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A Transient, Neuron-Wide Form of CREB-Mediated Long-Term Facilitation Can Be Stabilized at Specific Synapses by Local Protein Synthesis

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Summary

In a culture system where a bifurcated *Aplysia* sensory neuron makes synapses with two motor neurons, repeated application of serotonin (5-HT) to one synapse produces a CREB-mediated, synapse-specific, long-term facilitation, which can be captured at the opposite synapse by a single pulse of 5-HT. Repeated pulses of 5-HT applied to the cell body of the sensory neuron produce a CREB-dependent, cell-wide facilitation, which, unlike synapse-specific facilitation, is not associated with growth and does not persist beyond 48 hr. Persistent facilitation and synapse-specific growth can be induced by a single pulse of 5-HT applied to a peripheral synapse. Thus, the short-term process initiated by a single pulse of 5-HT serves not only to produce transient facilitation, but also to mark and stabilize any synapse of the neuron for long-term facilitation by means of a covalent mark and rapamycin-sensitive local protein synthesis.

Introduction

Neurons form synaptic connections that are at once precise but nevertheless modifiable in response to appropriate patterns of stimuli. This synaptic plasticity contributes to a variety of physiological and pathological processes in the adult brain—from learning and memory to amnesia and dementia, from drug dependence to epilepsy. Of these, the role of synaptic plasticity in the storage of memories has been particularly well studied.

There are at least two temporally distinct phases of memory storage: a short-term memory lasting minutes and a long-term memory lasting days or longer. These two phases differ not only in their time courses, but also in their molecular mechanisms: long-term memory, but not the short-term form, requires the synthesis of new proteins (Goelet et al., 1986). Recent studies in *Aplysia* and mice have revealed that these distinct stages in behavioral memory are reflected in distinct phases of synaptic plasticity (Bliss and Collingridge, 1993; Bailey et al., 1996). In *Aplysia*, these stages have been particularly well studied in the context of sensitization, a form of learning in which an animal learns to strengthen its reflex responses to previously neutral stimuli following the presentation of an aversive stimulus. The short- and long-term memory for sensitization are mirrored by the short and long-term facilitation of the synaptic connections between the sensory to motor neuron that mediate this reflex. This monosynaptic component can be examined not only in the intact animal (Frost et al., 1985), but also in a single sensory neuron cultured with its target postsynaptic motor neuron (Montarolo et al., 1986). At this cultured synapse, one pulse of 5-HT, a neurotransmitter released in vivo by interneurons activated by the sensitizing tail stimuli, produces a PKA- and PKC-mediated short-term facilitation lasting only minutes. By contrast, five spaced pulses of 5-HT elicit a long-term facilitation lasting more than 24 hr. With five pulses of 5-HT, PKA recruits MAP kinase and both translocate to the nucleus (Bacskai et al., 1993; Martin et al., 1997b). Here, they activate CREB1 and derepress CREB2, leading to the induction of a set of immediate-early genes (Dash et al., 1990; Alberini et al., 1994; Bartsch et al., 1995, 1998; Hegde et al., 1997). Long-term facilitation is further associated with the growth of new synaptic connections (Bailey and Kandel, 1993).

This set of mechanisms has proven to be quite general. A similar cascade of gene induction is involved in *Drosophila* and in mice (Bourtchuladze et al., 1994; Yin et al., 1994, 1995). The requirement for transcription explained why long-term memory requires the synthesis of new proteins. However, this requirement posed a cell biological problem: since long-term plasticity relies on the activation of genes in the nucleus, one might expect that long-lasting changes in the connectivity of the neuron have to be cell wide.

In a previous series of experiments, we modified the standard *Aplysia* sensory neuron–motor neuron culture by plating a single bifurcated sensory neuron in contact with two spatially separated postsynaptic motor neurons and found that each synapse or group of synapses can be modified independently in a protein synthesis–dependent manner (Martin et al., 1997a). This spatially restricted plasticity requires the activity of CREB1 in the nucleus as well as local protein synthesis in the 5-HT-treated processes of the sensory cell. In addition to synapse-specific facilitation, we observed a phenomenon we refer to as "synaptic capture." Once synapse-specific long-term facilitation has been initiated by microperfusing five pulses of 5-HT onto one branch of the sensory neuron, a single pulse of 5-HT, which per se induces only transient facilitation, is able to recruit long-term facilitation when applied to a second branch. This synaptic capture does not require local protein synthesis (see also Frey and Morris, 1997).

As is generally the case with neurons, the sensory neuron is a highly polarized cell with several functionally distinct and spatially separate compartments, each of which is selectively innervated. In fact, in the intact animal, the endings of different serotonergic interneurons

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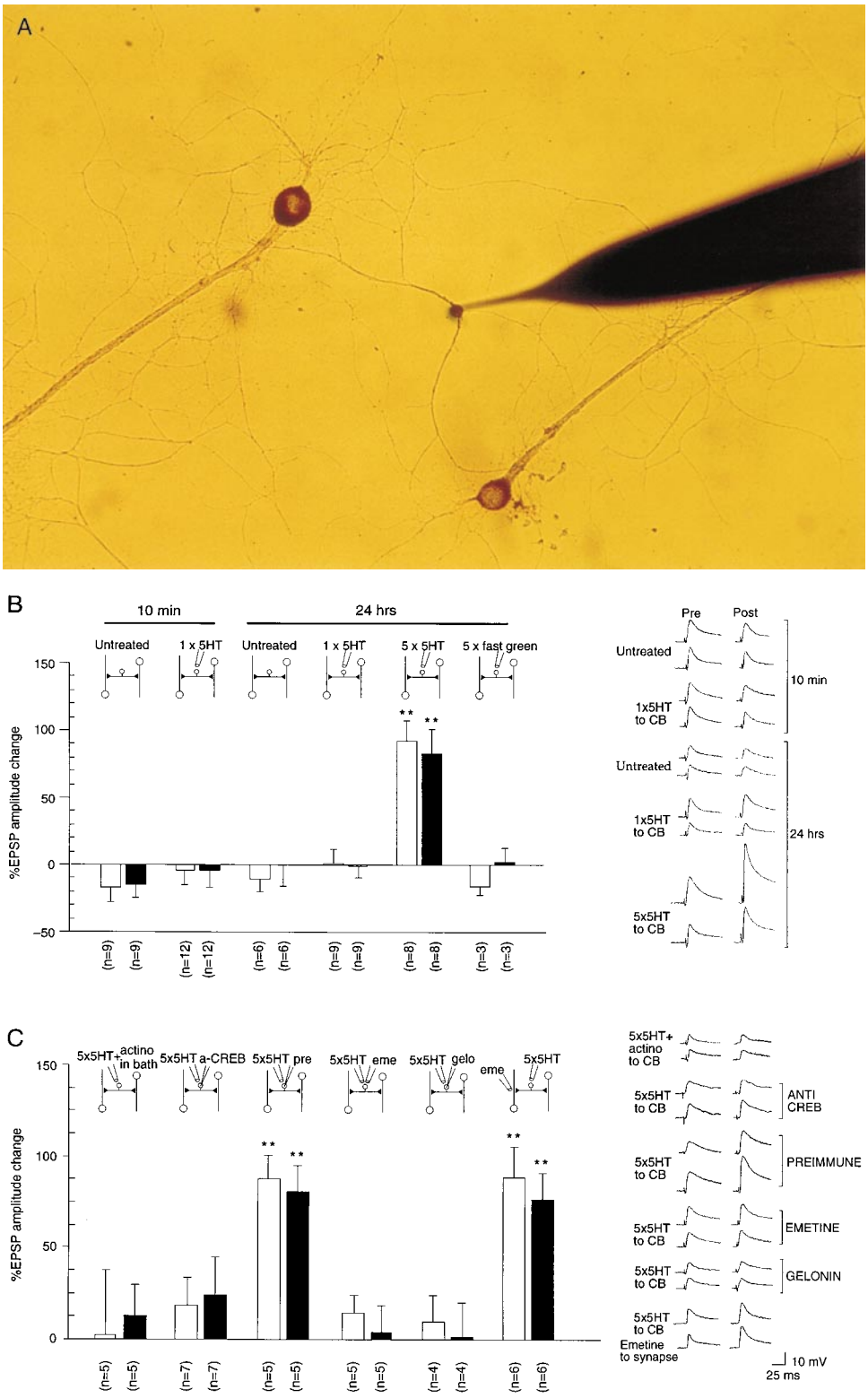

Figure 1. Neuron-Wide Long-Term Facilitation

(A) 5-HT was applied to the cell body of the sensory neuron using a perfusion microelectrode. Fast green (0.05%) was included to visualize the perfusion.

(B) Local application of a single pulse of 5-HT to the soma of the sensory cell did not produce any short-term (mean change in EPSP amplitude 10 min after 5-HT application was −4.29% ± 10.08% at one branch and −4.20% ± 12.82% at the other, n = 12) or long-term facilitation (mean change in EPSP amplitude 24 hr after 5-HT application was 1.51% ± 10.72% at one branch and 0.70% ± 8.64% at the other, n = 9). In contrast, local application of five spaced pulses of 5-HT to the cell body produced a cell-wide long-term facilitation, increasing the EPSP

contact the sensory neurons at different sites, including its pre- and postsynaptic terminals and processes and its cell body (Kistler et al., 1985; Zhang et al., 1991). What then are the functional consequences of applying 5-HT selectively to the cell body?

Here, we show that five pulses of 5-HT applied selectively to the soma of the sensory neuron produce a cell-wide long-term facilitation that requires CREB1-mediated transcription, but that does not persist and is not associated with growth. A similar cell-wide facilitation is obtained by injecting recombinant phospho-CREB1 protein into the sensory neuron. Cell-wide facilitation can be sustained and growth can be captured by a single pulse of 5-HT. Thus, a single pulse of 5-HT serves two functions. First, it allows for 24 hr facilitation and growth by means of a PKA-mediated covalent modification. Second, it stabilizes growth and facilitation in a protein synthesis–dependent way to produce facilitation that persists at least 72 hr. By systematically comparing cell-wide facilitation to synapse-specific facilitation and synaptic capture, we find that each represents a distinct form of CREB-dependent, long-lasting synaptic plasticity. Thus, a single neuron has multiple long-term mechanisms for temporally and spatially integrating stimuli to produce transcription-dependent long-lasting changes in synaptic strength.

