuli at different interstimulus intervals (ISIs = 1, 5, 10, 20 s) in the presence or absence of 5-HT. The paired-pulse ratios (PPR, i.e., EPSP₂ / EPSP₁) were examined at these different ISIs (denoted PPR₁, PPR₅, PPR₁₀, and PPR₂₀). To the extent that depletion underlies synaptic depression, the PPR could be taken as a measure of depletion of the RRP produced by the first release. Moreover, an increase in PPR with time or treatment with 5-HT could be taken as a measure of recovery of the RRP and an indication of maintenance of STF. Experiments were performed in the presence of 100 μM methiothepin or 20 μM chelerythrine to separate the effects of PKA- and PKC-dependent processes. Sample recordings are illustrated in Fig. 6A and summary data are illustrated in Fig. 6B.

One-way ANOVA revealed that the PPR in the absence of 5-HT (Fig. 6B, black trace) was not a function of ISIs between 1 s and 20 s (PPR ± SE: PPR₁, 0.41 ± 0.03, n = 5; PPR₅, 0.39 ± 0.04, n = 6; PPR₁₀, 0.44 ± 0.04, n = 6; PPR₂₀, 0.44 ± 0.02, n = 6;
A possible explanation for the lack of effects of ISIs on the PPR is that the basal level of vesicle mobilization is small and does not make a significant contribution to the refilling of RRP within a period of 20 s. In the presence of 5-HT, which was applied 20 s prior to EPSP, a two-way ANOVA indicated significant overall differences in the PPRs among the three treatment groups (5-HT, 5-HT + Met, 5-HT + Che; $F_{(2,55)} = 15.9, P < 0.001$) and among the four ISIs ($F_{(3,55)} = 22, P < 0.001$) and no significant interaction between treatments and ISIs ($F_{(6,55)} = 1.81, P = 0.12$). In the 5-HT treatment group, PPRs increased as the ISI increased (Fig. 6B, red trace) (PPRs ± SE: PPR₁, 0.29 ± 0.07, n = 4; PPR₅, 0.58 ± 0.06, n = 5; PPR₁₀, 0.72 ± 0.08, n = 6; PPR₂₀, 0.84 ± 0.07, n = 6). Pair-wise post hoc comparisons indicated significant differences among the groups (PPR₁ vs. PPR₅, $q₂ = 4.2, P < 0.01$; PPR₂₀ vs. PPR₅, $q₃ = 3.9, P < 0.05$). The 5-HT-induced increase in the PPRs was also observed in the presence of methiothepin (Fig. 6B, 5-HT + Met, green trace; PPR₁ vs. PPR₅, $q₂ = 3.9, P < 0.01$; PPR₂₀ vs PPR₅, $q₃ = 4.1, p < 0.05$; PPR₂₀ vs. PPR₁₀, $q₂ = 2.9, p < 0.05$). However, the increase in PPRs was not observed in the presence of chelerythrine (Fig. 6B, 5-HT + Che, blue trace; PPR₁ vs. PPR₂₀, $q₄ = 3.43, P = 0.08$). Moreover, addition of chelerythrine reduced the value of PRP₂₀, (5-HT vs. 5-HT + Che, $q₂ = 4.1, P < 0.01$), whereas the addition of methiothepin did not (5-HT vs 5-HT + Met, $q₂ = 1.56 P = 0.27$). Therefore, because chelerythrine, but not methiothepin, blocked the time-dependent increase of the PPRs, these data suggested that a PKC-dependent process facilitates the enhancement of the PPR during the 20 s period. However, some time is necessary for this process to be expressed because no significant differences were observed
among the PPR at ISI = 1s (PPR₁ ± SE: 5-HT, 0.29 ± 0.07, n = 4; 5-HT + Che, 0.32 ± 0.07, n = 5; 5-HT + Met, 0.36 ± 0.03, n = 4).

