A novel function for serotonin-mediated short-term facilitation in *Aplysia*: Conversion of a transient, cell-wide homosynaptic Hebbian plasticity into a persistent, protein synthesis-independent synapse-specific enhancement

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Studies of sensitization and classical conditioning of the gill-withdrawal reflex in *Aplysia* have shown that the synaptic connections between identified glutamatergic sensory neurons and motor neurons can be enhanced in one of two ways: by a heterosynaptic (modulatory input-dependent) mechanism that gives rise with repetition to long-term facilitation and by a homosynaptic (activity-dependent) mechanism that gives rise with repetition to a facilitation that is partially blocked by 2-amino-5-phosphonovaleric acid and by injection of 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate (BAPTA) into the postsynaptic cell and is similar to long-term potentiation in the hippocampus. We here have examined how these two forms of facilitation interact at the level of an individual synaptic connection by using a culture preparation consisting of a single bifurcated sensory neuron that forms independent synaptic contacts with each of two spatially separated motor neurons. We find that the homosynaptic facilitation produced by a train of action potentials is cell wide and is evident at all of the terminals of the sensory neuron. By contrast, the heterosynaptic facilitation mediated by the modulatory transmitter serotonin (5-HT) can operate at the level of a single synapse. Homosynaptic activation gives rise to only a transient facilitation lasting a few hours, even when repeated in a spaced manner. The heterosynaptic facilitation produced by a single pulse of 5-HT, applied to one terminal of the sensory neuron, also lasts only minutes. However, when one or more homosynaptic trains of spike activity are paired with even a single pulse of 5-HT applied to one of the two branches of the sensory neuron, the combined actions lead to a selective enhancement in synaptic strength only at the 5-HT-treated branch that now lasts more than a day, and thus amplifies, by more than 20-fold, the duration of the individually produced homo- and heterosynaptic facilitation. This form of synapse-specific facilitation has unusual long-term properties. It does not require protein synthesis, nor is it accompanied by synaptic growth.

The gill- and siphon-withdrawal reflex of *Aplysia* has proven a useful model system for studying the cellular and molecular basis of simple forms of learning and memory (1–4). The molecular mechanisms of memory storage have been particularly well studied in the context of sensitization, an elementary form of nonassociative learning in which an animal learns to strengthen its reflex responses to previously neutral stimuli after the presentation of an aversive stimulus. As is the case for other defensive withdrawal reflexes, the behavioral memory for sensitization of the gill- and siphon-withdrawal reflex is graded, and the duration of the memory is a function of the number of training trials. A single stimulus to the tail gives rise to short-term sensitization lasting minutes to hours. Repetition of the stimulus produces long-term behavioral sensitization that can last days to weeks (5, 6). The memory for both the short- and long-term forms of sensitization is represented on an elementary level by monosynaptic connections between identified mechanoreceptor sensory neurons and their follower cells. This monosynaptic pathway can be examined not only in the intact animal but also in a microculture consisting of a single sensory neuron and a single motor neuron (7). In this culture system, one brief pulse of serotonin (5-HT), a modulatory neurotransmitter normally released by sensitizing stimuli in the intact animal, produces a presynaptic increase in the strength of the synaptic connections between the sensory and motor cell that lasts minutes. This short-term facilitation, which accompanies short-term behavioral sensitization, is induced in part by increases in cAMP and the consequent activation of the cAMP-dependent protein kinase (PKA), as well as by activation of protein kinase C, leading to the covalent modifications of preexisting proteins that result in an enhancement of transmitter release (8–14). By contrast, five spaced applications of 5-HT designed to simulate the spaced training required to produce long-term behavioral sensitization lead to the recruitment of PKA and mitogen-activated protein kinase, and they both translocate to the nucleus. In the nucleus, these two kinases activate the transcription factor CREB (the *c*yclic AMP *r*esponse *e*lement-*b*inding *p*rotein), which in turn recruits a cascade of genes that leads to the growth of new synaptic contacts between the sensory and motor neuron and to a facilitation of synaptic strength that persists for days (15–24). One conclusion that emerged from these studies is that, even in the absence of activity in the presynaptic sensory neuron, the repeated presentation of a heterosynaptic modulatory input or the repeated presentation of modulatory transmitter on its own has the capability of activating transcription leading to the growth of new synaptic connections and to persistent changes in synaptic strength.

Martin *et al.* (25) extended this analysis of sensitization to examine how the long-term process initiated by a modulatory input becomes restricted to individual synaptic terminals of a sensory neuron (25–27). Toward this end, they cultured a single sensory neuron with bifurcated axonal branches with two spatially separated motor cells and found that when five brief pulses of 5-HT are applied to one branch of a bifurcated sensory neuron, that branch and not the other will undergo structural changes and a selective long-term enhancement in synaptic strength. This synapse-specific,

Abbreviations: 5-HT, serotonin; LTP, long-term potentiation; EPSP, excitatory postsynaptic potential; CREB, cAMP response element-binding protein; PKA, cAMP-dependent protein kinase; LTF, long-term facilitation.

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long-term facilitation (LTF) and the accompanying structural change can be captured at the second branch by the application to that branch of a single brief pulse of 5-HT.

In contrast to sensitization, classical conditioning in *Aplysia* recruits, in addition to heterosynaptic facilitation, a homosynaptic facilitation that resembles long-term potentiation (LTP) [refs. 28–30; I. Antonov, E.R.K. & R. D. Hawkins (2000) *Soc. Neurosci. Abstr.*, in press]. These several findings have raised the questions: Can homosynaptic facilitation be synapse specific? Can it, by itself, lead to persistence and to growth? How does this homosynaptic facilitation interact quantitatively with a short-term heterosynaptic process initiated by 5-HT?

