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Induction of long-term facilitation in *Aplysia* sensory neurons by local application of serotonin to remote synapses

(long-term facilitation/synapse-specific facilitation/sensitization)

GREGORY A. CLARK* AND ERIC R. KANDEL†

*Program in Neuroscience, Psychology Department, Green Hall, Princeton University, Princeton, NJ 08544; and †Howard Hughes Medical Institute, Center for Neurobiology and Behavior, College of Physicians and Surgeons of Columbia University, and New York State Psychiatric Institute, 722 West 168th Street, New York, NY 10032

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ABSTRACT **Long-term synaptic facilitation at the connections of *Aplysia* sensory neurons onto their target cells involves alterations in gene expression. How then are the relevant cellular signals for the induction and expression of long-term synaptic changes conveyed between the nucleus and remote synaptic terminals? We have explored this question using a set of remote, peripheral terminals of siphon sensory cells, which are ≈3 cm from the sensory cell body in the abdominal ganglion. We found that these remote synapses, like the proximal synapses previously studied in dissociated cell culture, can exhibit long-term facilitation 24 hr after cell-wide serotonin application. Furthermore, serotonin applications restricted to the remote synaptic terminals nevertheless produced long-term facilitation, indicating that signals generated in synaptic regions can trigger the long-term process, perhaps via retrograde signals to the nucleus to modify gene expression, followed by anterograde signals back to the terminal. Serotonin applications restricted to the cell body and proximal synapses of the sensory neuron also produced long-term facilitation at remote synapses, although to a lesser extent, suggesting that long-term facilitation is expressed cell-wide, but that superimposed on this cell-wide facilitation there appears to be a component that is synapse-specific.**

The gill- and siphon-withdrawal response to a tactile siphon stimulus in the marine mollusc *Aplysia californica* exhibits both short-term sensitization, lasting minutes to hours (1), and long-term sensitization, lasting days to weeks (2, 3). Morphological and physiological evidence indicates that both long-term and short-term sensitization involve presynaptic facilitation at connections from mechanosensory sensory cells onto their postsynaptic targets (3, 4) and that this facilitation involves activation by serotonin (5-HT) and possibly other facilitatory transmitters of the cAMP second-messenger pathway (5–14). But whereas short-term facilitation involves covalent modifications of preexisting proteins and is unaffected by inhibition of protein synthesis (5, 15, 16), long-term facilitation (≥24 hr) requires new protein synthesis and alterations in gene expression (5, 11, 16–23).

How are these genomic processes that are recruited for long-term facilitation coordinated with changes in synaptic regions? This question becomes particularly important for synapses remote from the nucleus. Does the induction of long-term facilitation require that 5-HT act directly on the cell body, which contains the machinery for mRNA and protein synthesis? Or can 5-HT produce long-term facilitation when applied selectively only to remote synaptic regions?

To explore these issues we took advantage of the fact that siphon sensory cells synapse not only onto central siphon and gill motor neurons, located in the abdominal ganglion near the cell body of the siphon sensory cells, but also onto peripheral siphon motor neurons, located at the distal end of the siphon nerve, 3–5 cm away (24). We previously had used this preparation to report (25) that 5-HT application restricted to either central or peripheral synapses was sufficient to produce facilitation and that this facilitation was specific to the exposed synapses. Synapse-specific facilitation was also produced by activation of facilitatory interneurons that projected selectively to one set of synapses, but not to the other set. More recently, we also found that restricted synaptic applications of the inhibitory neuromodulatory peptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) can produce short-term synapse-specific inhibition (26).

In the present studies, we have asked: Where must 5-HT be applied to induce long-term facilitation, which (unlike short-term facilitation) depends on changes in gene expression? We find that five pulses of 5-HT applied to the remote peripheral synapses of the siphon sensory cells leads to the expression of long-term facilitation at these synapses when tested 24 hr later, indicating that synaptic stimulation can induce long-term facilitation. By contrast, application of 5-HT directly onto the cell body and central synapses of sensory neurons produces less facilitation, suggesting that facilitation may be preferentially expressed at synapses previously exposed to 5-HT. Preliminary reports of some of these results have appeared (27, 28).