Simulations of PPRs were performed to examine the extent to which the PKC-dependent vesicle mobilization in the model could account for these data (Fig. 6C). Because in this series of experiments the empirical PPR₂₀ (0.44 ± 0.02, n = 6) in the absence of 5-HT in Fig. 6B (control) was smaller than that in Fig. 1B₂ (0.59 ± 0.03, n = 5; i.e., EPSP₂ / EPSP₁ at ISI = 20s), a recalibration of the model was necessary so that the model and empirical PPR₂₀ were similar. This recalibration was accomplished by simply adjusting the amount of initial depletion by increasing the synaptic release probability (KR) from 2.27 to 4.27 (see Table 1). The simulations of the PPRs in Fig. 6B were performed with all other parameters unchanged. Consistent with the empirical observations, the simulations showed that varying the ISI had little effect on the PPR in control conditions (Fig. 6C, Control, black trace; PPR₁ = 0.42, PPR₅ = 0.44, PPR₁₀ = 0.44 and PPR₂₀ = 0.45). In the 5-HT treatment group, PPRs increased as the ISI increased (Fig. 6C, red trace; PPRs ± SE: PPR₁ = 0.38, PPR₅ = 0.61, PPR₁₀ = 0.76 and PPR₂₀ = 0.9). With activation of PKC alone (Fig. 6C, 5-HT + Met, green trace), simulations showed that although PKC-dependent vesicle mobilization made little contribution to the PPR at ISI = 1 s compared with the control condition (Control PPR₁ = 0.42 vs. 5HT + Met PPR₁ = 0.48), it enhanced the PPR at ISI = 20 s (Control PPR₂₀ = 0.45 vs. 5HT + Met PPR₂₀ = 0.91). With activation of PKA alone (Fig. 6C, 5-HT + Che, blue trace), simulations showed that ISI had little effect on PPRs (PPR₁ = 0.31, PPR₅ = 0.36, PPR₁₀ = 0.37 and PPR₂₀ = 0.38).
Given that, as the above data suggested, PKC made little contribution to the maintenance of STF of nondepressed synapses stimulated at ISI = 1 s, we predicted that 5-HT would also have little effect on EPSPs that were depressed by continuous 1 Hz stimuli. Several previous studies examined STF of depressed synapses (Braha et al. 1990; Dumitriu et al. 2006; Emptage et al. 1996; Ghirardi et al. 1992; Leal and Klein 2009; Pieroni and Byrne 1992; Stark and Carew 1999). However, these studies did not examine STF and its maintenance during continuous 1 Hz stimulation. To determine whether 5-HT could facilitate EPSPs during continuous 1 Hz stimulation, SNs were given 50 stimuli with an ISI of 1 s or 20 s. The latter ISI was used as a positive control. In each case, 5-HT was applied at the end of the twentieth stimulus (Fig. 7). Similar levels of depression were produced by stimuli with either ISI (Fig. 7B). As predicted, 5-HT application facilitated synapses that were stimulated with an ISI of 20 s (Fig. 7B, red trace), whereas 5-HT induced very little facilitation of synapses that were stimulated with an ISI of 1 s (Fig. 7B, black trace). These empirical results could be simulated with the model using the same stimulus protocols (Fig. 7C). Parameters were identical to those in Fig. 2. Simulation of accumulated synaptic release and accumulated synaptic recovery showed that the release and recovery after 5-HT treatment was rapidly accelerated after 5-HT treatment at ISI = 20 s (Fig. 7D, note the steep deflection after treatment of 5-HT) but not at ISI = 1 s (Fig. 7E). The model suggested that the failure of 5-HT to facilitate depressed synapses stimulated at 1 Hz was due to a mobilization process that was insufficient to refill the RRP when it was being continuously depleted by high rates of stimulation.
**DISCUSSION**

**Contributions of PKC to STF.** Previous studies (Ghirardi et al. 1992; Manseau et al. 2001; for review see Byrne and Kandel 1996) found that PKC-dependent processes contribute to STF at highly depressed synapses. The results of the present study indicated a role of these processes in maintenance of STF of initially nondepressed synapses, which was revealed when the synapse was challenged repeatedly after application of 5-HT. Interestingly, this contribution to the maintenance of STF was dependent on the frequency of testing. The PKC-dependent process failed to maintain STF if the frequency of firing was 1 Hz (Fig. 6, ISI = 1 s), whereas PKC-dependent maintenance of STF was observed at 0.2, 0.1 and 0.05 Hz (Fig. 6, ISIs = 5, 10, and 20 s). In addition, 5-HT failed to produce STF of depressed EPSPs if the firing rate was 1 Hz (Fig. 7). The model indicates that these results could be explained by the inability of a PKC-dependent mobilization process to refill the RRP at high rates of depletion.