These questions are important from a behavioral perspective for understanding the relationships between nonassociative forms of long-lasting synaptic plasticity, like those that accompany sensitization, and associative forms, like those that accompany classical conditioning. Yet despite their behavioral importance, these questions have not been addressed directly on the level of individual synaptic connections, because there has not been until recently an appropriate cellular system for exploring long-term changes in either the strength or the structure of the different synaptic terminals of an individual neuron. The recent development of the bifurcated sensory neuron–two-motor neuron culture in *Aplysia* now provides an ideal system for examining the interaction of homo- and heterosynaptic mechanisms at the level of individual synaptic terminals.

Using this divergent culture system, we have found that homosynaptic tetanic activation of the presynaptic glutamatergic sensory neuron results in a cell-wide facilitation that is transient and lasts only 1 or 2 h, even in response to 4 repeated tetanic trains. By contrast, when these tetanic trains of homosynaptic spike activity in the sensory neuron are combined with the spatially restricted application of just a single pulse of 5-HT to one of the two branches of the bifurcated sensory neuron, there is a selective enhancement in the duration of the facilitation that now lasts more than 24 h, and that is restricted in its expression to the 5-HT-treated branch. Thus, the combination of short-term homo- and heterosynaptic mechanisms enhances, in a nonadditive fashion, the duration of the facilitation elicited by either mechanism alone. This form of long-lasting synapse-specific plasticity has novel properties in that it does not require protein synthesis and is not accompanied by synaptic growth.

Materials and Methods

***Aplysia* Cell Culture.** Culture dishes and medium were prepared as previously described (15). Bifurcated sensory neuron–motor neuron cultures were prepared as described in Martin *et al.* (25). Cultures were maintained for 5 days in an 18°C incubator.

Electrophysiology and Induction of Facilitation. After 5 days in culture, the strength of the synaptic connection between the sensory neuron and each of the motor neurons was tested. Each motor cell was impaled with a recording microelectrode (8–10 mΩ) containing 2.5 M KCl and held at a potential of −30 mV below its resting potential. Excitatory postsynaptic potentials (EPSPs) were evoked in both L7 motor neurons by stimulating the sensory cell with a brief depolarizing pulse by using an extracellular microelectrode. Cultures in which the initial EPSP amplitude was less than 4 mV were not used. When the posttreatment EPSP was a spike, a value of 60 mV was used for quantitation.

Homosynaptic LTP was achieved by stimulating the cell body of the sensory neuron with an extracellular electrode filled with recording medium and coated with silver paint to allow concentric bipolar stimulation. Cells were given either a single tetanus (20 Hz for 2 sec) or a series of 4 such tetani given at 10-min intervals. Stimulus intensity was increased 20% above that used to evoke the previous EPSP, which in pilot experiments ensured one-to-one EPSPs during the tetanus. To examine the contribution of homo- and heterosynaptic mechanisms to the total facilitation, we used paired training experiments. In these experiments, stimulation (either one or four tetani) was paired with the focal application of either a single episode of 5-HT (consisting of five 5-s low-pressure 1 psi puffs of 100 μM 5-HT in L15 containing 0.05% fast green and delivered to the synaptic region) or five separate episodes of 5-HT perfusion (each episode consisting of five 5-sec pulses at 10-s intervals) given at 10-min intervals. The bath was continuously perfused with 50% L15/50% artificial sea water (Instant Ocean) at a rate of approximately 0.5 ml per minute. The 5-HT and bath perfusions were adjusted so that the puffs of neurotransmitter (visualized by the fast green) selectively covered only the region of synaptic contact that the sensory neuron makes with the motor neuron (25, 26). The 5-HT treatments began 0.5 sec after the onset of the tetanizing stimulation with one-to-one pairing and approximately in the middle of pairing with four tetani. To measure short-term facilitation, EPSPs were measured in both motor neurons 10 min after the end of the 5-HT application; to measure LTF, EPSPs were recorded 24 h after 5-HT application. In time-course experiments, EPSPs were measured at the indicated time points. All data were recorded and analyzed with a computer by using AXOSCOPE software (Axon Instruments, Foster City, CA).

Treatments. *Protein synthesis inhibitor.* To examine the effect of blocking protein synthesis on synapse-specific activity-dependent LTF, anisomycin (10 μM; Sigma) was added to the cultures 30 min before the training and was present continuously during, and until 30 min after the end of, the training.

Dye injection, cell imaging, and quantification of structural changes. Dye injection, imaging, and analysis were done as previously described (18).

Analyses of data. All data are presented as mean percentage change ± SEM in the EPSP amplitude measured after treatment, as compared with its initial pretreatment amplitude. The significance of the EPSP changes was determined by using an unpaired Student's *t* test.

Results

Homosynaptic Activation Produces a Facilitation Similar to LTP That Is Cell Wide and Transient. To examine the duration of the facilitation elicited by homosynaptic activation alone, we first applied homosynaptic trains of stimuli to the cell body of the sensory neuron and measured the amplitude of the evoked glutamatergic EPSP at its synaptic contacts with each motor neuron at different time points after stimulation. We found that a single tetanus (20 Hz for 2 sec) produced a cell-wide facilitation of each connection between the sensory and motor neuron. This facilitation lasted approximately 1 h. At its peak, 10 min after the tetanus, the facilitation was 199.9 ± 52.1% at one branch and 181.6 ± 47.9% at the other branch ($n = 6$). The facilitation then gradually declined at 1 h to 55.2 ± 13.5% and 49.8 ± 19.9% ($n = 6$). At 2 h, no enhancement in synaptic strength was detectable (−0.9 ± 19.9% and −5 ± 9.4%, $n = 4$, Fig. 1*A*).