MATERIALS AND METHODS

Adult *Aplysia* were anesthetized by cooling and injection of isotonic $MgCl_2$. We then dissected the abdominal ganglion (containing the siphon sensory cell somata and central postsynaptic targets) and siphon nerve (including peripheral siphon motor neurons at its distal end) and pinned the preparation to the Sylgard-coated floor of a recording chamber. Dissections were done in a cooled, high-$MgCl_2$ (200 mM), low-Ca^{2+} (1 mM) solution. To facilitate subsequent intracellular recordings, the connective tissue sheath covering the abdominal ganglion was partially removed, and the distal end of the siphon nerve was treated with 0.2% porcine trypsin (type IX; Sigma) for 6–8 min at room temperature, followed by 10 min of 1% type II-S trypsin inhibitor. The dissecting medium was then replaced with a resting solution composed of *Aplysia* hemolymph, mixed 1:1 with modified L-15 medium that had been adjusted to appropriate salt concentrations and supplemented with antibiotics and glutamine (5). Preparations were kept at 18 ± 1°C throughout all subsequent resting and testing periods.

Physiological testing began ≈4 hr after the end of dissection. In brief, we measured the amplitudes of excitatory postsynaptic potentials (EPSPs) from individual sensory neu-

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Abbreviations: EPSP, excitatory postsynaptic potential; 5-HT, serotonin; ASW, artificial sea water.

rons onto peripheral motor neurons both before (day 1) and 24 hr after (day 2) application of 5-HT (in the form of 5-HT creatinine sulfate) or control applications of recording medium. Experiments were conducted in a recording medium of enriched artificial seawater (ASW) consisting of modified L-15 medium (without hemolymph or glutamine), mixed with equal parts of culture medium with the following composition: 460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, 10 mM Hepes·NaOH, supplemented with amino acids, vitamins, and sugars. For each preparation, we first impaled two peripheral motor neurons and then sampled connections from as many sensory neurons as possible (usually 10–20 cells), using conventional intracellular recording and stimulation techniques. (We did not record from central siphon motor neurons in these experiments.) To ensure that we measured the size of the first synaptic potential, we recorded all sensory neuron penetrations on magnetic tape to allow subsequent playback if necessary. After impalement, sensori–motor connections were tested by intracellular stimulation of the sensory neurons at 20- to 30-sec intervals. Synaptic potentials with initial amplitudes of 2–25 mV were accepted for further experimentation.

To examine whether the remote peripheral synapses exhibit long-term facilitation, we first compared the effects of five applications of 5-HT onto both the abdominal ganglion and siphon nerve (cell-wide 5-HT condition) to effects of sham additions of recording medium. 5-HT was bath-applied via pipette at 100 μM and mixed to a final concentration of 30 μM; each application lasted 5 min and was followed by a 15-min washout period (≈5 to 10 bath volumes). We next wished to determine more precisely the effective site of 5-HT application. For these experiments, during dissection the sensory cell soma in the abdominal ganglion was isolated from the peripheral sensori-motor synapses by threading the siphon nerve through a slit in a plastic wall that divided the recording chamber in two. The slit was then sealed with petroleum jelly, allowing the ganglion and siphon nerve to be perfused independently. After initial testing of synaptic connections, we applied 20–50 μM 5-HT selectively onto either (*i*) the abdominal ganglion, containing the sensory cell somata and central targets (somatic 5-HT application) or (*ii*) onto the distal end of the siphon nerve, containing the sensory cell connections onto peripheral motor neurons (synaptic 5-HT application). Control preparations received sham applications of recording medium, as did the alternate portion of the preparation in the somatic 5-HT and synaptic 5-HT conditions. At the end of each recording session, several nearby neurons were labeled with intracellular pressure injection of fast green dye to help identify the sensory neurons to be retested the following day, and recording solution was replaced with resting solution. Approximately 24 hr later (day 2), resting solution was replaced with recording solution, the same peripheral motor neurons and sensory neurons were reimpaled, and amplitudes of the same synaptic connections were retested.