Previous studies identified three major isoforms of PKC in *Aplysia*: a Ca\(^{2+}\)-dependent Apl I (PKC Apl I), a Ca\(^{2+}\)-independent Apl II (PKC Apl II), and an atypical Apl III (PKC Apl III) (Kruger et al. 1991; Sossin et al. 1993; Bougie et al. 2009). PKCs in *Aplysia* can be activated by conformational changes that expose the catalytic sites, or activated by cleavage of the link between the regulatory and the catalytic domain, which yields active PKC catalytic fragments, termed PKMs (for review see Sossin 2007). All three isoforms can be proteolyzed by calpain and generate the corresponding PKMs (PKM Apl I, PKM Apl II and PKM Apl III) (Sutton et al. 2004; Bougie et al. 2009).
We did not attempt to determine the specific isoform of PKC that is involved in the maintenance of STF. However, several lines of evidence suggest an involvement of PKM Apl II. Calpain inhibitors block the STF of depressed synapses (Khoutorsky and Spira 2006), indicating the involvement of a PKM. At low concentrations (< 30 µM), chelerythrine, the agent used in the present study, is an effective inhibitor of *Aplysia* isoforms of PKM, but not PKC (Sossin et al. 2011, Villarreal et al. 2009) presumably due to greater access to the substrate-binding pocket in the absence of the pseudosubstrate domain (for review see Sossin 2007). The dominant-negative form of PKC Apl II derived from mutations in its catalytic domain, but not the dominant-negative form of PKC Apl I, blocks 5-HT-induced STF of depressed synapses (Manseau et al. 2001), indicating the involvement of a PKC Apl II or PKM Apl II. Furthermore, bisindolylmaleimide-1 (Bis) (10 µM), a potent inhibitor of PKC Apl II (Villarreal et al. 2009), does not block STF of depressed synapses (unpublished observations), suggesting PKM Apl II rather than PKC Apl II underlies the 5-HT-induced STF. Finally, 5-HT induces the translocation of Apl II, but not Apl I, to the membrane where it is activated (Zhao et al. 2006). But it has not been possible to detect the activated PKM because the available antibody is not sufficiently sensitive to detect the endogenous PKMs in *Aplysia* (Bougie et al. 2009). Further studies are required to directly confirm the contribution of PKM Apl II to the 5-HT-induced maintenance of STF. These studies might require the use of siRNA techniques or the expression of dominant negative isoforms of PKMs.

Whereas the present results are consistent with PKM Apl II as the isoform underlying PKC-dependent maintenance of heterosynaptic (i.e., 5-HT-induced) STF, the
Ca^{2+}-dependent Apl I appears to be involved in a form of homosynaptic plasticity called burst-dependent protection from depression, in which homosynaptic depression of the sensorimotor synapse is reduced if the synapse is repeatedly challenged with bursts of 2-4 spikes rather than single spikes of the type used in the present study (Wan et al. 2012). Therefore, different isoforms of PKC may be used selectively for different forms of plasticity (homosynaptic vs. heterosynaptic) at the same synapse.

