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Serotonin Produces Long-Term Changes in the Excitability of *Aplysia* Sensory Neurons in Culture that Depend on New Protein Synthesis

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When isolated and grown in cell culture, the sensory and motor neurons of the gill withdrawal reflex of *Aplysia* readily form synaptic connections. Repeated exposures to 5-HT cause facilitation of the synaptic connections between co-cultured sensory and motor neurons lasting at least 24 hr. As a first step toward understanding the locus and the mechanisms underlying this long-term synaptic facilitation, we have examined the membrane excitability of the isolated presynaptic sensory neurons grown alone in dissociated cell culture. Four repeated applications of 1 μM 5-HT caused a significant increase in the excitability of sensory neurons, lasting at least 24 hr. This resembles the short-term changes in excitability seen in response to a single application of 5-HT. Unlike the short-term effect, this long-lasting change was blocked by exposure of the cells during the 5-HT treatment to 10 μM anisomycin, an inhibitor of protein synthesis. Thus, like the synaptic facilitation, the long-term change in excitability of the isolated presynaptic neurons differs from the short-term in requiring the synthesis of new protein. This finding suggests that the sensory neuron uses gene products to modulate membrane currents in its long-term response to repeated external stimuli that are not required in its short-term response to a single stimulus.

The siphon and gill withdrawal reflex in *Aplysia californica* can be sensitized in a long-lasting manner by the delivery of repeated noxious stimuli to the tail (Pinsker et al., 1973; Frost et al., 1985). Part of the mechanism underlying this long-term behavioral sensitization involves long-lasting changes in the efficiency of synapses between the sensory and motor neurons of the gill withdrawal reflex (Frost et al., 1985). These same synapses are facilitated in a short-lasting manner during short-term behavioral sensitization of the gill withdrawal reflex (Castellucci and Kandel, 1976).

The short-term synaptic facilitation of the sensory neuron-to-motoneuron synapse seems to be caused largely by the sensory neuron (Castellucci and Kandel, 1976; Klein and Kandel, 1978; Klein et al., 1982) and in part by the depression of a special class of K^+ current, the serotonin-sensitive K^+ current, or S current (Klein et al., 1982; Siegelbaum et al., 1982). Depression of this current leads to broadening of the action potential, allowing more Ca^{2+} to enter during each action potential and hence causing more transmitter to be released. The reduction in the S current also has other consequences: It contributes to a short-lasting increase in the input resistance of the sensory neuron, accompanied by an increase in the membrane excitability (Klein et al., 1986). As a result, a given intracellular stimulus generates a longer train of action potentials in the sensory neuron.

The mechanisms underlying long-term facilitation of the same synapse are unknown and could involve modification of either the pre- or postsynaptic cell. As a first step toward understanding the biophysical mechanisms underlying long-term synaptic facilitation, we have examined whether long-term synaptic facilitation was also accompanied by a corresponding long-term change in excitability. Such a change would suggest a long-lasting reduction in the outward currents of the sensory neuron, analogous to that occurring during short-term facilitation.

It is possible to reconstitute the monosynaptic component of the neuronal circuitry underlying the gill withdrawal reflex *in vitro* by co-culturing sensory neurons with gill motoneurons. The sensory neurons readily form synapses with the motor cells (Rayport and Schacher, 1986) and these connections can be facilitated in a short-lasting way by single applications of 5-HT (Rayport and Schacher, 1986) or in a long-lasting manner by repeated applications of 5-HT (Montarolo et al., 1986). The long-term synaptic facilitation obtained in these cultured cells