Statistical analyses were conducted on the amplitude of the first synaptic potential elicited for each synapse on days 1 and 2. The mean facilitation was computed across the different synapses for each preparation, so that each preparation contributed one score to the overall analysis. Overall analyses used *t* tests for repeated measures or independent means, as appropriate, with probability levels adjusted for multiple comparisons. Data are expressed as mean ± SEM. To quantify the number of individual preparations that showed increases in EPSP amplitude that were significantly greater than the nonspecific increases seen in controls, we counted preparations showing increases in EPSP amplitude >1.64 SDs above the relevant control group mean (i.e., increases greater than would occur in 95% of the theoretical population for the control group, $P < 0.05$, one-tailed test). We then compared the relative incidence of preparations showing significant facilitation for the different experimental conditions, using a proportions test, as indicated.

RESULTS

Neuron-Wide Application of 5-HT Induces Long-Term Facilitation at Remote Connections of Siphon Sensory Cells. Experiments in dissociated cell culture had demonstrated (5, 6, 8) that repeated applications of 5-HT can produce long-term facilitation at the connections made by sensory neurons onto nearby target cells. However, it was not known whether 5-HT application also produces long-term facilitation in the intact nervous system or whether long-term facilitation is expressed at synapses remote (≈3 cm) from the cell body. We therefore applied 5-HT onto the cell body of the sensory neuron, as well as on its proximal and distal synapses (cell-wide 5-HT application), and found that this application produced robust and consistent facilitation at the remote peripheral synapses [$\bar{x}$ = 74 ± 12% increase, n = 8 (32 synapses), t_7 (subscripts to t indicate statistical degrees of freedom) = 6.38, $P < 0.001$], which was significantly greater than changes in controls (t_{16} = 4.08, $P < 0.001$). Control preparations, which received sham applications of recording

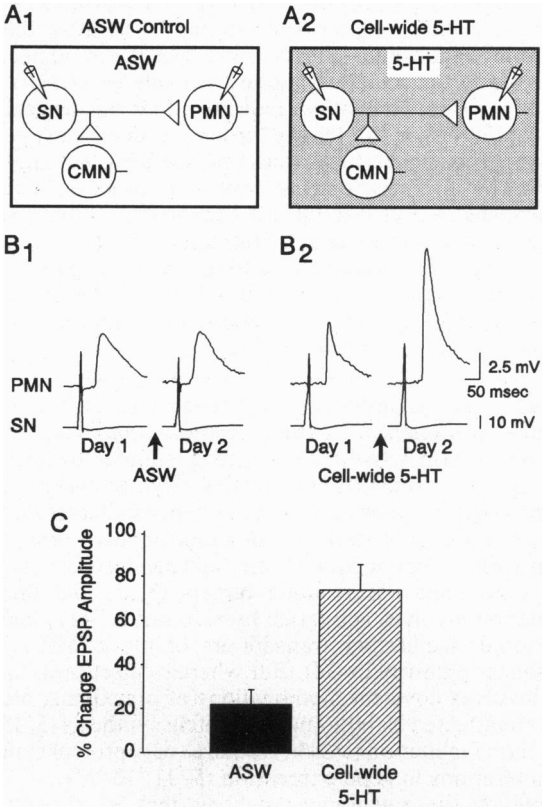

FIG. 1. Cell-wide applications of 5-HT produce long-term facilitation at remote peripheral synapses. (*A*) Schematic of preparation. Monosynaptic EPSPs evoked by stimulating individual siphon sensory cells (SN) were recorded in peripheral siphon motor neurons (PMN) before and 24 hr after application and washout of either ASW as control (A_1), or cell-wide 5-HT bath applied onto the entire preparation at 30 μM (A_2). CMN, central motor neuron. (*B*) Representative examples taken from preparations before (*Left*) and after (*Right*) receiving either ASW (B_1), or 5-HT (B_2). Bottom trace is the action potential triggered by intracellular stimulation in sensory neuron; top trace is the EPSP elicited in peripheral motor neuron (PMN). The long latency to the monosynaptic EPSP is due to conduction time of the siphon nerve. (*C*) Group data, indicating greater facilitation in preparations receiving 5-HT (n = 8) relative to ASW controls (n = 10, $P < 0.001$).