Mechanism of PKC-dependent processes contributing to STF. The PKC-dependent maintenance of STF is due at least in part to enhanced vesicle mobilization from the RP to the RRP (Byrne and Kandel 1996; Gingrich and Byrne 1985; Khoutorsky and Spira 2005). Perhaps the strongest evidence in support of this model comes from the use of sucrose to deplete the RRP. A single action potential depleted about 35% of the available vesicles in the RRP, which was restored after application of 5-HT (Zhao and Klein 2002, 2004). We extended these studies by finding that the refilling was dependent on PKC (Fig. 5). PKC-dependent phosphorylation of the synaptosome-associated protein of 25 kDa (SNAP-25) may be responsible for the increase of the refilling of the RRP (Houeland et al. 2007; Morgan et al. 2005; Nagy et al. 2002). In the sensorimotor synapse, PKC phosphorylates SNAP-25 at the C terminal Ser^{198}. Phosphomimetic mutants of Ser^{198} to Glu or Asp decrease rates of homosynaptic depression (Houeland et al. 2007). In contrast, non-phosphorylatable mutants of Ser^{198} to nonpolar Ala or Cys blocked increases in transmitter release due to PKC activation, but did not decrease the rate of depression (Houeland et al. 2007). These results suggest phosphorylation of SNAP-25 may contribute to the enhancement of vesicle mobilization.
Limitations of the model. In the present study, a simple depletion model (Gingrich and Byrne 1985), which assumes the existence of a homogenous RRP, was used to model homosynaptic depression. This model is likely an oversimplification because it cannot account for some additional features of homosynaptic plasticity (Jiang and Abrams 1998; Zhao and Klein 2002; Wan et al. 2012). A more complete model probably needs to include a heterogeneous population of vesicles (e.g., a two-pool model of the RRP; Zhou et al. 2009), which has been implicated in homosynaptic depression at other synapses (e.g., Sakaba and Neher 2001; Trommershauser et al. 2003; von Gersdorff and Borst 2002; Wu and Borst 1999). But despite the lack of a complete model of the mechanisms underlying homosynaptic depression, a relatively simple lumped parameter model of a PKC-dependent mobilization process, appears to capture the salient features of heterosynaptic facilitation and makes predictions that were validated experimentally.

The results of the present study indicate that PKC-dependent processes contribute to the maintenance of STF and that the view of a dichotomy between the preferential actions of PKA and PKC on facilitation of nondepressed and depressed synapses is probably an over-simplification. Both PKA and PKC contribute to the facilitation of nondepressed synapses, with the effects of PKC becoming important when a facilitated synapse is challenged with more than one release event. The relative contribution of PKC also depends on the rate at which the synapse is challenged. Consequently, the degree of heterosynaptic plasticity exhibited by a synapse is a complex function of its current release state and the dynamics of the multitude of kinases that are be engaged by modulatory transmitters. An additional level of
complexity is introduced when activity and associated activity-dependent kinases interact with those activated by modulatory transmitters.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
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Figure Legends

Fig. 1. Contribution of PKC to the maintenance of short-term facilitation (STF) at a nondepressed synapse.  
**A:** Representative traces of pre-test (EPSP\textsubscript{0}) and EPSPs elicited by eight repetitive stimuli at 0.05 Hz. A 5 min interval separated EPSP\textsubscript{0} and EPSP\textsubscript{1}. From left to right: EPSP\textsubscript{0}, EPSP\textsubscript{1}, EPSP\textsubscript{2}, EPSP\textsubscript{5}, EPSP\textsubscript{8}. Four treatment conditions were examined: 1) vehicle control; 5-HT applied 20 s prior to evoking EPSP\textsubscript{1} either 2) alone (5-HT); 3) in the presence of methiothepin (5-HT + Met), an inhibitor of adenylyl cyclase-coupled 5-HT receptors; or 4) in the presence of chelerythrine (5-HT + Che), a PKC inhibitor.  
**B1:** Summary data on the STF induced by 5-HT treatment. STF was examined by the normalized EPSP (EPSP\textsubscript{n} / EPSP\textsubscript{0}, where n denotes the n\textsuperscript{th} EPSP).  
**B2:** Summary data on the maintenance of STF induced by 5-HT treatment 20 s prior to evoking EPSP\textsubscript{1}.  