Results

Application of 5-HT to the Sensory Cell Body Produces a Novel Cell-Wide CREB-Dependent Facilitation at Both Branches that Does Not Require a Marking Signal

In the intact animal, serotonergic interneurons contact not only the processes but also the cell bodies of the sensory cells (Kistler et al., 1985; Zhang et al., 1991). We therefore asked, Does the application of 5-HT restricted to the soma of the sensory neuron initiate facilitation even in the absence of a marking signal? We find that a single pulse of 5-HT restricted to the cell body of the sensory cell (Figure 1A) did not produce any short-term facilitation (Figure 1B), confirming that the 5-HT application was indeed restricted spatially. Five pulses of 5-HT applied to the cell body did, however, induce cell-wide long-term facilitation; measured 24 hr later, the synaptic strength of both branches increased equally (Figure 1B). Thus, a single presynaptic cell can undergo a 24 hr cell-wide facilitation.

Like its synapse-specific counterpart, cell-wide facilitation requires transcription and translation. Cell-wide long-term facilitation was blocked by bath incubation with the transcriptional inhibitor actinomycin D (see Figure 1C). It was also blocked by perfusing the cell body with emetine, a protein synthesis inhibitor. Similarly, microinjection of gelonin, a membrane-impermeant protein synthesis inhibitor, into the sensory cell blocked cell-wide facilitation. Importantly, perfusion of the connections made by the sensory neuron on one of the motor neurons with emetine did not affect cell-wide facilitation, indicating that local protein synthesis in the distal sensory cell processes is not required for cell-wide facilitation (see Figure 1C).

The transcription required for cell-wide facilitation is mediated by CREB. Microinjection of sensory cells with a polyclonal anti-CREB antibody blocked the facilitation induced by cell body application of 5-HT, whereas microinjection of preimmune serum had no effect (see Figure 1C). Thus, application of five pulses of 5-HT to the sensory cell body produces a cell-wide facilitation that lasts 24 hr, depends on CREB-mediated transcription, and occurs in the absence of any short-term facilitation. This indicates that synaptic tagging is not necessary for the expression of certain forms of long-term synaptic plasticity.

Cell-Wide Facilitation Differs from Synapse-Specific Facilitation and Synaptic Capture in Both Its Onset and Time Course

The finding that long-lasting facilitation produced by application of 5-HT to the cell body of the sensory neuron occurred in the absence of any short-term facilitation indicated that synapse-specific facilitation and synaptic capture differed from cell-wide facilitation in the time course for the onset of facilitation. To analyze this difference further, we carried out a more detailed analysis of the time course of facilitation during synapse-specific facilitation, synaptic capture, and cell-wide facilitation. As shown in Figure 2A, application of five pulses of 5-HT to the synapse produced short-term synaptic facilitation at that branch (Figure 2A). The EPSP amplitude then decreased at 1 hr, rose gradually at 4 hr and 12 hr, and then reached a peak at 24 hr. The time course of synaptic capture was indistinguishable from that of synapse-specific facilitation (Figure 3A). By contrast, when five pulses of 5-HT were applied to the cell body of the sensory cell, there was no short-term

amplitude at both branches after 24 hr (92.11% ± 15.58% at one branch and 82.87% ± 18.30% at the other, n = 8). (**, $p < 0.01$, change in EPSP amplitude in cells treated with five pulses of 5-HT to the cell body versus untreated cells, ANOVA and Neuman-Keul's multiple range test.) In control experiments, perfusion of fast green alone did not change the EPSP. Shown are representative recordings of EPSPs and a histogram of the mean change in EPSP amplitude ± SEM.

(C) Bath application of actinomycin D (50 μg/ml) (actino) for 30 min prior to, during, and 30 min after local application of five pulses of 5-HT blocked neuron-wide long-term facilitation (mean change in EPSP amplitude at 24 hr was 2.42% ± 34.91% at one branch and 12.83% ± 16.38% at the other, n = 5). Microinjection of anti-CREB antibodies (α-CREB) into the presynaptic sensory neuron 1 hr prior to local application of five pulses of 5-HT also blocked neuron-wide long-term facilitation, while injection of the preimmune serum (pre) had no effect. Local somatic application of 100 μM emetine (eme) or presynaptic injection of 25 μM gelonin (gel) also blocked neuron-wide long-term facilitation (mean change in EPSP amplitude at 24 hr was 14% ± 9.79% at the one branch and 4.33% ± 13.66% at the other [n = 5] in emetine-treated cell and 9.21% ± 15.10% at the one branch and 1.08% ± 17.85% at the other [n = 4] in gelonin-injected cells). Neuron-wide long-term facilitation was not affected by synaptic application of 100 μM emetine (eme) for 30 min prior to, during, and 30 min after local application of five pulses of 5-HT to the cell. (**, $p < 0.01$, change in EPSP amplitude in cells injected with preimmune serum and receiving five pulses of 5-HT to the soma versus cells treated with emetine, actinomycin, or injected with anti-CREB antibodies or gelonin; ANOVA and Neuman-Keul's multiple range test.) Shown are representative recordings of EPSPs and a histogram of the mean change in EPSP amplitude ± SEM.

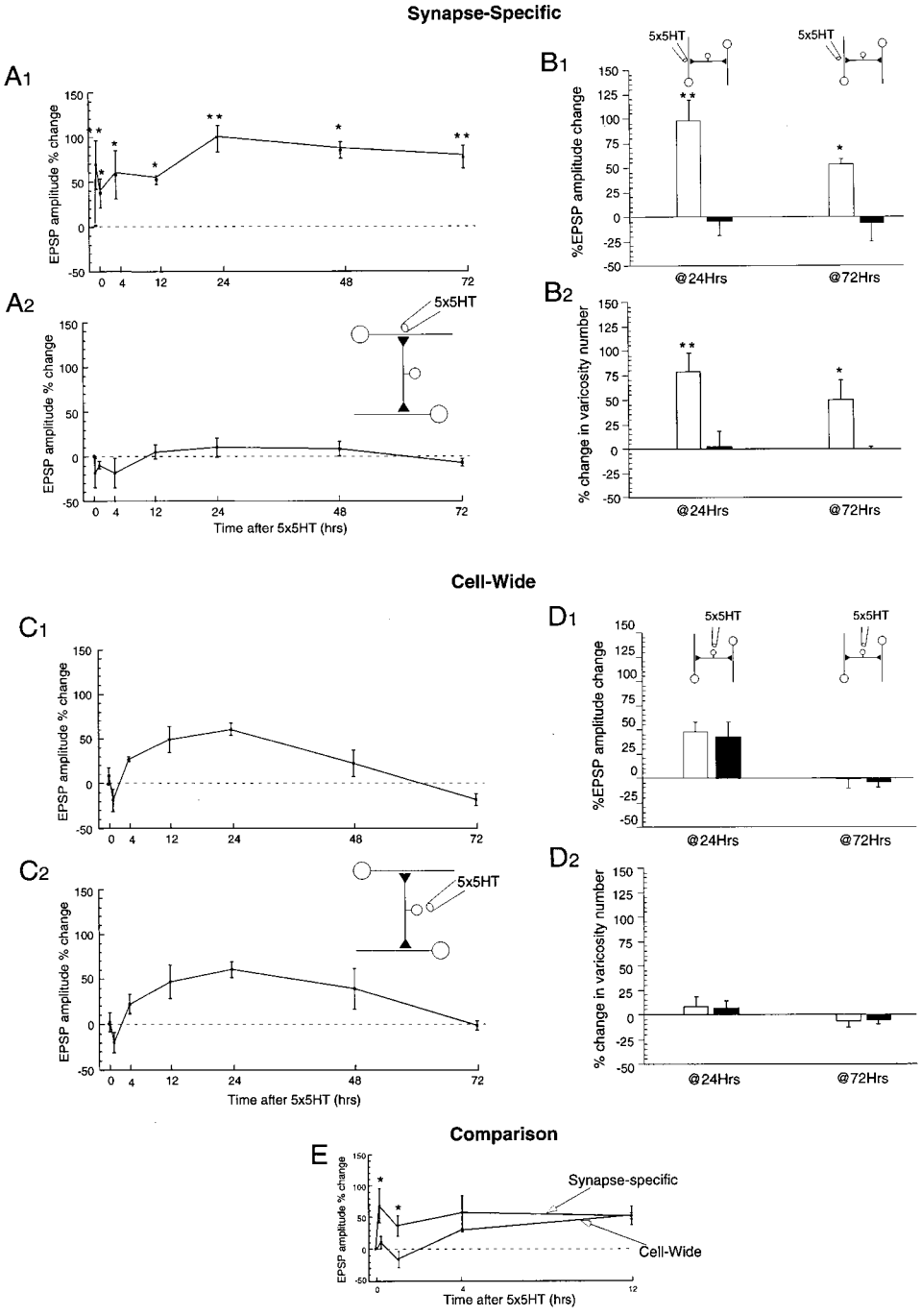

Figure 2. Two Major Forms of Long-Term Plasticity: Synapse-Specific and Neuron-Wide Long-Term Facilitation

Synapse-specific facilitation (A_1 and A_2) differs from neuron-wide facilitation (C_1 and C_2) in both its onset and time course. In addition, synapse-specific facilitation (B_2), but not the neuron-wide form (D_2), is accompanied by the growth of new synapses.

Application of five spaced pulses of 5-HT to one set of terminals induced a short-term facilitation at that branch (A_1). This facilitation decreased at 1 hr, rose gradually at 4 hr, continued to increase, reaching a peak at 24 hr and persisted for at least 72 hr (A_1). A_2 shows the time course of the EPSP changes at the branch that did not receive any 5-HT (A_1 and A_2: 10 min, n = 5; 1 hr, n = 5; 4 hr, n = 5; 12 hr, n = 5; 24 hr, n = 7; 48 hr, n = 4; 72 hr, n = 8). (*, $p < 0.05$; **, $p < 0.01$, change in EPSP amplitude at the one branch versus the opposite branch, Student's t test.) To study the morphological changes accompanying synapse-specific facilitation, carboxyfluorescein-filled sensory neurons were imaged before and 24 or 72 hr after 5-HT treatment. The number of varicosities was counted using a blind procedure. B_1 and B_2 show histograms of the mean change in EPSP amplitude ± SEM and of the varicosity number ± SEM in each branch, respectively (B_1 and B_2: at 24 hr, n = 5; at 72 hr, n = 5). Synapse-specific facilitation involves synapse-specific growth of new varicosities 24 hr after treatment. Most of the newly grown varicosities persist after 72 hr (*, $p < 0.05$; **, $p < 0.01$, change in EPSP amplitude in the branch treated with five 5-HT pulses versus the untreated branch, Student's t test, and change in varicosity number in the branch treated with five 5-HT pulses versus the untreated branch, Student's t test).

facilitation, and facilitation only began with a delay of 4 hr and gradually increased between 12 and 24 hr (Figure 2C).