medium, showed a smaller nonspecific increase in the amplitude of the synaptic connections from day 1 to day 2 [$\bar{x}$ = 22 ± 7% increase, n = 10 (42 synapses), $t_9 = 3.33$, $P < 0.01$] (Fig. 1), which may represent either recovery from depression induced by the dissection procedure, a response to our resting medium, or, most likely, a delayed injury-induced enhancement that occurs after axonal injury (29). The input resistance of the peripheral motor neurons was not significantly changed in either the control group ($\bar{x}$ = 9 ± 7% increase, $t_9 = 1.28$, ns) or the cell-wide applications of 5-HT group ($\bar{x}$ = 5 ± 8% decrease, $t_7 = 0.58$, ns), and there was no significant between-group difference ($t_{16} = 1.28$, ns), indicating that facilitation could not be accounted for by a generalized increase in input resistance of the postsynaptic cell (although other postsynaptic mechanisms are possible). These results show that the remote peripheral connections of sensory cells can exhibit long-term facilitation in response to 5-HT, comparable to that reported (5, 8) for proximal synapses in dissociated cell culture.

Application of 5-HT Restricted to Distal Synapses Is Sufficient to Induce Long-Term Facilitation. We next determined where 5-HT had to be applied to induce long-term facilitation. Because the peripheral synaptic terminals of the sensory neurons are physically remote from the sensory neuron soma, it is possible to apply 5-HT selectively onto either the somatic or peripheral synaptic regions. Local application of 5-HT onto the remote peripheral terminals and their targets produced robust facilitation at these connections [$\bar{x}$ = 98 ± 15% increase, n = 14 (73 synapses), $t_{13} = 6.44$, $P < 0.001$], and this facilitation was significantly greater ($t_{26} = 3.10$, $P < 0.01$) than changes that occurred in the control condition [$\bar{x}$ = 43 ± 9% increase, n = 14 (79 synapses), $t_{13} = 4.65$, $P < 0.001$] (Fig. 2). This finding indicates that 5-HT application onto synaptic regions alone, without direct application onto the sensory cell soma, is sufficient to induce long-term facilitation.

When we restricted application of 5-HT to the cell body and central synapses and did not apply 5-HT distally, a relatively modest effect was produced at the unexposed distal synapses [$\bar{x}$ = 73 ± 26% increase, n = 14 (92 synapses), t_{13} = 2.85, $P < 0.05$]. Now only three preparations showed significant facilitation; all of these were also outside the control range. Between-group comparisons indicated that this incidence was higher than that which occurred in the control group (proportions test, $z = 1.95$, $P < 0.05$, one-tailed test). However, synaptic 5-HT application produced a higher incidence of facilitation (8 of 14 preparations) than did somatic 5-HT application ($z = 2.08$, $P < 0.05$, proportions test) and overall was more apt to result in larger increases in EPSP amplitudes (11 of the 14 largest increases, $P < 0.02$, Kolmogorov–Smirnov test). There were no significant changes in input resistance of the postsynaptic motor neurons in any condition and no significant between-group differences (ASW: $\bar{x}$ = 2 ± 5% decrease; somatic 5-HT: $\bar{x}$ = 5 ± 8% decrease; synaptic 5-HT: $\bar{x}$ = 0 ± 9% change). These results indicate that 5-HT application restricted to distal synapses produces reliable long-term facilitation at these connections. Application of 5-HT restricted to the cell body and central synapses may also produce some facilitation but appears somewhat less effective.

DISCUSSION

Induction of Long-Term Facilitation at Remote Synapses. Long-term presynaptic facilitation examined at other synapses of siphon sensory cells requires the synthesis of protein and mRNA (5, 11, 21, 22). Because macromolecular synthesis is almost exclusively somatic, it raises questions about how communication occurs between the cell soma and its synapses, the sites where facilitation is expressed. In particular, where must 5-HT act to induce long-term facilitation? Must it be applied to the cell body, which contains the machinery for macromolecular synthesis, or can 5-HT be applied directly to the synaptic terminals? Will remote synapses, in fact, show long-term facilitation?