Increasing the number of tetani from one to four (each separated by 10-min intervals) produced less rather than more facilitation and did not prolong the facilitation. Ten minutes after the application of four tetani, the facilitation was only 51.4 ± 9.3% ($n = 4$); this declined to 36.5 ± 21.5% ($n = 4$) at 1 h and was back to baseline at 2 h (6.6 ± 14.2%, $n = 5$, Fig. 1*B*). With both one and four trains there was no enhancement in synaptic strength at 24 h (−4.9 ± 9.2% vs. 7.7 ± 25.1%, $n = 10$ and 4.8 ± 9.8% vs. 5.3 ± 12.4%, $n = 12$), compared with unstimulated control cells (−1.1 ± 10.7% and 7.7 ± 4.5%, $n = 6$, Figs. 1 *A* and *B* and 2*B*).

This synapse is glutamatergic (31), and homosynaptic LTP to repeated trains has been shown to depend on activation of an NMDA-type receptor (28). Even the potentiation to a single train is blocked by injecting BAPTA into the postsynaptic cell

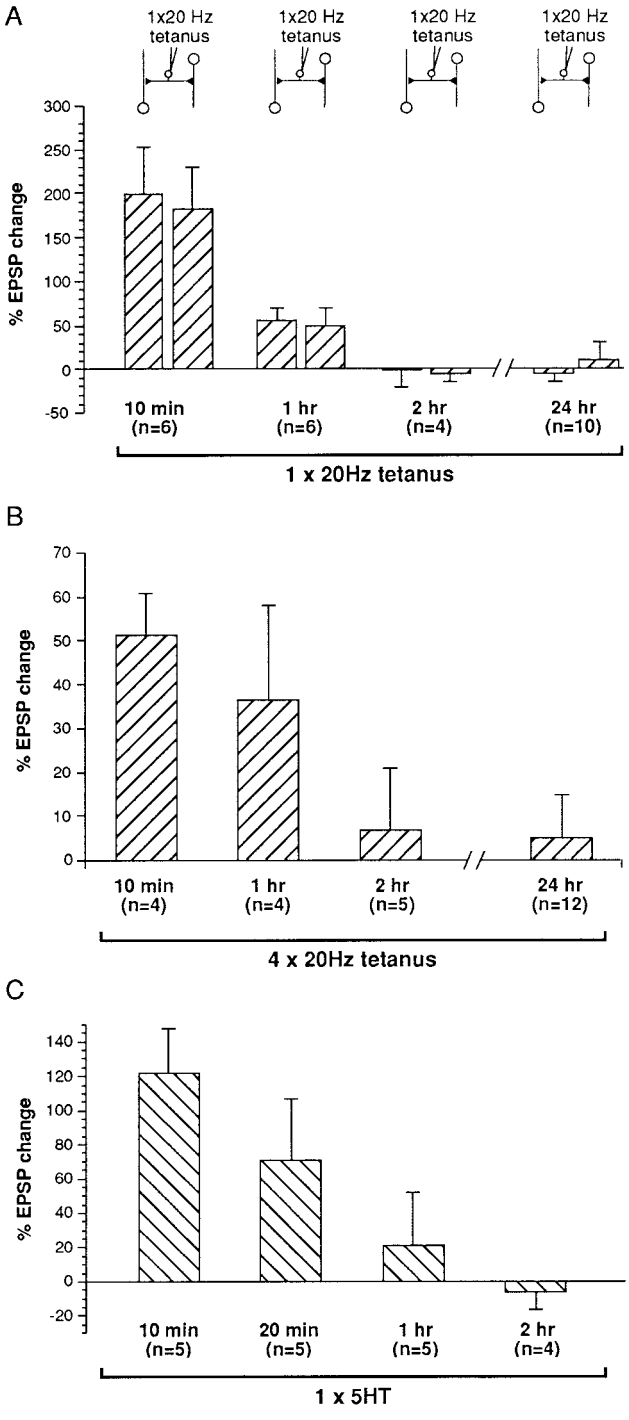

Fig. 1. Homosynaptic activation produces a facilitation that is cell wide and transient. (*A*) A single tetanus (20 Hz for 2 sec) applied to the soma of the sensory neuron produces a cell-wide increase in the amplitude of the evoked EPSP recorded in both postsynaptic motor neurons that peaks at 10 min. This homosynaptic facilitation was transient: the increase in the EPSP amplitude was greatly reduced 1 h after training, returned to baseline by 2 h, and no facilitation was present at 24 h. (*B*) As was the case with a single tetanus, the increase in the EPSP amplitude at sensory-to motor-neuron connections evoked by 4 trains lasted about 1 h and was no longer present at 2 and 24 h. (*C*) Application of a single pulse of the modulatory neurotransmitter 5-HT produced only short-term facilitation. The amplitude of the EPSP peaked at 10 min and decayed rapidly, returning to baseline at 1 h. The data in *A* and the 24-h time point in *B* were taken from bifurcated cultures. The remaining data points in *B* and *C* were taken from regular cocultures. No differences were found in the magnitude of response of each type of culture to homo- or heterosynaptic stimulation.