Cell-Wide Facilitation Is Not Associated with Synaptic Growth and Is Less Persistent Than Synapse-Specific Facilitation or Synaptic Capture

As previously described (Martin et al., 1997a), when synapse-specific facilitation was induced by applying five 5-HT pulses to a synapse, the growth of new varicosities was observed at 24 hr exclusively at the stimulated connection (see also Figure 3B). We found that synaptic capture was also accompanied by synaptic growth, both at the branch receiving five pulses of 5-HT and at the branch receiving a single pulse of 5-HT (Figures 3B and $7A_2$). In contrast to synapse-specific facilitation and capture, when cell-wide facilitation was induced by applying five 5-HT pulses to the cell body, there was no significant increase in varicosity number at 24 hr (Figures 2D and $7B_2$), despite the increase in synaptic amplitude that occurred at both connections (Figure 2D). These findings indicate that despite the dependence of both types of facilitation on CREB-mediated transcription, cell-wide facilitation and synapse-specific facilitation differ from one another. As a corollary, these results indicate that transcription-dependent long-lasting increases in synaptic strength can occur in the absence of a local marking signal at the synapse and in the absence of detectable synaptic growth.

The fact that cell body application of 5-HT produced a 24 hr increase in synaptic strength in the absence of synaptic growth led us to examine the long-term persistence of the functional changes in synaptic strength. We found that whereas synapse-specific long-term facilitation and capture persisted for 3 or more days, as long as we followed it (in some cases to 96 hr; data not shown) (Figure 2A), cell-wide facilitation began to decay to baseline levels after 24 hr (Figure 2C).

The observation that only the persistent forms of synaptic plasticity—synapse-specific long-term facilitation and synaptic capture—are accompanied by growth led us to examine growth more systematically. To do this, we imaged carboxyfluorescein-filled sensory cells, treated them according to the synapse-specific capture or cell-wide protocol, and reimaged them at 72 hr. Both synapse-specific long-term facilitation and synaptic capture were accompanied by growth of new varicosities at 72 hr (Figure 2B). On the contrary, cell-wide synaptic plasticity was not accompanied by growth of new synapses at 72 hr (Figure 2D). These findings provide further evidence that there are at least two different forms of CREB-mediated transcription-dependent, long-lasting synaptic plasticity: one of which is associated with growth and persists beyond 72 hr, and the other of which is not associated with growth and does not persist beyond 48 hr.

A Single Pulse of 5-HT at the Synapse Can Convert Transient Cell-Wide Facilitation into a Persistent Facilitation Accompanied by the Growth of New Synapses

We next asked whether the cell-wide process can be converted into a persistent facilitation associated with growth by giving a single pulse of 5-HT to one set of connections immediately after applying five pulses of 5-HT to the cell body. As shown in Figure 3C, when a sensory cell receives five pulses of 5-HT at its soma and then a single pulse of 5-HT at the connections made onto one motor neuron, the synaptic amplitude at 24 hr was elevated at both the marked synapse and at the unmarked synapse. However, while the synaptic amplitude at the unmarked synapse decreased by 48 hr, the synaptic amplitude at the marked synapse remained elevated at 48 hr and 72 hr, indicating that the single pulse of 5-HT had marked the synapse for persistent facilitation. Indeed, when five pulses of 5-HT were given to the sensory cell body and a single pulse of 5-HT was given to one branch, there was growth of new varicosities at the marked branch but no growth at the unmarked branch (Figures 3D and 7B). Thus, as is the case with synapse-specific synaptic capture, a single pulse of 5-HT can recruit both growth and persistence at a synapse when five pulses of 5-HT are given at another site in the neuron, be it the cell body or another synapse.

These experiments indicate that cell-wide facilitation requires CREB much as does synapse-specific facilitation and that the products of CREB activation can also be captured by a synapse to which a single pulse of 5-HT is applied.

Repeated Subthreshold Pulses of 5-HT to the Cell Body Can Produce Long-Term Facilitation at a Marked Synapse

What then distinguishes cell-wide from synapse-specific facilitation mechanistically? Why does activation of CREB from a distant synapse not also lead to the activation of other synapses at 24 hr in the absence of a marking signal? One possibility is that the difference between synapse-specific and cell-wide facilitation is quantitative. Application of repeated pulses of 5-HT to a single synapse requires the sending of a retrograde signal from the synapse to the nucleus and might therefore be less effective quantitatively in inducing nuclear transcription than direct application of 5-HT to the cell body. Moreover, five pulses of 5-HT might cause substantial increases in cAMP in the cell body, and this

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In contrast, five spaced pulses of 5-HT to the cell body induced a neuron-wide facilitation at both branches, which occurred in the absence of any short-term facilitation at 10 min. The EPSP amplitude began to increase only at 4 hr, peaked at 24 hr but returned to the baseline at both branches by 72 hr (C_1 and C_2: 10 min, $n = 5$; 1 hr, $n = 4$; 4 hr, $n = 4$; 12 hr, $n = 5$; 24 hr, $n = 10$; 48 hr, $n = 4$; 72 hr, $n = 7$). (D_1) and (D_2) show histograms of the mean change in EPSP amplitude $\pm$ SEM and of the varicosity number $\pm$ SEM in each branch, respectively, obtained as described above (D_1 and D_2: at 24 hr, $n = 7$; at 72 hr, $n = 4$). Unlike the synapse-specific form, neuron-wide facilitation was not accompanied by the growth of new varicosities either at 24 or 72 hr.

(E) Comparison between the first 12 hr in the time courses of synapse-specific and neuron-wide facilitation, respectively. (*, $p < 0,05$, change in EPSP amplitude at the branch treated with five 5-HT pulses versus the branch treated with one 5-HT pulse at any given time point, Student's t test.)

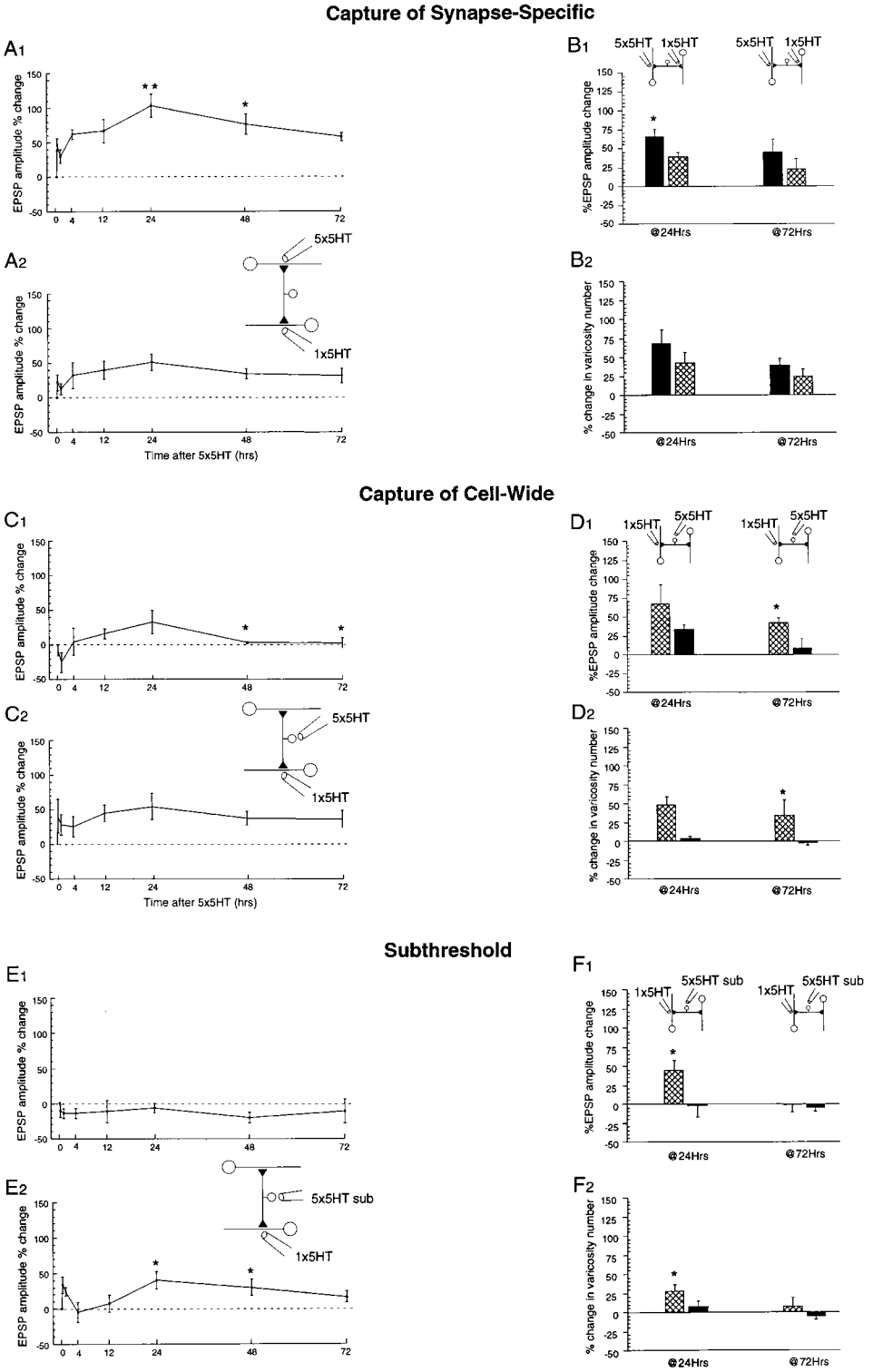

Figure 3. Both Synapse-Specific and Neuron-Wide Facilitation Can Be Captured by Synaptic Application of a Single Pulse of 5-HT

A single pulse of 5-HT, which usually only produces transient facilitation, given to one branch immediately after five spaced pulses of 5-HT to the other induced long-term facilitation at that branch. Synaptic capture (A_2 showed a time course that is indistinguishable, though smaller in amplitude from that of synapse-specific facilitation [A_1] for any given time point). (A_1 and A_2: 10 min, n = 5; 1 hr, n = 4; 4 hr, n = 4; 12 hr, n = 5; 24 hr, n = 10; 48 hr, n = 6; 72 hr, n = 7). (*, $p < 0.05$; **, $p < 0.01$, change in EPSP amplitude at the one branch versus the opposite one at any given time point, Student's t test.) Carboxyfluorescein-filled sensory neurons treated according to the capture protocol were imaged as described above. (B_1) and (B_2) show histograms of the mean change in EPSP amplitude ± SEM and of the varicosity number ± SEM in each branch, respectively (B_1 and B_2: at 24 hr, n = 5; at 72 hr, n = 5). Both synapse-specific facilitation and synaptic capture involved

might diffuse to the terminals. If the difference were quantitative, then even a weaker set of five pulses of 5-HT to the cell body might set up long-term facilitation that could be captured.