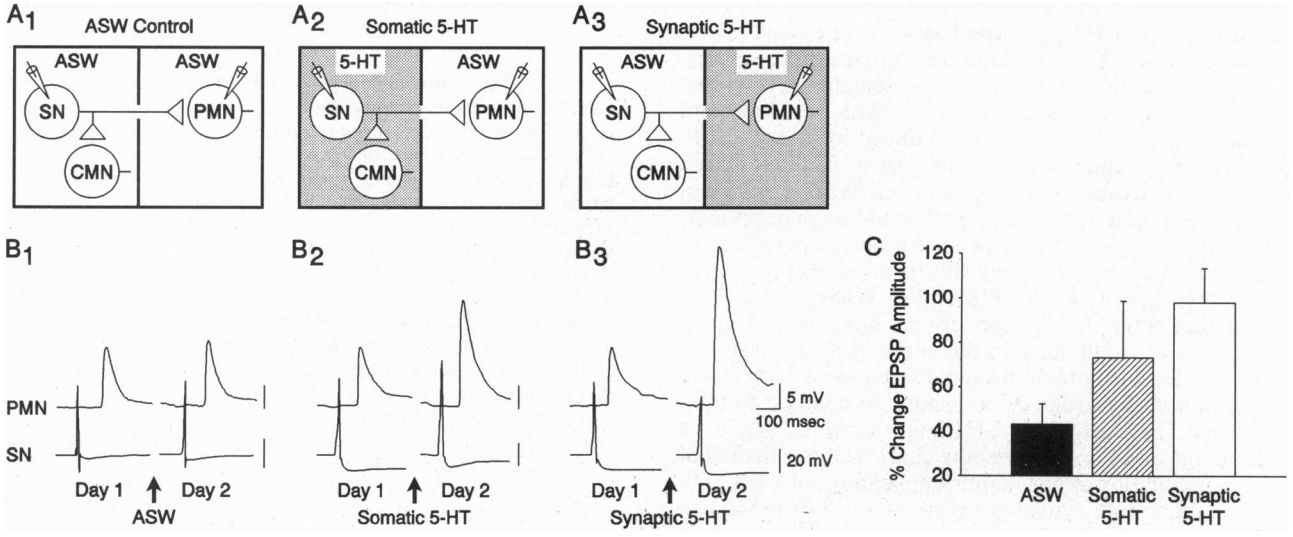

FIG. 2. Local applications of 5-HT restricted to the sensory cell soma or synapses produce long-term facilitation at peripheral synapses. (*A*) Schematic of preparation. EPSPs at peripheral sensori–motor connections were examined before and 24 hr after three different experimental treatments: control applications of ASW (A_1); 5-HT bath-applied selectively onto the abdominal ganglion, which contains the sensory cell somata and synapses onto central postsynaptic targets (somatic 5-HT) (A_2); or 5-HT bath-applied selectively onto the peripheral nervous system, containing sensory cell connections onto peripheral motor neurons (synaptic 5-HT) (A_3). CMN, central motor neuron; SN, sensory neuron; PMN, peripheral motor neuron. (*B*) Representative examples from each of the three experimental conditions. The relatively large hyperpolarizations after some sensory cell action potentials are from negative current injections used to prevent repetitive firing, as some sensory cells became hyperexcitable on day 2. (*C*) Group data, indicating greater facilitation at exposed peripheral synapses after synaptic applications of 5-HT (n = 14), relative to ASW controls (n = 14, $P < 0.001$). Applications of 5-HT onto the sensory neuron soma and central synapses (n = 14) produced intermediate effects, with significant facilitation occurring more frequently than in the ASW control condition ($P < 0.05$, one-tailed test) but less frequently than after synaptic 5-HT applications ($P < 0.05$).

In the present studies, we found that 5-HT applied cell-wide onto sensory neurons produced long-term facilitation at remote peripheral synapses measured 24 hr later. These findings demonstrate that 5-HT can induce long-term facilitation at mature sensory neuron connections in the intact nervous system, as well as at synapses in dissociated cell culture as reported (5, 6, 8). These results further indicate that long-term facilitation can be expressed within 24 hr at synapses some distance from the cell soma.