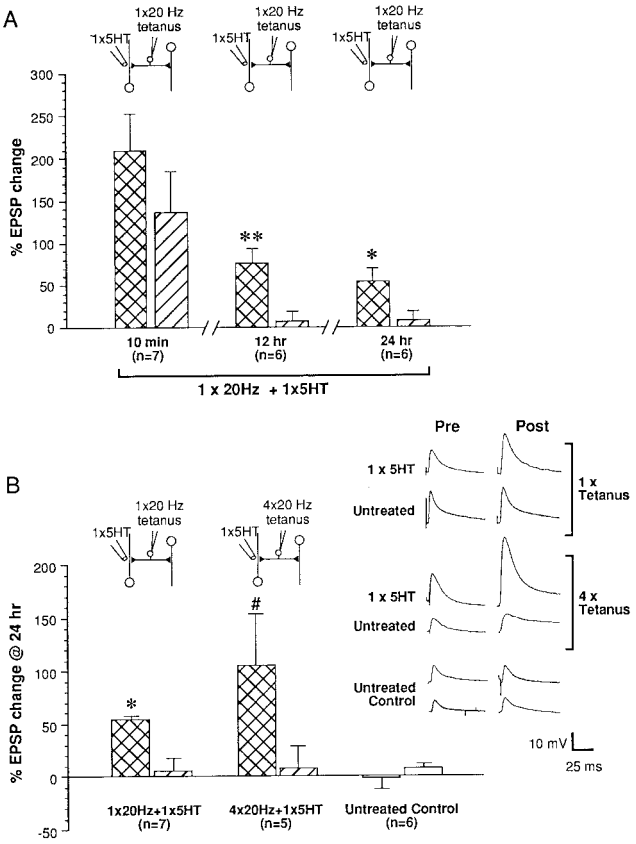

Fig. 2. A single heterosynaptic modulatory input enhances the duration of cell-wide homosynaptic facilitation in a synapse-specific way. (*A*) The time course of the LTF produced by pairing a single homosynaptic tetanus to the cell body with a single pulse of 5-HT to the synapse is shown. Combined homo- and heterosynaptic activation produces robust synaptic facilitation that peaks at 10 min. This facilitation persists only at the branch treated with 5-HT and remains stable from 12 h after training (**, $P < 0.01$ change in EPSP amplitude in 5-HT-treated vs. untreated branch) up to at least 24 h (*, $P < 0.05$ change in EPSP amplitude in 5-HT-treated vs. untreated branch). (*B*) The combination of a single homosynaptic tetanus with a single heterosynaptic pulse of 5-HT produced a long-term enhancement in the amplitude of the EPSP that lasted at least 24 h. This increase in synaptic strength was restricted in its expression to the 5-HT-treated branch compared with the untreated branch (*, $P < 0.05$). Increasing the number of tetani from one to four also produced an increase in the magnitude of the LTF expressed at the 5-HT-treated branch compared with the untreated branch (#, $P < 0.1$). In control experiments, untreated cells showed no change in the EPSP amplitude at 24 h. Shown are representative recordings of EPSPs and histograms of the mean change in EPSP amplitude ± SEM. The histograms for the 4 × 20 Hz + 1 × 5-HT illustrated here are taken from the experiments described below in Fig. 3.

(30, 32) and has many properties similar to LTP in the Schaffer collateral pathway of the hippocampus.

A Single Heterosynaptic Modulatory Input Produces Facilitation That Lasts Less Than 1 Hour. In contrast to the transience of cell-wide facilitation induced by homosynaptic activation, heterosynaptic facilitation produced by 5-HT can give rise to either transient or persistent changes depending on the number of repetitions. Thus, whereas one pulse of 5-HT gives rise to transient changes lasting minutes (22, 25), five repeated pulses of 5-HT give rise to persistent changes that last more than 24 h (25, 26). We have focused here on the effects of a single pulse. Ten minutes after the application of a single pulse of 5-HT, the facilitation was 121.9 ± 25.4% ($n = 5$). This declined to 70.7 ± 36% ($n = 5$) at 20 min and 21.4 ± 30.7% ($n = 5$) at 1 h. At 2 h, no enhancement

in synaptic strength was detectable ($-6.1 \pm 10.5\%$, $n = 4$, Fig. 1*C*).

A Single Heterosynaptic Modulatory Input Enhances the Duration of Cell-Wide Homosynaptic Facilitation in a Nonadditive Way. How do these homo- and heterosynaptic processes interact? We have found that the combination of spike activity in the presynaptic sensory neuron with the spatially restricted application of a single pulse of 5-HT to one of the two branches of the bifurcated sensory neuron–two-motor neuron culture preparation leads to a selective enhancement in the amplitude of the evoked EPSP only at the 5-HT-treated branch that can last at least 24 h. The combination of a single homosynaptic tetanus with a single heterosynaptic pulse of 5-HT has two consequences (Fig. 2). First, it prolongs the facilitation so that now there is an increase of synaptic strength ($54 \pm 2.9\%$, $n = 7$) that lasts 24 h. Second, this enhancement in the amplitude of the evoked EPSP is restricted to the treated branch with no change in the EPSP at the untreated branch ($5.4 \pm 13.1\%$, $n = 7$) (Fig. 2*B*). At this restricted branch, the facilitation produced by combining a single pulse of 5-HT with a single homosynaptic tetanus to the cell body is $209.9 \pm 43.5\%$ ($n = 7$) at 10 min after paired stimulation vs. $136.4 \pm 47.9\%$ ($n = 7$) at the untreated branch and declines to $75.8 \pm 16.9\%$ vs. $7.1 \pm 11.9\%$ ($n = 6$) at 12 h and $54.3 \pm 15.6\%$ vs. $7.7 \pm 11.2\%$ ($n = 6$) at 24 h (Fig. 2*A*).

Moreover, when four electrical tetani, which produce only a modest and transient cell-wide facilitation on their own, are paired with a single pulse of 5-HT, they produce an even greater enhancement in LTF than that seen when a single tetanus is paired with a single pulse of 5-HT. This facilitation is $106 \pm 48\%$ ($n = 5$) at the treated branch compared with the untreated branch ($7.7 \pm 20.8\%$, $n = 5$) and also persists at least for 24 h (Fig. 2*B*). These results indicate that a single heterosynaptic stimulus at one synapse that normally gives rise only to short-term facilitation can enhance dramatically the duration of cell-wide homosynaptic activity in a synapse-specific way.