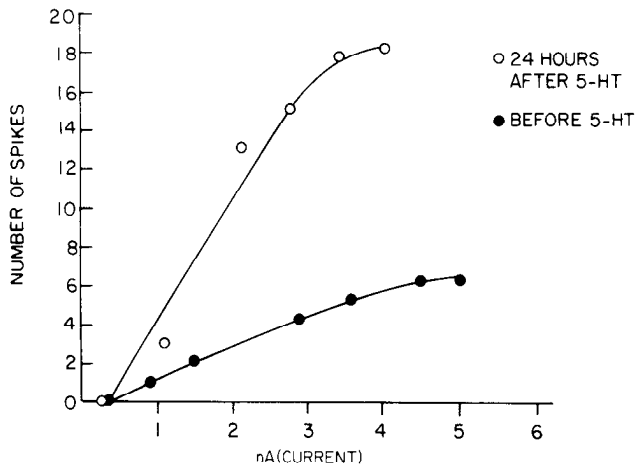

Figure 1. Repeated application of 5-HT causes a long-term change in the excitability of sensory neurons in synaptic contact with L7 motor neurons. The excitability of a sensory neuron recorded before and 24 hr after 5-HT treatment.

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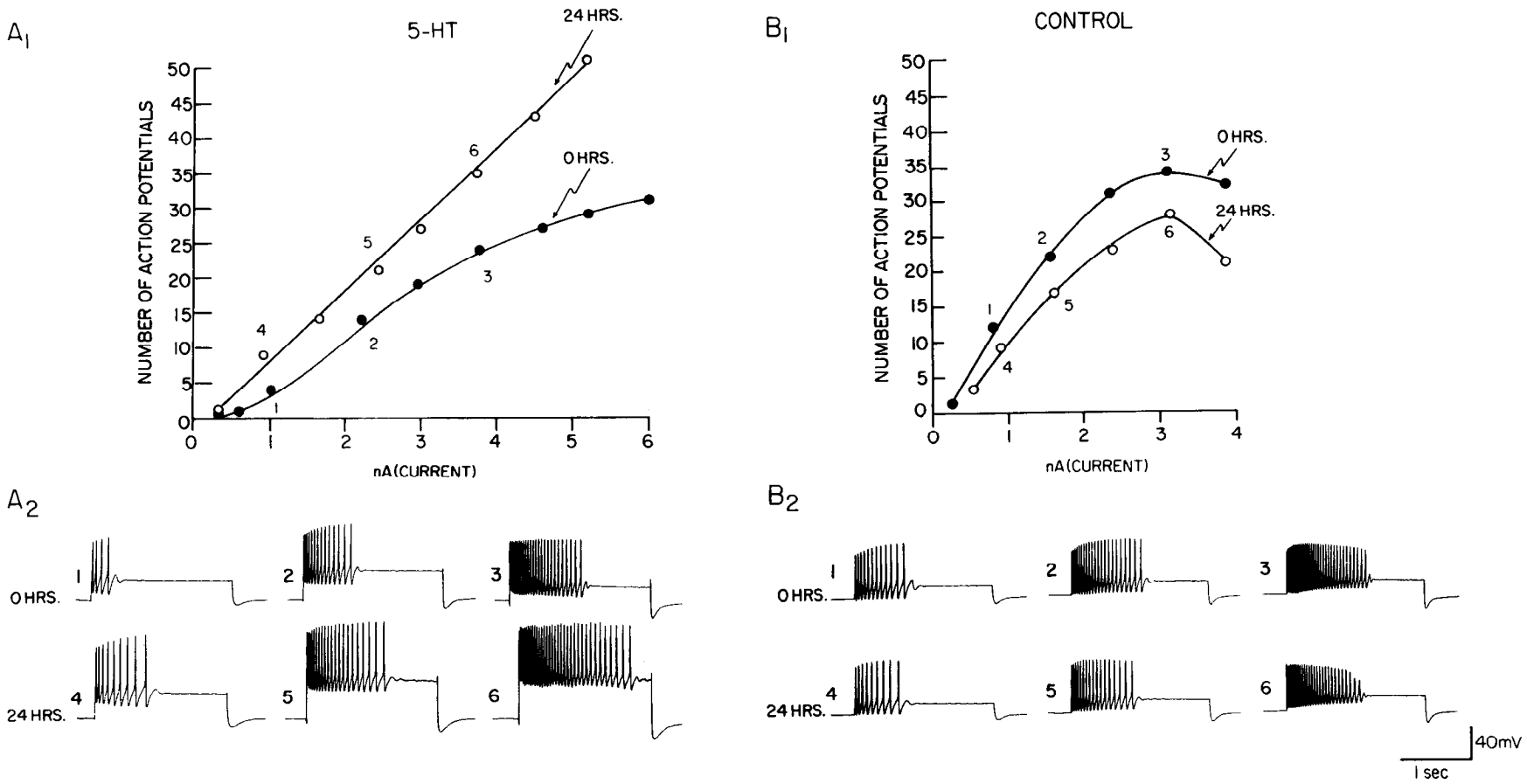