How might this facilitation occur? Our experiments do not answer this question directly. One possibility is that long-term facilitation at remote synapses (unlike proximal synapses) requires only local cellular processes, either independent of alterations in gene expression or dependent only on genes expressed in the postsynaptic cell. Alternatively, facilitation might involve transport of newly synthesized protein from the cell body to the distal synapse. Thus, 5-HT applications onto the abdominal ganglion might recruit gene expression by acting directly on the cell body (or on local synaptic processes) to elevate intracellular cAMP and activate the cAMP-dependent protein kinase. The kinase would, in turn, translocate to the nucleus and regulate gene expression (11, 12). The fact that facilitation occurred at distal synapses after somatic 5-HT applications suggests that cellular signals (presumably newly synthesized proteins) may be transported from the cell body to distal synapses, even synapses not previously exposed to 5-HT. In the intact nervous system, there is serotonergic input directly to the sensory neuron soma (30), which could contribute to this process. More recently, Emptage and Carew (31, 32) have also found that somatic 5-HT applications can produce long-term facilitation at unexposed synapses of pleural sensory cells but that this facilitation is not expressed until several hours after 5-HT application, consistent with a time requirement for synthesizing new protein and transporting it down the axons to the terminals. Black (33) has also found that reserpine-induced increases in tyrosine hydroxylase activity in the terminals of locus coeruleus cells exhibit a temporal–spatial gradient, moving with time from proximal to distal synapses.

Applications of 5-HT Restricted to Remote Synaptic Loci Also Produce Long-Term Facilitation. Perhaps more surprising than the induction of facilitation by somatic 5-HT is that 5-HT applications restricted to distal synapses and their postsynaptic motor neurons also produced long-term facilitation. We do not know whether this form of facilitation also requires gene activation in the sensory cell nucleus, but if so, active transport of a retrograde signal would seem necessary. Simple diffusion of cAMP is probably not responsible, as diffusion of cAMP is a relatively slow process (12) compared with the breakdown of cAMP (34, 35). Walters *et al.* (29) reported that after distal axon crush, there is a delayed enhancement of excitability in the soma of pleural sensory cells and facilitation at their proximal synapses, which would be consistent with retrograde transport. In contrast to their original report in which lower 5-HT concentrations were used (31), Emptage and Carew have now confirmed our finding of long-term facilitation after synaptic applications of 5-HT (32).

How could remote synapses signal the nucleus in the cell body? Ambron *et al.* (36) have recently found that the sequence for the nuclear importation signal also allows proteins to access the rapid retrograde transport system. Moreover, retrograde transport of peptides containing the importation signal is enhanced after axonal injury (37). These findings raise the possibility that synaptic stimulation by 5-HT might also elicit retrograde transport of proteins with nuclear importation signals (including perhaps the cAMP-dependent protein kinase) to the nucleus, where they would regulate gene expression. Neurite outgrowth in response to nerve growth factor operates via a similar process, involving retrograde transport of the nerve growth factor–receptor complex from terminal regions to the soma, followed by synthesis and transport of proteins to the neurites (38).

In our experiments, the round-trip distance for anterograde and retrograde transport was ≈4 cm. This distance is consistent with facilitation being mediated by fast axonal transport (39). To examine these notions, it will be important to determine whether long-term facilitation at distal synapses is blocked by inhibitors of axonal transport or by inhibitors of protein and RNA synthesis applied selectively to either the sensory neuron soma or to its synaptic connections (and postsynaptic cells).

Is Long-Term Facilitation Cell-Wide or Synapse-Specific? Synapse-specific facilitation provides a spatially precise means of modifying neural pathways. We had shown (25) in the siphon sensory cells that short-term facilitation, which involves covalent modifications of preexisting proteins, can be synapse-specific. But can long-term facilitation involving gene regulation also be synapse-specific? If so, how might new gene products be used selectively at facilitated synapses but not at other synapses?