The LTF Produced by Combining Homosynaptic Activation and a Single Heterosynaptic Modulatory Input Is Novel: It Does Not Require Protein Synthesis and Is Not Accompanied by Synaptic Growth. Previous studies of the sensory-to-motor-neuron synapse in *Aplysia*, both in the intact animal and in culture, have shown that the heterosynaptic plasticity that underlies LTF has three properties: (*i*) it requires repeated heterosynaptic modulatory input; (*ii*) it requires the synthesis of new proteins; and (*iii*) when initiated at the synapse itself, these long-term processes are accompanied by the growth of new synaptic connections (15, 17, 18, 25, 26, 33–37).

To test whether this form of synapse-specific LTF produced by combining only a single heterosynaptic input with homosynaptic activity can also recruit new protein synthesis and growth, we first examined the effect of anisomycin (10 μM) applied for a period extending from 30 min before the initial recording until 30 min after training. We found that, when given during this time domain (see also ref. 38), the inhibitor of protein synthesis did not block LTF produced by combining a single heterosynaptic input with one homosynaptic train (mean change of EPSP at 24 h of $52.8 \pm 11.3\%$ at the 5-HT-treated branch and $12.9 \pm 13.8\%$ at the untreated branch, $n = 11$, Fig. 3*A*).

To determine whether the LTF induced by the interaction of homosynaptic activity and a single heterosynaptic modulatory input was accompanied by synaptic growth, we have examined the consequences of this interaction on the number of fluorescently labeled sensory neuron varicosities contacting each motor neuron. We have found that, although the paired training induced facilitation evident at 24 h, this was not accompanied by the growth of new sensory neuron synapses. The combination of a single pulse of 5-HT with a single tetanus produced a $52.4 \pm 22.5\%$ ($n = 5$) enhancement in the amplitude of the evoked EPSP at the treated branch at 24 h vs. the untreated control branch ($5.6 \pm 6.6\%$, $n = 5$) but no increase in varicosity number ($0.86 \pm 5.5\%$, $n = 5$) compared with the untreated branch ($-4 \pm 4\%$, $n = 5$, Fig. 3*A*). Pairing a single pulse of 5-HT with four tetani increased the amount of facilitation present at 24 h ($106.3 \pm 47.9\%$ vs. $7.7 \pm 21\%$, $n = 5$) but still induced no synaptic growth ($-3.6 \pm 3.6\%$ vs. $-5 \pm 5\%$, $n = 4$, Fig. 3*B*). These results show that the recruitment of new protein synthesis and growth requires repeated applications of 5-HT. Under the range of conditions that we here examined, the recruitment of protein synthesis and synaptic growth cannot be achieved by repeated homosynaptic tetani alone.

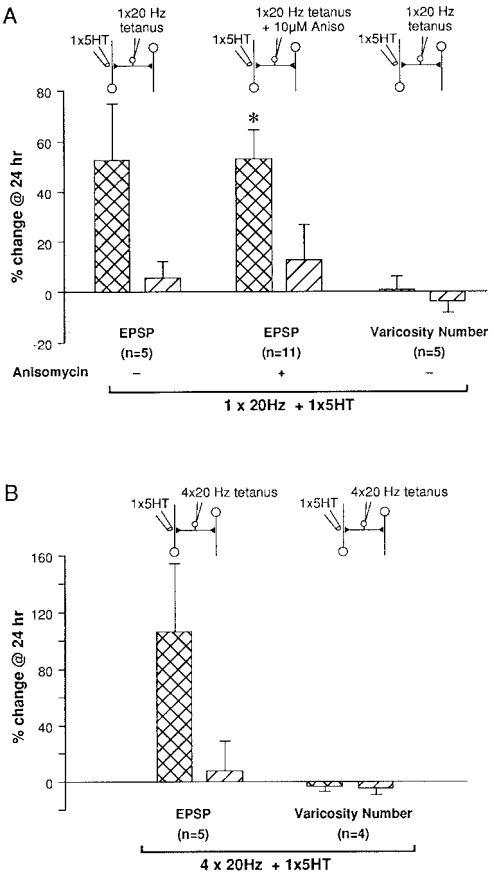

Fig. 3. Pairing homosynaptic activation with a single heterosynaptic modulatory input produces a form of LTF that is protein synthesis independent and is not associated with synaptic growth. (*A*) Bath application of 10 μM anisomycin, a translational inhibitor, for 30 min before, during, and 30 min after branch-specific paired training did not block LTF at 24 h (*, $P < 0.05$, change in EPSP amplitude in 5-HT-treated vs. untreated branch). To test whether the LTF induced by branch-specific paired homo- and heterosynaptic training was accompanied by synaptic growth, we examined the consequences of combining a single tetanus and a single pulse of 5-HT on the number of fluorescently labeled sensory neuron varicosities contacting each motor neuron. We found that although the paired training produced LTF measured at 24 h at the 5-HT-treated branch, it produced no increase in varicosity number. (*B*) Pairing a single pulse of 5-HT with 4 tetani also produced facilitation present at 24 h but still induced no synaptic growth.

Discussion

A New Form of LTF in *Aplysia* Achieved By Combining Homosynaptic Activation with a Single Heterosynaptic Modulatory Input. Cellular studies of associative synaptic plasticity in higher invertebrates and in mammals suggest that both homosynaptic activation and heterosynaptic modulation can interact, and that both processes contribute importantly to associative memory storage [3, 28–30, 39–43; see also ref. 44; I. Antonov, E.R.K. & R. D. Hawkins

(2000) *Soc. Neurosci. Abstr.*, in press]. However, an unresolved question in the biology of learning-related synaptic plasticity that we have here tried to address is: What is the quantitative relationship between these two types of facilitation in situations where they interact? This question is difficult to address in complex mammalian systems where homosynaptic activation during LTP often recruits fibers of passage from one or another modulatory pathway (45, 46). However, the technical advantages of the neural circuit of the gill- and siphon-withdrawal reflex in *Aplysia*, both in the intact animal and in culture, allow independent activation of the same synapses either homosynaptically, heterosynaptically, or in combination.