To test this idea, we applied five weak pulses of 5-HT to the cell body using a subthreshold concentration of 5-HT (0.1 μM rather than 100 μM), which by itself failed to induce any detectable facilitation. Nevertheless, when these five subthreshold pulses were applied to the cell body and followed by a single threshold pulse of 5-HT to one set of terminals, the one pulse induced a synapse-specific long-term facilitation at that branch (see Figure 3E). These functional changes were accompanied by morphological growth of the capturing terminal both at 24 and 72 hr without any change in the untreated branch (Figure 3F). These results are consistent with the idea that applications of 5-HT to the cell body might lead to activation of synapses throughout the cell because it activates more CREB-mediated transcription.

Cell-Wide Facilitation Can Be Phenocopied by Injection of Phospho-CREB1a Protein into the Sensory Neuron

It still is possible, however, that the application of 5-HT to the cell body marks the cell body in much the way that synapse-specific application of 5-HT marks a synapse. Since the cell body is closer to either set of synapses than they are to each other, some of the mark may diffuse from the cell body down the axon and reach the presynaptic terminals of synapses. However, the amount of the marking signal that diffuses down to the synapse may not be comparable to that produced by a local mark and may not be sufficient for growth and maintenance. To test this possibility, we attempted to produce cell-wide facilitation in the absence of any marking signal by activating the cell-wide process downstream from 5-HT, cAMP, and PKA. We therefore injected recombinant phospho-CREB1a protein into the cell body of the sensory neuron to turn on transcription (Bartsch et al., 1998). Injection of phospho-CREB1a alone generated a transient cell-wide long-term facilitation at 24 hr at both branches (Figure 4A). We have previously shown through mutagenesis experiments that this action of CREB1a is specific (Bartsch et al., 1998). CREB-induced cell-wide facilitation, like the facilitation induced by applying 5-HT to the cell body, did not persist and was not accompanied by morphological changes (Figure 4B).

If this minimalist model is correct, then applying a single pulse of 5-HT locally to one set of synaptic terminals immediately after phospho-CREB1 injection would convert the transient cell-wide process at the capturing branch into a persistent synapse-specific facilitation accompanied by synaptic growth. Indeed, this is what we found (Figures 4C and 4D). Although the changes were quantitatively smaller, they resembled qualitatively those typical of the branch-specific long-term process induced by five pulses of 5-HT at the synapse.

These several findings indicate that the activation of CREB is necessary for both synapse-specific and cell-wide facilitation. They also show that cell-wide facilitation does not require 5-HT, cAMP, or any other marking signal upstream from CREB. Finally, the experiments reveal that CREB is necessary but insufficient for persistent facilitation and the growth of new connections. For persistence and growth, a synaptic mark is needed in addition to CREB activation.

The finding that a local synaptic mark is required for the persistence of each of the three persistent long-term processes—synapse-specific facilitation, synaptic capture, and capture of cell-wide facilitation—raised the question, What is the nature of the synaptic mark? To examine the synaptic mark, we turned to the simplest case—synaptic capture at one branch following synapse-specific facilitation at the other branch—and addressed two questions related to the marking signal: (1) What is the time course during which the mark must be present? and (2) What is the molecular nature of the mark?

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the growth of new varicosities at 24 hr, which still persisted after 72 hr (*, $p < 0.05$, change in EPSP amplitude in the branch treated with five 5-HT pulses versus the branch treated with one 5-HT pulse, Student's t test, and change in varicosity number in the branch treated with five 5-HT pulses versus the branch treated with one 5-HT pulse, Student's t test).

A single pulse of 5-HT given to one branch immediately after five spaced pulses of 5-HT to the cell body of the sensory neuron was also able to capture the cell-wide process at that set of synapses, converting the neuron-wide facilitation (C_1) into a synapse-specific facilitation that persisted for at least 72 hr (C_2). (C_1 and C_2: 10 min, $n = 5$; 1 hr, $n = 4$; 4 hr, $n = 4$; 12 hr, $n = 5$; 24 hr, $n = 7$; 48 hr, $n = 6$; 72 hr, $n = 6$). The time course of the capture of the neuron-wide process was identical to that of synapse-specific facilitation (*, $p < 0.05$, change in EPSP amplitude at the one branch versus the opposite branch, Student's t test). Carboxyfluorescein-filled sensory neurons treated according to the capture protocol were imaged as described above. (D_1) and (D_2) show histograms of the mean change in EPSP amplitude ± SEM and of the varicosity number ± SEM in each branch, respectively (D_1 and D_2: at 24 hr $n = 5$; at 72 hr, $n = 5$). Capture of the neuron-wide process was accompanied by the growth of new synapses at 24 and 72 hr (*, $p < 0.05$, change in EPSP amplitude in the branch treated with one 5-HT pulse versus the opposite branch, Student's t test, and change in varicosity number the branch treated with one 5-HT pulse versus the opposite branch, Student's t test).

Repeated subthreshold pulses of 5-HT to the cell body can produce long-term facilitation at a marked synapse. A single pulse of 5-HT given to one branch immediately after five spaced pulses of 5-HT given to the cell body of the sensory neuron at a subthreshold concentration (0.1 μM) normally unable to induce any facilitation was also able to capture long-term facilitation at that branch (E_1 and E_2: 10 min, $n = 5$; 1 hr, $n = 4$; 4 hr, $n = 4$; 12 hr, $n = 4$; 24 hr, $n = 5$; 48 hr, $n = 3$; 72 hr, $n = 8$). (*, $p < 0.05$, change in EPSP amplitude at the one branch versus the opposite branch, Student's t test.) Carboxyfluorescein-filled sensory neurons treated according to this protocol were imaged as described above. (F_1) and (F_2) show histograms of the mean change in EPSP amplitude ± SEM and of the varicosity number ± SEM in each branch, respectively. (F_1 and F_2: at 24 hr, $n = 5$; at 72 hr, $n = 4$.) Synaptic capture of the subthreshold process was accompanied by the growth of new synapses at 24 and at 72 hr (*, $p < 0.05$, change in EPSP amplitude in the branch treated with one subthreshold 5-HT pulse versus the opposite branch, Student's t test, and change in varicosity number in the branch treated with one subthreshold 5-HT pulse versus the opposite branch, Student's t test).

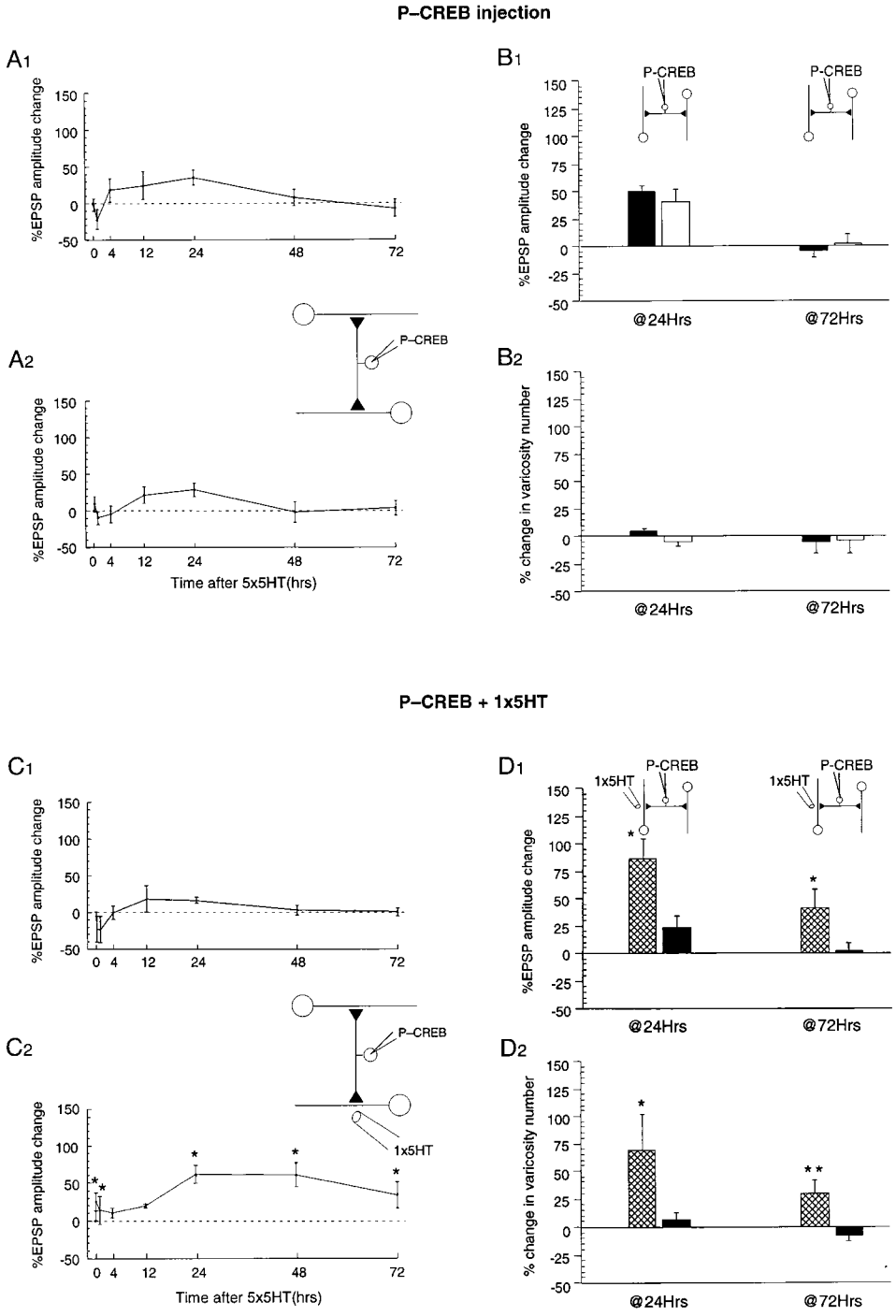

Figure 4. Cell-Wide Facilitation Can Be Phenocopied by Injection of Phosphorylated CREB1a Protein into the Sensory Neuron

Microinjection of phosphorylated CREB1a (phospho-CREB1a) protein into the sensory neuron generated a transient cell-wide facilitation at both branches at 24 hr, which did not persist at 72 hr. (A_1 and A_2: 10 min, n = 5; 1 hr, n = 7; 4 hr, n = 6; 12 hr, n = 5; 24 hr, n = 11; 48 hr, n = 7; 72 hr, n = 11). Facilitation produced by microinjection of phospho-CREB1a was not accompanied by growth of new varicosities at 24 or 72 hr. Carboxyfluorescein-filled sensory neurons injected with phospho-CREB1a protein were imaged as described above. (B_1) and (B_2) show histograms of the mean change in EPSP amplitude ± SEM and of the varicosity number ± SEM in each branch, respectively, obtained as described above. (B_1 and B_2: at 24 hr, n = 7; at 72 hr, n = 5).