Fig. 2. Simulations of the maintenance of short-term facilitation (STF) of nondepressed synapses.  
**A:** Schematic of the modified Gingrich-Byrne (G-B) model, which was extended by incorporating PKC-dependent vesicle mobilization. In the original G-B model, vesicle mobilization from the reserve pool (RP) to the readily releasable pool (RRP) is regulated by three fluxes (\(I_{F1}\), \(I_{S1}\), \(I_{VD1}\), see Material and Methods). PKC-dependent vesicle mobilization was incorporated by adding a new flux (\(I_{PKC}\)). PKA-dependent processes enhanced Ca\textsuperscript{2+} influx which in turn regulated both Ca\textsuperscript{2+}-dependent fluxes of vesicle mobilization (\(I_{F1}\) and \(I_{S1}\)) and exocytosis (\(I_{ER}\)). The RP is refilled by a flux of vesicle \(I_{IN}\). Lines with arrowheads indicate positive interactions or activation, whereas lines with filled circles indicate negative interactions or inhibition.  
**B1:** Simulations of 5-HT (or vehicle control) treatment applied 20 s prior to EPSP\textsubscript{1}: 1) vehicle control; 2) 5-HT simulated by combined activation of PKA and PKC; 3) 5-HT with the PKA cascade disabled to mimic the effects of methiothepin (5-HT + Met) (i.e., simulations performed with contribution of PKC alone); or 4) 5-HT with the PKC cascade disabled to mimic the effects of chelerythrine (5-HT + Che) (i.e., simulations performed with contributions of PKA alone).  
**B2:** Simulation of the maintenance of STF induced by 5-HT treatment 20 s prior to EPSP\textsubscript{1} in the presence or absence of inhibitors.
Maintenance of STF of nondepressed synapses was quantified by the magnitude of the re-normalized EPSP (EPSP\(_n\) / EPSP\(_1\)).

**Fig. 3. Empirical analysis and simulation of the effects of PKC on STF of depressed synapses.** A: Representative recordings of STF of depressed synapses. Synaptic depression was elicited with a 0.05 Hz train of eight stimuli in the presence of 100 μM methiothepin (EPSP\(_1\) - EPSP\(_8\)). 5-HT was applied immediately after EPSP\(_8\) and another 0.05 Hz train of 8 stimuli were delivered to examine STF of EPSP\(_9\) – EPSP\(_{16}\). B: Comparison of empirical data and simulations. Green squares indicate means of empirical data (n = 5). Agreement between model (filled circles) and data suggested that the same fundamental PKC-dependent process could account for the dynamics of 5-HT-induced STF of both depressed and nondepressed synapses.

**Fig. 4. Dynamics of PKC- and PKA- dependent processes in the maintenance of STF of nondepressed synapse.** A1-A4, Model predictions of maintenance of STF using the same stimulus protocol as Fig. 1. A1: non-5-HT treated control group (control). Traces from top to bottom are: Ca\(^{2+}\) influx (\(I_{Ca}\)) triggered by the repetitive stimuli; concentration of vesicles in RRP (\(C_{RRP}\)); total vesicle mobilization from RP to RRP (\(I_{Replenish}\); see Eq. 1); contribution of PKC-dependent process to vesicle mobilization (\(\beta_{PKC}\)). A2: 5-HT application 20 s prior to the EPSP\(_1\) without disabling either the PKA or PKC cascades (5-HT). Effects of PKA on the Ca\(^{2+}\) influx are highlighted in the inset. A3: 5-HT application 20 s prior to EPSP\(_1\) with the PKA cascade disabled to simulate treatment with a PKA cascade inhibitor (5-HT + Met). A4, 5-HT application 20 s prior to EPSP\(_1\) with the PKC cascade disabled to simulate treatment with a PKC cascade inhibitor (5-HT + Che). B: Normalized release of the first EPSP (initial release) and recovery between first and the second EPSPs (initial recovery) in the above four simulated conditions. Initial release (blue bars) and initial recovery (red bars) in each of the four simulations were normalized to the upper bound of the RRP (see text for details).