To examine these interactions at the level of individual synaptic connections, we therefore have used the bifurcated sensory neuron culture system developed by Martin *et al.* (25), in which a single glutamatergic sensory neuron with a bifurcated axon is cocultured with two spatially separate motor neurons. Using this system, we have asked: Can homosynaptic activity in the presynaptic cell lead to growth of new synaptic connections and to persistence of synaptic facilitation? How does homosynaptic activity interact with a short-term heterosynaptic process initiated by 5-HT at only a single synapse? What is the relative contribution of the homo- and heterosynaptic processes to the time course of LTF?

We find that the homosynaptic facilitation produced by both a single and repeated trains of homosynaptic activity in *Aplysia* has properties that resemble an NMDA-dependent form of LTP, as described by Lin and Glanzman (28). As is the case with LTP in the Schaffer collateral pathway of the hippocampus, this homosynaptic facilitation in *Aplysia* to repeated tetanic stimulation is partially blocked by inhibitors of the NMDA receptor and by injection of BAPTA into the postsynaptic cell (28, 30, 32). The homosynaptic facilitation is cell wide and, surprisingly, the homosynaptic activation by itself—even when repeated—produces a facilitation that lasts only a few hours and is not sufficient to produce the persistent synaptic changes that underlie long-term memory storage in *Aplysia*. However, if one or more homosynaptic trains of spike activity in the sensory neuron are combined with the spatially restricted application of even a single pulse of 5-HT to one of the two sensory neuron branches, this leads to a selective enhancement in synaptic strength only at the 5-HT-treated branch, and that synapse-specific enhancement now persists for more than a day.

The combination of homosynaptic (activity-dependent) facilitation and heterosynaptic (modulatory input-dependent) facilitation leads to a form of long-term plasticity with emergent properties that are more than the sum of the individual components. First, the duration of the facilitation so produced greatly extends—by more than 20-fold—the duration of each of the facilitatory processes expressed alone. Second, when homo- and heterosynaptic mechanisms are combined, the spatial distribution of the plasticity now becomes restricted to their point of overlap, resulting in a greater level of synapse specificity than that produced by either alone. Third, even though the facilitation lasts more than 24 h, it does not recruit new protein synthesis and growth.

The Activity-Dependent Synapse-Specific LTF Is a Direct Extension of Short- and Intermediate-Term Facilitation. The activity-dependent synapse-specific LTF that we describe here is unusual not only in its amplified duration and synaptic restriction but also in its mechanisms. This long-term process is, to our knowledge, the first example of LTF in *Aplysia* that lasts more than 24 h yet does not require CREB-mediated gene expression. In fact, these experiments illustrate that it is possible to obtain some persistence, lasting more than just a few hours, in ways that do not require any protein synthesis. Consistent with these findings, this facilitation also is not accompanied by the growth of new synaptic connections (Table 1). Rather, the long-term process is a direct extension of the short- and intermediate-term processes (47) and emerges from combining two stimulus protocols that on their own produce only a short-term process. This form of LTF, therefore, differs from four other forms of LTF that have been described in *Aplysia*: synapse specific, capture of synapse specific, cell wide, and capture of cell wide (25, 26).

Table 1. Five different forms of long-term facilitation in *aplysia* (25, 26)

	CREB	72-hr time course	Local protein synthesis 24 hr	Local protein synthesis 72 hr	Growth
Synapse specific	+	+	+	+	+
Capture of synapse specific	+	+	−	+	+
Cell wide	+	−	−	−	−
Capture of cell wide	+	+	ND	+	+
Activity dependent, synapse specific	−	ND	−	ND	−

ND, not determined.

Potential Behavioral Significance of Different Forms of LTF. The modern study of memory storage can be traced to 1885, when Hermann Ebbinghaus transformed the study of human memory from a subject of philosophical speculation into a laboratory science (48). One of Ebbinghaus' key findings—a cornerstone of modern studies on learning—was that memory is graded. Repetition of a task during learning increases both the strength and duration of retention in memory. This has raised the question: What determines this relationship? Is memory a single process whose duration is simply related to the number of training trials, or does repetition during learning activate fundamentally different memory stores with different time courses of retention?

During the 20th century, serious efforts were made to address these questions in cognitive psychological terms, giving rise to three very different views. According to one view, short- and long-term memory is a single process that varies in duration with repetition, because repetition leads to a greater variation in the depth of processing (49). This is consistent with the finding made repeatedly in the literature of cognitive psychology that the more extensive, elaborate, and deep the encoding of memory at the time of initial learning, the more enduring the memory. These elaborative encoding events are thought to endow the short-term process with greater depth and duration (50). According to the second view, short-term memory and long-term memory represent two different processes that are in series: a short-term memory lasting minutes to hours that precedes and causally leads to a long-term memory lasting days, weeks, or even the lifetime of the organism (51, 52). A third view is that short-term memory and long-term memory are different processes, but they are in parallel, not in series (52–54).

Whether memory stores are single or multiple, in series or in parallel, could not be resolved by behavioral studies alone. These questions fundamentally concern how nerve cells distribute, handle, and store information. The study of memory, therefore, has benefited from the attempt to combine behavioral with reductionist approaches designed to analyze the neurobiological mechanisms of memory in cellular terms (50). Reductionist studies have been particularly effective with simple vertebrate behaviors and several tractable, higher invertebrate systems (2–4, 55). Of the several invertebrate systems, *Aplysia* and *Drosophila* have been particularly informative, because memory in these animals has been shown to have both short- and long-term components (56). In *Aplysia*, transformation can be studied at the level of a single synapse (25).