A single pulse of 5-HT given to one branch immediately after microinjection of phospho-CREB1a protein into the sensory neuron was also able to capture the cell-wide process, converting neuron-wide facilitation (C_1) into a synapse-specific facilitation accompanied by growth, which persisted for at least 72 hr (C_2 and D_2). (C_1 and C_2: 10 min, n = 5; 1 hr, n = 4; 4 hr, n = 4; 12 hr, n = 8; 24 hr, n = 19; 48 hr, n = 7; 72 hr, n = 8). The time course of the capture of the phospho-CREB1a-induced facilitation was identical to that of synapse-specific facilitation (*, $p < 0.05$, change in EPSP amplitude at the one branch versus the opposite branch at any given time point, Student's t test). Carboxyfluorescein-filled sensory neurons treated according to this protocol were imaged as described above. (D_1) and (D_2) show histograms of the mean

The Time Course of the Marking Signal: Synaptic Tagging Can Capture Long-Term Facilitation during a Time Period of Several Hours

For synaptic tagging or capture to occur, both the tag and the products of gene expression induced by repeated pulses of 5-HT must persist long enough to interact with one another. The time it takes for these products of gene expression to reach a tagged synapse can be broken down into four components: retrograde signaling from the synapse that is specifically activated to the nucleus to turn on gene expression, gene expression itself (transcription and translation), anterograde transport of the products of gene expression to the synapse that captures the gene products, and functional incorporation of these newly synthesized molecules. To begin to define the kinetics of each of these processes, we carried out two complementary sets of experiments. In the first set, we asked, How long then does the marking signal persist? To address this question, we gave one pulse 5-HT immediately, 1 hr or 2 hr before the five pulses of 5-HT, and found that it produced successful capture of long-term facilitation (see Figure 5A). However, when one pulse preceded the five pulses by 4 hr, no facilitation occurred. These findings indicate that the "tag" set up by the single pulse of 5-HT persists for between 4 and 5 hr.

In the second set of experiments, we asked, What is the time window during which the single pulse of 5-HT is effective in recruiting synapse-specific facilitation? As shown in Figure 5A, if given either immediately or 1 hr after five pulses of 5-HT, a single pulse can capture the facilitation fully at 24 hr. If, however, the single pulse was given 4 hr after the five pulses, no long-term facilitation occurred. These findings indicate that the products of gene expression induced by 5-HT persist between 1 and 4 hr.

In our cultures, the average distance between each sensory cell synapse and nucleus is 500 μm. From the rate of fast retrograde and anterograde axonal transport in *Aplysia* neurons (Goldberg and Schwartz, 1980), one can estimate that approximately 15 min would be needed for the retrograde signal to reach the nucleus, and only 6–15 min would be needed for the products of gene expression to be transported from the nucleus back to the synapse. These data suggest that retrograde and anterograde transport are not temporally limiting.

The Mark for Synaptic Growth Is Dependent on PKA and Is Stabilized by Local Protein Synthesis

The 5-HT receptor in sensory neurons is positively coupled to adenylate cyclase, and the 5-HT-induced activation of PKA in sensory cells is required for short- and long-term facilitation. Thus, to begin to analyze the molecular nature of the mark produced by a single pulse of 5-HT, we asked whether the mark required PKA. As shown in Figure 5B, the PKA inhibitor RpcAMP completely blocked the synaptic capture normally produced at 24 hr by a single pulse of 5-HT. We also asked whether synapse-specific facilitation depended on PKA activation. As shown in Figure 5C, Rp-cAMP also blocked synapse-specific facilitation at 24 hr.

We have previously shown that synapse-specific facilitation at 24 hr requires local protein synthesis, but that synaptic capture at 24 hr does not (Martin et al., 1997a). We now examined whether local protein synthesis was required to stabilize synaptic capture at 72 hr. When the protein synthesis inhibitor emetine was applied to the synapse that received a single 5-HT pulse, there was an increase in EPSP amplitude at the "marked" synapse at 24 hr, but that facilitation relaxed to baseline by 72 hr (Figure 6A). Thus, whereas local protein synthesis is not required for synaptic capture at 24 hr, it is required at 72 hr.

These findings suggested, as one possibility, that synaptic growth required local protein synthesis, and that facilitation at 72 hr was blocked because the growth was blocked by inhibiting protein synthesis. To test this possibility, we imaged carboxyfluorescein-filled sensory cells and then treated one branch with five pulses of 5-HT and the other branch with a single pulse of 5-HT in the presence of inhibitors of protein synthesis. To our surprise, we found that although local protein synthesis inhibition blocked facilitation at 72 hr, it did not block synaptic growth at 24 hr (Figures 6B and $7C_2$). However, at the captured synapse the growth did not persist but returned to baseline by 72 hr (Figure $7C_3$). These results indicate that local protein synthesis is not required to initiate synaptic growth but is required for the stabilization and persistence of that growth.

The Signal for Synaptic Stabilization Involves Rapamycin-Sensitive Protein Synthesis

To begin to analyze the molecular mechanisms whereby 5-HT regulates translation, we were struck by the finding that in marking the synapse for synaptic growth, 5-HT, cAMP, and PKA act like growth factors. Growth factors are known to stimulate protein synthesis and frequently do so by means of a particular signal transduction pathway that is sensitive to the immunosuppresant rapamycin (Brown and Schreiber, 1996). 5-HT-induced increases in translation have been shown to be rapamycin sensitive in dissected *Aplysia* pleural ganglia (Yanow et al., 1998). To test whether translation in isolated sensory cell neurites was also sensitive to rapamycin, we cultured 30–40 sensory cells, removed their cell bodies, and performed metabolic labeling. As shown in Figure 5C, a 1 hr incubation with 5-HT induced a 2.5-fold increase in translation ($p < 0.01$, Student's t test). Incubation of untreated processes with rapamycin reduced basal translation by 36% and reduced 5-HT-stimulated translation by 50%. To estimate how much rapamycin reduced the 5-HT-stimulated portion of translation, we subtracted the amounts attributable to basal translation from 5-HT-induced translation and found that 60% of the 5-HT-stimulated translation was rapamycin sensitive.

change in EPSP amplitude ± SEM and of the varicosity number ± SEM in each branch, respectively (D_1 and D_2: at 24 hr, $n = 5$; at 72 hr, $n = 6$) (**, $p < 0.001$; *, $p < 0.05$, change in EPSP amplitude in the branch treated with one 5-HT pulse versus the opposite branch, Student's t test, and change in varicosity number the branch treated with one 5-HT pulse versus the opposite branch, Student's t test).

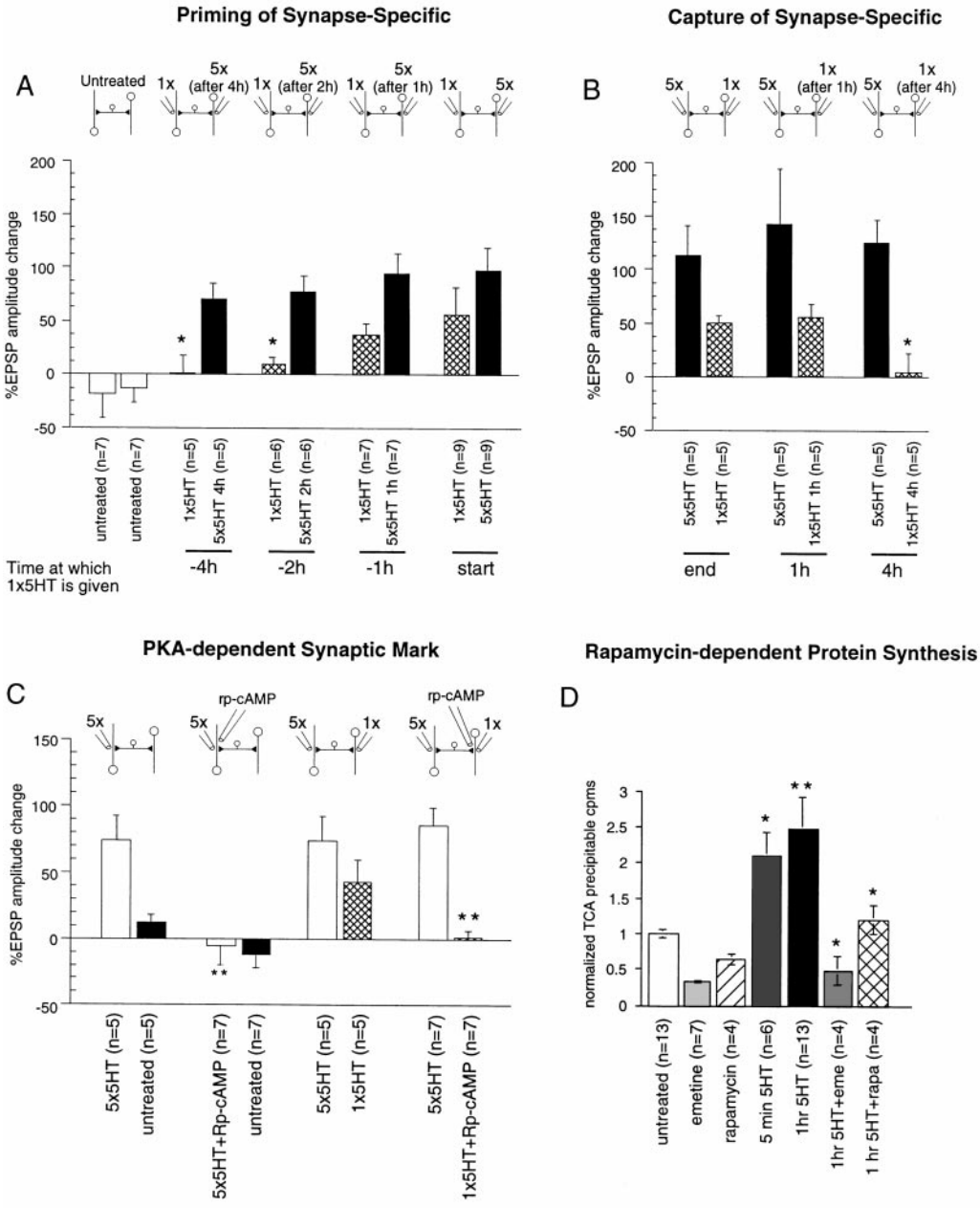

Figure 5. A Single Pulse of 5-HT Can Capture Long-Term Facilitation Only during a Discrete Time Window of Several Hours and in a PKA-Dependent Manner