**Fig. 5. PKC-dependent process enhanced the recovery of the RRP.** A1: Schematic of the experimental protocol in the control condition (top), 5-HT alone...
(middle), and 5-HT in the presence of the PKC inhibitor chelerythrine. Vehicle 1 (H₂O) or chelerythrine was applied 15 min before the first treatment of hypertonic solution sucrose. Vehicle 2 (L15-ASW) or 5-HT was applied 1 min before the second treatment of hypertonic solution. **A2**: Representative traces of the asynchronous release between 5 and 7.5 s after the treatment with hypertonic solution in the control condition. Top trace, first treatment of hypertonic solution; bottom trace: second treatment. Note the decrement of asynchronous release triggered by the second treatment of hypertonic solution. **B1-B3**: Percentage of recovery of the RRP in the control condition (**B1**), 5-HT alone (**B2**) and 5-HT + chelerythrine (**B3**). Ratio of the size of RRP of second treatment and first treatment of hypertonic solution indicated the recovery of the RRP. Dotted lines represent individual experiments and solid lines represent the average of all experiments. **C1**: Summary data. 5-HT alone enhanced the recovery of the RRP, which was blocked by the PKC inhibitor. **C2**: Simulation of the PKC-dependent recovery of RRP. Simulations indicated that PKC-dependent vesicle mobilization could account for the 5-HT-enhanced recovery of RRP.

**Fig. 6. Contribution of PKC-dependent processes to the maintenance of STF was dependent on stimulus frequency.** **A**: Representative EPSPs elicited by paired-pulse stimuli in four conditions (Control, black traces; 5-HT, red traces; 5-HT + Chelerythrine, blue traces; 5-HT + Methiothepin, green traces) and at two different interstimulus intervals (ISIs = 1, and 20 s). Although not shown, ISIs of 5 s and 10 s were also tested. **B**: Summary data. In the control condition, the PPR was stable despite the increases in ISI, suggesting that the basal level of recovery of the RRP was small. 5-HT increased the PPR compared with control at ISI = 5, 10 and 20 s. The effect was blocked by chelerythrine, but not methiothepin, suggesting that PKC enhanced the recovery of RRP and contributed to the maintenance of STF. In addition, 5-HT had little effect on the PPR at ISI = 1 s compared with control, suggesting that the contribution of PKC to maintenance of STF is dependent on stimulus frequency. **C**: Simulations indicated that PKC-dependent vesicle mobilization could account for the 5-HT-induced maintenance of STF at different stimulus frequencies.
Fig. 7. 5-HT-induced STF of depressed synapses was dependent on the stimulus frequency. **A:** Representative traces of the experiment at an ISI of 1 s (top) and an ISI of 20 s (bottom). 5-HT was added at the end of the 20th stimulus. **B:** Summary data. After 20 stimuli, synapses were depressed to the same level at both ISIs. However, 5-HT application only facilitated synapses that were stimulated with an ISI of 20 s. **C:** Simulation of the facilitation of depressed synapses. Simulations indicated that PKC-dependent vesicle mobilization is insufficient to overcome the depletion associated with the high frequency stimulation (ISI = 1s). **D, E:** Simulation of accumulated release and recovery at ISI = 20 s (**D**) and ISI = 1 s (**E**). Simulations indicated that the release and recovery after 5-HT treatment was rapidly accelerated after 5-HT treatment at ISI = 20 s (**D**), but not at ISI = 1 s (**E**).

**Table 1. Original and new parameters in the model.** Numbers in parenthesis adjacent to each parameter represent the equation number from Gingrich and Byrne (1985) or from the present paper for the equations on the PKC-dependent vesicle mobilization. NC refers to no change from original G-B model. Bold fonts highlight the new values.
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<th>New value</th>
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<tr>
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Figure 1

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B1

\[ \frac{\text{EPSP}_n}{\text{EPSP}_0} \]

B2

\[ \frac{\text{EPSP}_n}{\text{EPSP}_1} \]
Figure 2

A

B1

B2

EPSP_n

EPSP_n

control
S-HT
S-HT + Met
S-HT + Che
Figure 4

A1 Control

- $I_{Ca}$
- $C_{RRP}$
- $I_{Replenish}$
- $\beta_{PKC}$

A2 5-HT

- $I_{Ca}^{ctrl}$
- $PKA$
- $I_{Ca}$
- $C_{RRP}$
- $I_{Replenish}$
- $\beta_{PKC}$

A3 5-HT + Met

- $I_{Ca}$
- $PKA$
- $I_{Replenish}$
- $\beta_{PKC}$

A4 5-HT + Che

- $I_{Ca}^{ctrl}$
- $PKA$
- $I_{Replenish}$
- $\beta_{PKC}$

0 50 100 150 200

B

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# of Vesicles (Normalized to $C_{RRP \max}$)