It recently has been proven possible in *Aplysia* to obtain cellular

and molecular evidence at the level of the single synapse for each of the three major mechanisms of memory postulated on behavioral grounds and to find, in addition, two subsidiary mechanisms (Table 1). These studies suggest the interesting possibility that the cellular mechanisms contributing to behavioral long-term memory may not themselves be unitary but may involve a family of at least five related long-term storage mechanisms that can be used alone and in various combinations (Table 1).

First, Martin *et al.* (25) found that short- and LTF can be in series. A single synapse of the presynaptic sensory neuron can undergo long-lasting functional and structural plasticity that both depends on transcription and is synapse specific. Synapse-specific LTF, initiated by five repeated pulses of 5-HT at a synapse or group of synapses, is accompanied by the growth of new synaptic connections, persists for at least 72 h, and requires local protein synthesis for both a retrograde signal from the synapse to the nucleus and stabilization of growth at the site of initiation. Here, inhibiting CREB blocks the long-term process without blocking the short-term process, showing that LTF differs fundamentally from short-term facilitation, even though both involve PKA and are, to a degree, in series (for discussion, see refs. 13 and 52). Second, this long-term process can be captured by any other synapse of the neuron by applying a single pulse of 5-HT. Here, the short-term process at the captured synapse is in parallel with the long-term process. Third, in applying 5-HT selectively to the cell body, Casadio *et al.* (26) found that the same sensory neurons can also undergo a LTF that is cell wide. Cell-wide LTF, generated by repeated pulses of 5-HT at the cell body, also requires CREB but is distinctive in two ways. It is associated neither with short-term facilitation nor with synaptic growth and does not persist beyond 48 h. Fourth, to obtain persistent facilitation and specifically synaptic growth, one needs, in addition to CREB-mediated transcription, a parallel short-term marking signal produced by a single pulse of 5-HT applied to the synapse (25, 26). Here, the long- and the short-term processes also are in parallel.

The fifth form of the long-term process is the one that we have here described, where one or more homosynaptic trains are paired with a single pulse of 5-HT. Here the long-term process is in series with and a direct extension of a preceding short- and intermediate-term process (47). This fifth form provides a direct cellular confirmation of Ebbinghaus' original idea of the graded nature of memory.

The Importance of Short-Term Modulatory Input in Memory Storage. In a more general sense, these results provide a new insight into the importance of modulatory inputs in memory storage. The data in *Aplysia* suggest that Hebbian homosynaptic (activity-dependent) plasticity can provide some specificity and short-term synaptic changes that might be relevant for the acquisition of learning and for short-term memory, but it does not, when initiated by itself, appear to ensure the persistence necessary for long-term memory storage.

However, the combination of even a single heterosynaptic modulatory input with homosynaptic activation can dramatically enhance the duration of the resultant synaptic changes and thus provide persistence that can extend for at least 24 h. This illustrates that short-term synaptic plasticity produced by a single modulatory input may itself have multiple functions: (*i*) Acting by itself, a single pulse of 5-HT produces a short-term facilitation that contributes to short-term memory (25, 57). (*ii*) Acting in conjunction with a long-term process expressed at any other synapse of the neuron, the short-term process can select which additional synapses of the neuron are recruited for the long-term process (25, 26). And (*iii*) when combined with homosynaptic activation, which by itself produces only transient synaptic changes lasting 1 or 2 h, a single heterosynaptic modulatory input can enhance, in a nonadditive way, the duration of the combined interactive plasticity 20-fold to give rise to a form of LTF that does not recruit protein synthesis and growth and seems to be a direct prolongation of the short-term process itself. As a corollary, these results indicate that to recruit protein synthesis and synaptic growth, repeated heterosynaptic pulses of 5-HT are required (26).