Figure 2. 5-HT causes a long-term change in the excitability of isolated neurons. A_1, Excitability of an isolated sensory neuron recorded before (●) and 24 hr after (○) 5-HT treatment. A_2, Examples of the spike trains evoked by current injection corresponding to the *numbered points* on the graph. *B*, In a control sensory neuron, the excitability declines over 24 hr.

can be distinguished from short-term facilitation at the same synapse not only by its time course but also by its requirement for protein synthesis (Montarolo et al., 1986). Whereas short-term facilitation can occur in the absence of new protein synthesis, long-term facilitation requires new protein synthesis. By using sensory neurons in isolated cell culture, we have found that 4 repeated applications of 5-HT will also cause long-lasting changes in their excitability. These excitability changes occur in sensory neurons connected to their motoneurons as well as in isolated sensory neurons. Like the long-lasting synaptic facilitation, these excitability changes also require the synthesis of new proteins.

Materials and Methods

Cell culture. The techniques for isolating and maintaining the neurons of *Aplysia* in cell culture have been described previously (Schacher and Proshansky, 1983; Schacher, 1985). Abdominal ganglia were isolated from animals weighing 1–3 gm and bathed for 2 hr in a 1% solution of the proteolytic enzyme, protease (Sigma type IX). After proteolytic digestion, the ganglia were pinned down in a Sylgard dish and desheathed. The gill motoneuron (L7) was identified by its position, size, pigmentation, and axonal projections, as seen during removal from the ganglion. Mechanosensory neurons, from either the LE cluster of the abdominal ganglion or the VC cluster of the pleural ganglion, were obtained from ganglia that had been removed from animals weighing 70–150 gm and were treated in 1% protease solution for 2.5 hr. After removal from the ganglion, the neurons were transferred to polylysine-coated culture dishes containing *Aplysia* hemolymph and L15 (Flow Laboratories, McLean, VA), with salts added to adjust the medium to marine saline conditions (400 mM NaCl, 11 mM $CaCl_2$, 10 mM KCl, 27 mM $MgSO_4$, 27 mM $MgCl_2$, and 2 mM $NaHCO_3$).

Electrophysiology and excitability measurements. Standard electrophysiological techniques were used to make intracellular recordings from sensory neurons and motoneurons (L7). Results were stored on a 4-channel FM tape recorder (Hewlett-Packard) and permanent records made with a Gould pen recorder. Only those sensory neurons with resting potentials greater than −40 mV and with an input resistance (measured at rest by 1-sec-long 0.1–0.3 nA hyperpolarizing current pulses) greater than 25 MΩ (mean, 189 MΩ; SD, 125 MΩ) were selected for analysis. The temperature of the bathing medium (a 1:1 mixture of L15 and artificial seawater) was maintained between 17 and 19°C.

The excitability of sensory neurons was measured by injecting, through the recording electrode, a graded series of constant depolarizing current pulses 2 sec long, each separated by 1 min of rest. To generate an input–output curve for each neuron, the number of spikes evoked by each current pulse was counted and plotted against the amplitude of the injected current pulse.