In the present experiments, somatic 5-HT applications produced some facilitation, even at remote synapses not exposed to 5-HT. This finding provides initial evidence that a component of long-term facilitation is cell-wide. Once the cell body has been activated by 5-HT, even synapses not exposed to 5-HT may show some long-term facilitation. However, we also found that local applications of 5-HT restricted to distal synapses produced long-term facilitation

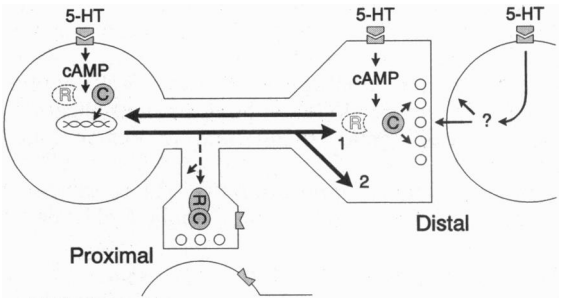

FIG. 3. Possible mechanisms for long-term synaptic facilitation at remote synapses of sensory neurons. Stimulation of distal synaptic regions by 5-HT might produce long-term changes either through entirely local processes, or via retrograde transport (back arrow) of cellular signals that would trigger gene expression. Genomic regulation could also occur from direct stimulation of the soma or proximal synapses. Gene products would then be distributed to synaptic regions by anterograde transport (forward arrows). Although facilitation might be, in part, cell-wide, synapse-specific modifications could nonetheless occur if these gene products were preferentially transported to (44, 45), or selectively used by, synapses previously exposed to 5-HT. One mechanism that could contribute to selective utilization is enhanced proteolysis of regulatory (R) subunits of the cAMP-dependent kinase (21, 46, 47), leaving the catalytic (C) subunits free to maintain long-term facilitation (arrow 1). Because proteolysis occurs preferentially when the regulatory and catalytic subunits are dissociated, newly expressed proteases transported from the soma might produce greater degradation in local areas where this dissociation was triggered by 5-HT, particularly when 5-HT stimulation was repeated at relatively long intervals relative to axonal transport time. Alternatively (arrow 2), anterograde transport of new gene products might help stabilize or induce morphological or other physiological changes at previously exposed synapses. Finally, certain long-term structural changes in sensory cells, such as growth of new varicosities, require a postsynaptic target (9), suggesting that postsynaptic target cells could also contribute to synapse-specific changes, either by a retrograde intercellular signal, by providing a greater postsynaptic target area, or through other means. In principle, postsynaptic contributions to long-term facilitation could occur preferentially at synapses onto targets stimulated by 5-HT.

and that this facilitation was greater than that produced by 5-HT application to the cell body and central synapses. This differential enhancement suggests that long-term facilitation is preferentially expressed at synapses specifically exposed to 5-HT, and that long-term facilitation, like short-term facilitation, may be partially synapse-specific, although other explanations are possible. Long-term synapse-specific facilitation *in vivo* would require that facilitators project to certain synapses but not to others, and there is both anatomical (40) and physiological (25) evidence for this specificity. To investigate these possibilities more directly, it will be important to record synaptic potentials elicited by a single sensory cell to both central and peripheral motor neurons and to examine their responses to local 5-HT applications restricted to one site or the other.

Although the dependence on new proteins imposes important constraints (17, 25, 32, 41–43), long-term synapse-specific facilitation could nevertheless arise by one of several mechanisms (Fig. 3). Synapse-specific, long-term modifications have been also demonstrated in several other systems. For example, nerve growth factor can stimulate local outgrowth specifically at exposed neurites, despite the involvement of retrograde transport mechanisms (48). Synaptic reorganization is, in fact, a common theme during development: some axonal processes of a cell proliferate, while others retract (49, 50). Finally, long-term potentiation in hippocampus is, at least partially, synapse-specific during the early and possibly later phases (51–53), although these later phases that are more dependent on protein synthesis (54) have not been well explored at the single-cell level. Our findings suggest that *Aplysia* siphon sensory cells may provide an attractive preparation for investigating long-term synapse-specific modifications, as well as their cellular mechanisms and functional significance during learning.

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