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1. Kandel, E. R. (1976) *Cellular Basis of Behavior: An Introduction to Behavioral Biology* (Freeman, San Francisco).
2. Carew, T. J. & Sahley, C. L. (1986) *Annu. Rev. Neurosci.* **9,** 435–487.
3. Byrne, J. H. (1987) *Physiol. Rev.* **67,** 329–439.
4. Hawkins, R. D., Kandel, E. R. & Siegelbaum, S. A. (1993) *Annu. Rev. Neurosci.* **16,** 625–665.
5. Pinsker, H. M., Hening, W. A., Carew, T. J. & Kandel, E. R. (1973) *Science* **182,** 1039–1042.
6. Frost, W. N., Castellucci, V. F., Hawkins, R. D. & Kandel, E. R. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8266–8269.
7. Rayport, S. G. & Schacher, S. (1986) *J. Neurosci.* **6,** 759–763.
8. Brunelli, M., Castellucci, V. & Kandel, E. R. (1976) *Science* **194,** 1178–1181.
9. Ghirardi, M., Braha, O., Hochner, B., Montarolo, P. G., Kandel, E. R. & Dale, N. (1992) *Neuron* **9,** 479–489.
10. Schacher, S., Castellucci, V. F. & Kandel, E. R. (1988) *Science* **240,** 1667–1669.
11. Scholz, K. P. & Byrne, J. H. (1988) *Science* **240,** 1664–1666.
12. Byrne, J. H. & Kandel, E. R. (1995) *J. Neurosci.* **16,** 425–435.
13. Carew, T. J. (1996) *Neuron* **16,** 5–8.
14. Müller, U. & Carew, T. J. (1998) *Neuron* **21,** 1423–1434.
15. Montarolo, P. G., Goelet, P., Castellucci, V. F., Morgan, J., Kandel, E. R. & Schacher, S. (1986) *Science* **234,** 1249–1254.
16. Dash, P. K., Hochner, B. & Kandel, E. R. (1990) *Nature (London)* **345,** 718–721.
17. Glanzman, D. L., Kandel, E. R. & Schacher, S. (1990) *Science* **249,** 799–802.
18. Bailey, C. H., Montarolo, P., Chen, M., Kandel, E. R. & Schacher, S. (1992) *Neuron* **9,** 749–758.
19. Bacskai, B. J., Hochner, B., Mahaut-Smith, M., Adams, S. R., Kaang, B.-K., Kandel, E. R. & Tsien, R. Y. (1993) *Science* **260,** 222–226.
20. Kaang, B.-K., Kandel, E. R. & Grant, S. G. N. (1993) *Neuron* **10,** 427–435.
21. Alberini, C., Ghirardi, M., Metz, R. & Kandel, E. R. (1994) *Cell* **76,** 1099–1114.
22. Bartsch, D., Ghirardi, M., Skehel, P. A., Karl, K. A., Herder, S. P., Chen, M., Bailey, C. H. & Kandel, E. R. (1995) *Cell* **83,** 979–992.
23. Martin, K. C., Michael, D., Rose, J. C., Barad, M., Casadio, A., Zhu, H. & Kandel, E. R. (1997) *Neuron* **18,** 899–912.
24. Bartsch, D., Casadio, A., Karl, K. A., Serodio, P. & Kandel, E. R. (1998) *Cell* **95,** 211–223.
25. Martin, K. C., Casadio, A., Zhu, H., Yaping, E., Rose, J., Bailey, C. H., Chen, M. & Kandel, E. R. (1997) *Cell* **91,** 927–938.
26. Casadio, A., Martin, K. C., Giustetto, M., Zhu, H., Chen, M., Bartsch, D., Bailey, C. H. & Kandel, E. R. (1999) *Cell* **99,** 221–237.
27. Martin, K. C., Barad, M. & Kandel, E. R. (2000) *Curr. Opin. Neurobiol.*, in press.
28. Lin, X. Y. & Glanzman, D. L. (1994) *Proc. R. Soc. London Ser. B* **255,** 215–221.
29. Murphy, G. G. & Glanzman, D. L. (1997) *Science* **278,** 467–470.
30. Bao, J.-X., Kandel, E. R. & Hawkins, R. D. (1998) *J. Neurosci.* **18,** 458–466.
31. Dale, N. & Kandel, E. R. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7163–7167.
32. Bao, J.-X., Kandel, E. R. & Hawkins, R. D. (1997) *Science* **275,** 969–973.
33. Castellucci, V. F., Blumenfeld, H., Goelet, P. & Kandel, E. R. (1989) *J. Neurobiol.* **20,** 1–9.
34. Mauelshagen, J., Sherff, C. H. & Carew, T. J. (1998) *Learn. Mem.* **5,** 246–256.
35. Bailey, C. H. & Chen, M. (1983) *Science* **220,** 91–93.
36. Bailey, C. H. & Chen, M. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2373–2377.
37. Bailey, C. H. & Chen, M. (1989) *J. Neurosci.* **9,** 1774–1780.
38. Sherff, C. M. & Carew, T. J. (1999) *Science* **285,** 1911–1917.
39. Carew, T. J., Hawkins, R. D. & Kandel, E. R. (1983) *Science* **219,** 397–400.
40. Hawkins, R. D., Abrams, T. W., Carew, T. J. & Kandel, E. R. (1983) *Science* **219,** 400–405.
41. Walters, E. T. & Byrne, J. H. (1983) *Science* **219,** 405–408.
42. Walters, E. T. & Byrne, J. H. (1985) *J. Neurosci.* **5,** 662–672.
43. Schacher, S., Wu, F. & Sun, Z.-Y. (1997) *J. Neurosci*. **17,** 597–606.
44. Huang, Y.-Y., Nguyen, P. V., Abel, T. & Kandel, E. R. (1996) *Learn. Mem.* **3,** 74–85.
45. Huang, Y.-Y. & Kandel, E. R. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2446–2450.
46. Huang, Y.-Y. & Kandel, E. R. (1996) *Neuron* **16,** 611–617.
47. Sutton, M. A. & Carew, T. J. (2000) *Neuron* **26,** 219–231.
48. Ebbinghaus, H. (1885) *Über das Gedächtnis* (Duncker, Leipzig); reprinted in Ebbinghaus, H. (1985) *Memory: A Contribution to Experimental Psychology* (Dover, New York).
49. Wickelgren, W. A. (1973) *Psychol. Bull.* **80,** 425–438.
50. Squire, L. & Kandel, E. R. (1999) *Memory: From Mind to Molecules* (Scientific, New York).
51. Atkinson, R. C. & Shiffrin, R. M. (1971) *Sci. Am.* **225,** 82–92.
52. Goelet, P., Castellucci, V. F., Schacher, S. & Kandel, E. R. (1986) *Nature (London)* **322,** 419–422.
53. Emptage, N. J. & Carew, T. J. (1993) *Science* **262,** 253–256.
54. Weisenkrantz, L. (1970) in *Short-Term Changes in Neural Activity and Behavior*, eds. Horn, G. & Hinde, R. A. (Cambridge Univ. Press, Cambridge, U.K.), pp. 63–74.
55. LeDoux, J. E. (1998) *Biol. Psychiatry* **44,** 1229–1238.
56. Yin, J. C. & Tully, T. (1996) *Curr. Opin. Neurobiol.* **6,** 264–268.
57. Castellucci, V., Pinsker, H., Kupfermann, I. & Kandel, E. R. (1970) *Science* **167,** 1745–1748.