(A and B) A single pulse of 5-HT can capture long-term facilitation only during a discrete time window of several hours. To study the kinetics of the marking signal necessary for capture, we performed two complementary series of experiments, shown in (A) and (B). To measure how long the marking signal for capture persisted, we applied a single pulse of 5-HT to one branch before applying five pulses of 5-HT to the opposite branch (A). A single pulse of 5-HT to one branch was able to capture long-term facilitation when applied 2 hr, 1 hr, or immediately but not 4 hr before five pulses of 5-HT to the opposite branch (A) (*, $p < 0.05$, change in EPSP amplitude in the branch treated with one 5-HT pulse versus the branch treated with five 5-HT pulses, ANOVA and Neuman-Keul's multiple range test). To measure the time window during which the single pulse of 5-HT is effective in capturing the long-term process, we applied a single pulse of 5-HT to one branch after applying five pulses of 5-HT to the opposite branch. A single pulse of 5-HT was able to capture long-term facilitation when applied immediately or 1 hr but not 4 hr after five pulses of 5-HT to the opposite branch (B) (*, $p < 0.05$, change in EPSP amplitude in the branch treated with one 5-HT pulse versus the branch treated with five 5-HT pulses, ANOVA and Neuman-Keul's multiple range test).

(C) Synapse-specific facilitation and synaptic capture require local PKA activity. Perfusion of the PKA inhibitor Rp-cAMP for 20 min prior to, during, and for 20 min after the five pulses of 5-HT blocked synapse-specific facilitation. Perfusion of Rp-cAMP for 20 min prior to, during, and for 20 min after the single pulse of 5-HT blocked capture (**, $p < 0.01$, change in EPSP amplitude in the branch treated with 5-HT and Rp cAMP versus the branch treated with five 5-HT pulses, ANOVA and Neuman-Keuls multiple range test).

(D) Translation in isolated presynaptic sensory cell neurites stimulated by a 1 hr or a 5 min application of 5-HT is inhibited by emetine and partially inhibited by rapamycin. Sensory cells were cultured for 5 days. The cell body of each neuron was severed and removed, and the processes were labeled with ^{25}S methionine. Emetine (200 μm) or rapamycin (20 nM) was present for 30 min prior to and during the metabolic

To determine whether this rapamycin-dependent component of local protein synthesis was required to stabilize the mark for long-term facilitation and growth, we perfused rapamycin onto the set of synapses treated with a single pulse of 5-HT. Rapamycin alone had no effect on basal synaptic transmission (data not shown) but blocked the marking component affected by emetine. Synaptic capture was not affected at 24 hr but was completely blocked at 72 hr (Figure 6C). Rapamycin perfusion also blocked growth in the captured branch at 72 hr but not at 24 hr (Figure 6D). This rapamycin effect was presynaptic: gelonin injected into the postsynaptic motor neuron did not block synaptic capture (data not shown). These results demonstrate that the stabilization of growth requires a rapamycin-sensitive component of local protein synthesis. They also imply that a single pulse of 5-HT is able to induce translation in sensory cell processes. As shown in Figure 5C, a single pulse of 5-HT produced a 2-fold increase in TCA-precipitable counts as compared with untreated cultures ($p < 0.05$, Student's t test).

We previously found that the establishment of synapse-specific facilitation was blocked even at 24 hr by inhibiting local protein synthesis. This suggested that the retrograde signal back to the nucleus necessary to turn on transcription might require local protein synthesis (Martin et al., 1997a). To test whether the component of local protein synthesis required for retrograde signaling also was rapamycin sensitive, we perfused rapamycin onto the serotonin-treated synapses and found that while long-term facilitation was not affected at 24 hr, it was completely blocked at 72 hr (Figure 6E). Similarly, rapamycin did not block the growth of varicosities at 24 hr but completely blocked them at 72 hr (Figure 6F). These results show that the retrograde signal necessary to induce synapse-specific long-term facilitation is generated by an emetine-sensitive, rapamycin-insensitive component of local protein synthesis. In contrast, the stabilization of synaptic growth and the persistence of long-term facilitation are generated by a rapamycin-sensitive component of local protein synthesis.

Taken together, these data demonstrate that 5-HT is able to activate local protein synthesis and that this local synthesis has two functions. One function is to stabilize plastic changes. This is achieved by one pulse of 5-HT and requires rapamycin-sensitive protein synthesis. In addition, local application of five pulses of 5-HT induces a rapamycin-insensitive protein synthesis that turns on, perhaps via the generation of a retrograde signal to the nucleus, a transcription-dependent long-term facilitation.

Discussion

Four Forms of CREB-Mediated Facilitation

Long-lasting forms of synaptic plasticity, as well as long-lasting behavioral changes, require the transcription of genes in the nucleus. We previously found that a single presynaptic cell can undergo a transcription-dependent, synapse-specific, long-term plasticity and that this plastic process can be captured at a second stimulated (marked) synapse (Martin et al., 1997a). To explore further the process of capture and the family of mechanisms that give rise to facilitation, we now show that stimulation restricted to the cell body of a sensory neuron is sufficient to induce long-term facilitation and can do so in the complete absence of short-term facilitation at any other synapse of the neuron (see also Clark and Kandel, 1993; Emptage and Carew, 1993). Like the synapse-specific process, cell-wide facilitation requires CREB-mediated transcription. However, whereas branch specific facilitation is associated with the growth of new synaptic connections and persists beyond 72 hr, cell-wide facilitation is not associated with significant growth and decays within 48 hr. However, once the cell-wide process has been triggered by five pulses of 5-HT at the cell body, a single pulse of 5-HT applied to any one synapse can capture growth at that synapse, thereby converting a 24 hr cell-wide process into a more persistent synapse-specific form.

These studies demonstrate that CREB-mediated long-term plasticity is not a unitary phenomenon. In fact, the same neuron can undergo four different CREB-mediated forms of long-term synaptic plasticity, depending on the site and the number of repetitions of modulatory input: synapse-specific long-term facilitation, capture of the synapse-specific form, cell-wide long-term facilitation, and capture of the cell-wide form. Although all four processes share at least one common nuclear mechanism (CREB-mediated transcription), each has distinct properties. Synapse-specific long-term facilitation initiated by five repeated pulses of 5-HT stimuli at a synapse is accompanied by growth, persists for at least 72 hr, and requires local protein synthesis for both its retrograde signal and for the stabilization of growth. Capture of the synapse-specific long-term facilitation by a marked synapse is smaller in amplitude than synapse-specific facilitation but is also accompanied by growth, is persistent, and requires local protein synthesis only for stabilization. Cell-wide long-term facilitation, generated by repeated modulatory stimuli at the cell body, is transient. This cell-wide long-term facilitation, however, can be captured by a marking signal that stimulates growth and converts the facilitation to a persistent form.

Dissociation of Functional Synaptic Plasticity from the Growth of New Synaptic Connections

The finding that cell-wide, CREB-dependent facilitation can occur in the absence of any synaptic growth was unexpected, since long-lasting increases in synaptic strength and long-term facilitation have been thought to result, at least in part, from the growth of new synaptic connections. How, then, might an increase in synaptic

labeling, and 5-HT (10 μm) was present during the metabolic labeling for the 1 hr incubation and for the final 5 min of the metabolic labeling for the 5 min incubation. The cells were then TCA precipitated, and the TCA insoluble counts were counted on a scintillation counter. Total cpm were divided by the number of cells in each dish. The mean total cpms/untreated dish was 231,300 ± 62,600. Shown is a histogram of the mean counts ± SE, normalized to the counts in the untreated dishes. (Planned t tests: **, $p < 0.01$, untreated versus 1 hr 5-HT-treated processes; *, $p < 0.05$, untreated versus 5 min 5-HT-treated processes, 1 hr 5-HT-treated versus 1 hr 5-HT plus emetine-treated processes and versus 1 hr 5-HT− and rapamycin-treated processes.)

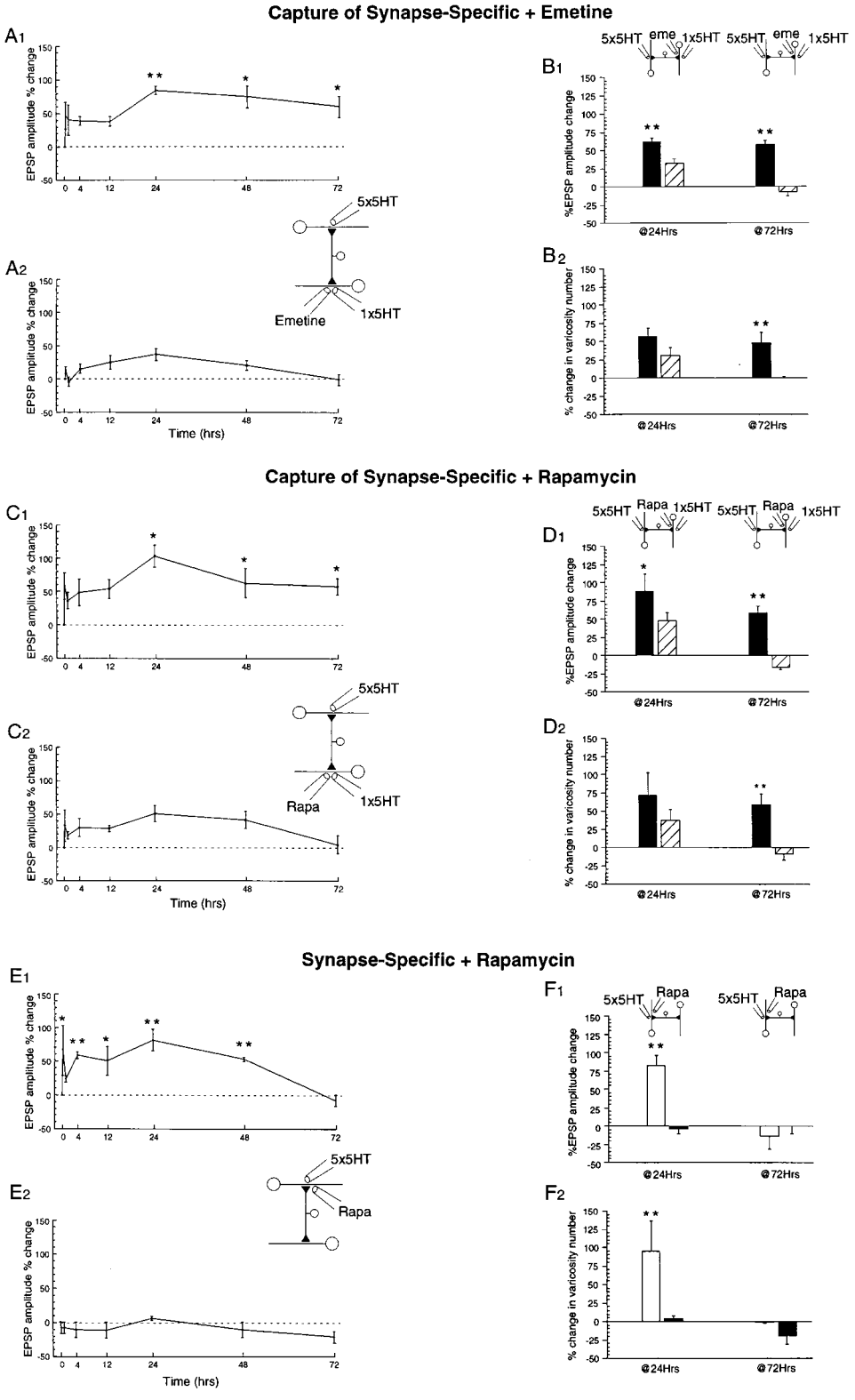

Figure 6. The Marking Signal for Synapse Stabilization Involves Rapamycin-Sensitive Protein Synthesis, While the Retrograde Signal Requires Rapamycin-Insensitive Protein Synthesis

To test whether local protein synthesis was required to mark synapses for stabilization of long-term facilitation and growth, we continuously perfused emetine (eme) or rapamycin (rapa) onto the set of synapses treated with a single pulse for 20 min prior to, during, and 20 min following 5-HT application, immediately after five spaced pulses of 5-HT to the opposite branch. We found that neither emetine nor rapamycin

strength that requires transcription and translation persist for 24 hr in the absence of synaptic growth?

One possibility is the recruitment of "silent synapses" (Bailey and Chen, 1983; Isaac et al., 1995). In the intact animal, approximately 60% of the presynaptic varicosities of the sensory neurons do not have active release sites. When long-term sensitization is produced in the animal, it leads to both an increase in the number of varicosities and to an increase in the percentage of varicosities that have active zones (Bailey and Chen, 1983, 1988). Whereas the increase in varicosity number persists as long as the memory, changes in the size of active zones only last a few days (Bailey and Chen, 1989). Since only 50% of the varicosities in cultured sensory neurons have active zones (Glanzman et al., 1990), cell body application of 5-HT might change synaptic strength at 24 hr by increasing the incidence of active zones (see also Sun and Schacher, 1998).

CREB Is Necessary but Not Sufficient for Persistent Facilitation and Synaptic Growth

CREB-mediated transcription appears to be necessary for the initial establishment of all four forms of synaptic plasticity, but it is not sufficient for the self-maintained stabilization of the plastic changes. To obtain persistent facilitation and the growth of new synaptic connections, one also needs a single pulse of 5-HT applied to the synapse. This single pulse by itself is only able to induce short-term facilitation. But in a cell where CREB-mediated transcription is induced by repeated stimuli, the single pulse also marks a synapse so as to allow long-term facilitation to become persistent and to be accompanied by growth of new terminals. Thus, one of the surprising functions of the short-term process is to convert a transient long-term process into a persistent one. These results illustrate again, as did our earlier studies, that the short-term process serves two functions (Figure 8). First, a single pulse of 5-HT alone produces a selective synapse-specific enhancement of synaptic strength that contributes to short-term memory lasting minutes. Second, in conjunction with the activation of CREB by repeated stimuli given to any other synapse or to the cell body, a single pulse of 5-HT will also act to "mark" that synapse for persistent synaptic facilitation and growth.

There Are Two Components to the Marking Signal: A Covalent PKA-Mediated Component for the Initiation and a Rapamycin-Sensitive Protein Synthesis–Dependent Component for Stabilization

Studies of the molecular nature of the mark necessary for synapse-specific facilitation and for capture suggest that there are two components to the marking signals: a covalent mark for the initiation of synapse-specific plasticity, mediated by PKA, and a mark for its stabilization, mediated by local protein synthesis (Figure 8). The initiation of synapse-specific facilitation is reflected by the facilitation (both functional and morphological) expressed at 24 hr, while the stabilization is reflected by the facilitation (both functional and morphological) expressed at 72 hr.

Translation of messenger RNAs can be regulated in a number of ways that act on either the 5′ or 3′ end of the message. In response to growth factors such as cytokines and insulin, there is a 2- to 3-fold increase in protein synthesis, yet only a small subset of transcripts (10%–15%) are preferentially stimulated (Brown and Schreiber, 1996). The translation of this specific subset of mRNAs is enhanced by means of a signaling pathway that is blocked by rapamycin. Rapamycin acts on the kinase RAFT/TOR, which is critical for the phosphorylation of the S-6 kinase. Through inactivation of the S-6 kinase, rapamycin reduces the translation of a set of mRNAs containing an oligopyrimidine tract immediately after the N^7 methyl guanosine cap. The family of mRNAs that contain the polypyrimidine tract includes proteins that are themselves important components of the translational machinery, such as ribosomal proteins (S3, S6, S14, and S24) and translational elongation factors eF-1α and eF-1β (Jeffries and Thomas, 1996). Rapamycin also blocks the phosphorylation of eIF4EBP, which affects the translation of another class of mRNAs containing a high degree of secondary structure in their 5′ ends, such as cyclin D (Rousseau et al., 1996). Rapamycin has recently been shown to cause degradation of

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affected synaptic capture at 24 hr but that both completely blocked it at 72 hr. The time course of emetine-treated cells is shown in (A_1) and (A_2). (A_1 and A_2: 10 min, n = 5; 1 hr, n = 4; 4 hr, n = 4; 12 hr, n = 4; 24 hr, n = 8; 48 hr, n = 4; 72 hr, n = 8.) (*, $p < 0{,}05$; **, $p < 0.01$, change in EPSP amplitude at the one branch versus the opposite branch, Student's t test.) The time course of rapamycin-treated cells is shown in (C_1) and (C_2). (C_1 and C_2: 10 min, n = 5; 1 hr, n = 4; 4 hr, n = 4; 12 hr, n = 5; 24 hr, n = 7; 48 hr, n = 4; 72 hr, n = 5.) (*, $p < 0.05$, change in EPSP amplitude at the one branch versus the opposite branch, Student's t test.) Carboxyfluorescein-filled sensory neurons treated according to the capture protocol and perfused with emetine or rapamycin on the capturing synapse were imaged as described above. (B_1), (B_2), (C_1), and (C_2) show histograms of the mean change in EPSP amplitude ± SEM and of the varicosity number ± SEM in each branch of emetine- or rapamycin-treated cells, respectively. (B_1 and B_2: at 24 hr, n = 6; at 72 hr, n = 7. D_1 and D_2: at 24 hr, n = 6; at 72 hr, n = 4). Both emetine and rapamycin did not affect the capture related growth of new varicosities at 24 hr, but completely blocked it at 72 hr. (*, $p < 0.05$; **, $p < 0.01$, change in EPSP amplitude in the branch treated with one 5-HT pulse and rapamycin or emetine versus the branch treated with five 5-HT pulses, Student's t test).

We continuously perfused rapamycin onto the set of synapses treated with five pulses of 5-HT for 20 min prior to, during, and 20 min following 5-HT application, and we found that rapamycin did not affect long-term facilitation at 24 hr, but completely inhibited it at 72 hr. The time course of rapamycin-treated cells is shown in (E_1) and (E_2). (E_1 and E_2: 10 min, n = 5; 1 hr, n = 4; 4 hr, n = 3; 12 hr, n = 4; 24 hr, n = 7; 48 hr, n = 3; 72 hr, n = 8.) (*, $p < 0{,}05$; **, $p < 0.01$, change in EPSP amplitude at the one branch versus the opposite branch, Student's t test.) Carboxyfluorescein-filled sensory neurons treated with five synaptic pulses of 5-HT and perfused with rapamycin were imaged as described above. (F_1) and (F_2) show histograms of the mean change in EPSP amplitude ± SEM and of the varicosity number ± SEM in each branch of rapamycin-treated cells, respectively. (B_1 and B_2: at 24 hr, n = 6; at 72 hr, n = 7. D_1 and D_2: at 24 hr, n = 6; at 72 hr, n = 4.) Rapamycin did not affect the growth of new varicosities at 24 hr, but completely blocked it at 72 hr. (**, $p < 0.01$, change in EPSP amplitude in the branch treated with five 5-HT pulses and rapamycin versus the untreated branch, Student's t test).

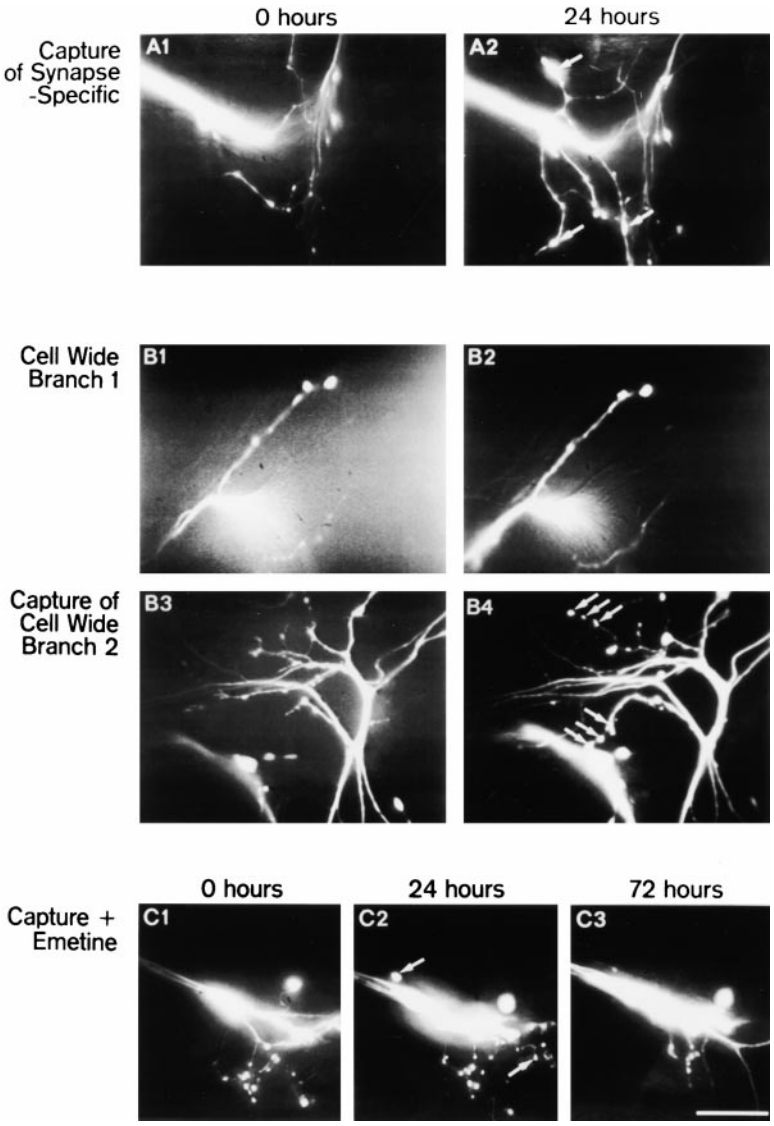


Figure 7. Structural Plasticity Associated with Synaptic Capture

Carboxyfluorescein-filled sensory neurons were imaged at their region of contact with the axon hillock of L7 motor neurons before (A_1, B_1, B_3, and C_1) and 24 hr after 5-HT treatment (A_2, B_2, B_4, and C_2). Clear synaptic growth (arrows point to new varicosities) occurs 24 hr following the capture of synapse-specific long-term facilitation (A_2). By contrast, cell-wide long-term facilitation is not associated with synaptic growth (compare B_1 and B_2). However, a single pulse of 5-HT given to one branch (branch 2) following five pulses of 5-HT at its soma now induces the growth of new varicosities at the captured branch (compare B_3 and B_4). The growth associated with synaptic capture can be induced in the presence of the protein synthesis inhibitor emetine and is present at 24 hr (C_2) but returns to baseline by 72 hr (C_3). These results suggest that local protein synthesis is not required for synaptic growth but rather for the stabilization and persistence of the structural change.

All the micrographs are composed of superimpositions of labeled sensory neurite images taken from all focal planes of the view area. As a result, the shape of individual varicosities may be obscured. Bar, 40 μm.

eIF4G, a component of mRNA cap recognition complex eIF4F (Berset et al., 1998). Unfortunately, rapamycin is not completely specific and can have effects on cell functions other than translation.

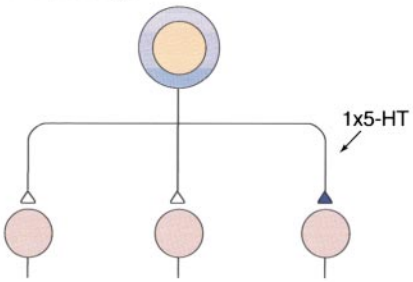
When emetine is continuously perfused onto the synapses that receive five pulses of 5-HT, the block of long-term facilitation already is evident at 24 hr, indicating that unlike synaptic capture, local protein synthesis for the induction of long-term facilitation is required early and may therefore be necessary to initiate the retrograde signal from the synapse to the nucleus. This presynaptic retrograde signal is likely to be important for activating CREB-dependent transcription in the nucleus and for the translation of the new proteins required for the expression of the long-term plasticity. These newly expressed proteins are then shipped back not only to the original stimulated synapse (where they are captured and incorporated into functional release sites), but to all synapses. This component of protein synthesis can be blocked by emetine but not by rapamycin.

Thus, local protein synthesis has two components that serve two distinct functions at two different sites. At the site of initiation for synapse-specific facilitation, a non-rapamycin-sensitive component of local protein synthesis is required for the retrograde signal. At both this site and at the site of capture, rapamycin-sensitive local protein synthesis is required to mark the synapse for persistence of the functional and structural change.

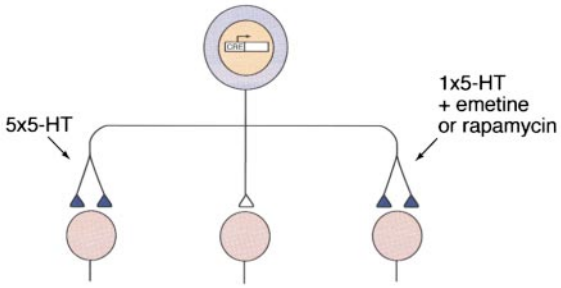
Synaptic Capture Reveals a New Dimension to the Integrative Capabilities of Neurons: Nuclear-Mediated Temporal and Spatial Summation

Our finding suggests that activation of transcription by one set of synapses in a synapse-specific way activates downstream genes that affect all nonactivated synapses, any one of which presumably can now be recruited by a single pulse of 5-HT. Moreover, this period of capture persists in time over a period of between 1 and 4 hr (see also Frey and Morris, 1998) and extends in space over distances between synapses of hundreds of microns. Retrograde signals, nuclear loops, marking

1. Short term memory storage



2. Covalent marking for 24 hr transient process with growth of new synapses



3. Marking for local protein synthesis-dependent persistence of new synapses

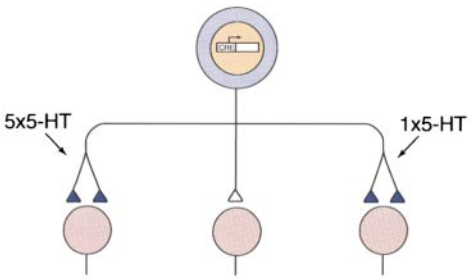


Figure 8. The Three Different Functions of Short-Term Facilitation

signals, and time windows of 1 to 4 hr for capture introduce a new temporal and spatial dimension in the integrative actions of the neuron. To give but one example, the classical temporal and spatial summative processes of neurons typically involve the associative interaction of signals separated by seconds. This is evident, for example, in the associative activation of the NMDA receptor by glutamate and voltage, or the associative enhancement of facilitation by 5-HT and activity. These forms of temporal summation allow short-term associative influences of the sort required for behavioral associative events such as Pavlovian conditioning (Hawkins et al., 1983). As these arguments make clear, capture, which can persist for 1 to 4 hr, adds a new dimension to the temporal summative capabilities of a single neuron.

What might be the function of such long-range and long-term associations? One possibility is that they are important for arousal or for flashbulb memories, for charged events that produce a memory for relevant events that occurred during a period of hours surrounding an important event. Similarly, there are forms of learning such as bait shyness with a long conditioned stimulus–unconditioned stimulus interval that could be accommodated by this mechanism.

This new level of integration raises a further question: Will this integrative action also coordinate the action of inhibitory or facilitatory input? The sensory neurons undergo presynaptic inhibition, induced by the peptide FMRFamide. Is inhibition also branch specific, and if so, what are the interactive mechanisms that coordinate long-term inhibition and facilitation?

Experimental Procedures

Aplysia Cell Culture

Culture dishes and medium were prepared as previously described (Montarolo et al., 1986). Bifurcated sensory neuron–motor neuron cultures were prepared as described in Martin et al. (1997a). Cultures were maintained for 5 days in an 18°C incubator.

Electrophysiology

After 5 days in culture, the strength of the synapse between the bifurcated sensory neuron and each one of the postsynaptic motor neurons was measured as previously described (Martin et al.,

1997a). Bath application of 5-HT was performed as previously described (Montarolo et al., 1986). Local application of 5-HT was performed as previously described (Martin et al., 1997a). To measure short-term facilitation, EPSPs were measured in both motor neurons 10 min after the end of the 5-HT application; to measure long-term facilitation, EPSPs were recorded 24 hr after 5-HT application. In time course experiments, EPSPs were measured at the indicated time points.

In experiments using actinomycin D, the drug was included in the perfusion medium at a concentration of 50 μg/ml for 30 min before, during, and 30 min after 5-HT treatment. Emetine (100 μM) and rapamycin (20 nM) were locally applied to the area of interest using a second perfusion microelectrode connected to a second picospritzer using very low pressure (1 PSI) for 20 min before, during, and 20 min after 5-HT application. Gelonin (25 μM), anti-CREB antibodies (described in Bartsch et al., 1998), and preimmune serum were diluted in 0.5 KCl, 10 μM Tris (pH 7.6) and injected into sensory neurons with microelectrodes (15–20 MΩ). Recombinant phospho-CREB1 was prepared and microinjected as previously described (Bartsch et al., 1998). All microinjections were performed 1 hr before the beginning of the 5-HT treatment.

All data are presented as mean percentage change ± SEM in the EPSP amplitude measured after treatment. A one-way analysis of variance and Newman Keuls' multiple range test were used to determine the significance of the changes in EPSP amplitude.

Dye Injection, Cell Imaging, and Quantitation of Structural Changes

Dye injection, cell imaging, and quantitation of structural changes were performed as previously described (Bartsch et al., 1995).

Metabolic Labeling

Metabolic labeling experiments were performed as previously described (Martin et al., 1997a). In experiments in which a single 5-HT pulse was given, it was given at the end of a 1 hr labeling with [^{35}S]methionine. In rapamycin experiments, processes were incubated with 20 nM rapamycin for 30 min before and during the 1 hr 5-HT application.

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