By comparing the input–output curves of individual cells before and 24 hr after any treatment, the long-term effects of pharmacological manipulations on sensory neuron excitability could be assessed. The overall excitability change in a group of cells was measured in 2 ways. For the first measure, the maximum number of spikes generated in the excitability input–output curve by a cell before any treatment was subtracted from the maximum number generated in the excitability curve for the same cell 24 hr after treatment. These values were then averaged to give the mean difference between the maximum number of spikes generated before and 24 hr after treatment (Fig. 7). The second method involved interpolating from the excitability curve the number of spikes generated at 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 nA. The difference in number of spikes at each interpolated current level before and 24 hr after any treatment was calculated for each cell, and these values were then averaged. This gave a mean difference in excitability throughout a range of current levels (cf. Figs. 3 and 5). Since different neurons generated their maximum number of spikes at different current levels, the variance of each mean is high, and this second method is a more conservative way of estimating the excitability change than the first.

Results

Initial experiments were carried out on sensory neurons co-cultured and in synaptic contact with L7 motor cells. Four 5 min applications of 1 μM 5-HT, separated by 15 min of washing, caused an increase in excitability lasting at least 24 hr (Fig. 1). To see whether isolated sensory neurons could also show excitability changes in the absence of their postsynaptic partners, and to produce an experimentally simpler preparation more favorable for study, we next examined the effects of 5-HT on isolated sensory neurons. Like their co-cultured counterparts, isolated sensory neurons also showed an excitability change lasting at least 24 hr in response to 4 applications of 1 μM 5-HT separated by 15 min of washing (Fig. 2). The mean difference between the maximum number of spikes generated before and 24 hr after the 5-HT treatment (see Materials and Methods) was 9.9 spikes (SEM = 1.72; n = 20). Thus, even sensory neurons alone show a long-term change in excitability in response to repeated 5-HT applications. This suggests that the 5-HT was acting directly on the sensory neuron, rather than indirectly via the motor cell. The 5-HT treatment also caused an increase in the input resistance of the sensory neurons (mean, 68 MΩ; SEM = 43 MΩ; range, −166 to +309 MΩ). However, the change in the input resistance was not statisticaly significant (paired t test).

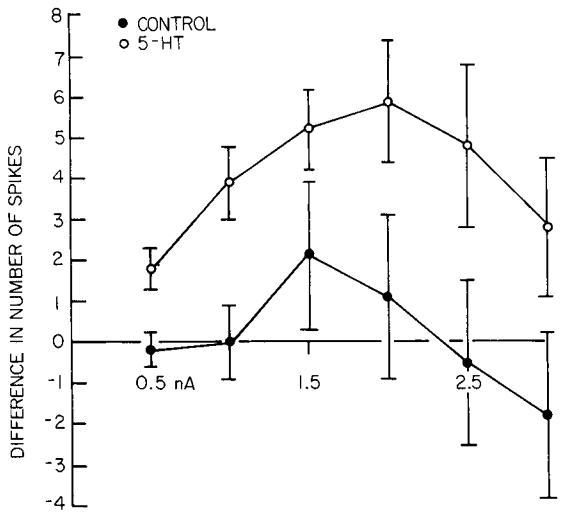

Figure 3. Mean excitability change calculated at each interpolated level of current for the 5-HT-treated and control groups of sensory neurons. Each point is the mean of 11–20 measurements; *bars* show 1 SEM.

The change in excitability was not simply a consequence of the 24-hr time interval between the excitability measurements: a group of control cells that received no 5-HT treatment showed no significant change in excitability over the 24 hr (Fig. 2; mean, 0.44; SEM = 2.30; n = 18). Overall, the control cells showed a small but not statistically significant decrease in input resistance (mean, −18 MΩ; SEM = 20; range, −161 to +99 MΩ). The maximal change in the excitability of the experimental group was significantly greater than that of the control group ($p < 0.01$; see below). Furthermore, the excitability change of the 5-HT-treated group over 24 hr was greater than that of the control group at all interpolated levels of current injection (see Materials and Methods; Fig. 3).

The effect of protein synthesis inhibition

The long-term facilitation caused by 5-HT of synaptic connections between co-cultured sensory neurons and motor cells requires new protein synthesis. Indeed, there appears to be a critical time period during the 5-HT treatment in which new protein synthesis must occur in order for long-term facilitation to take

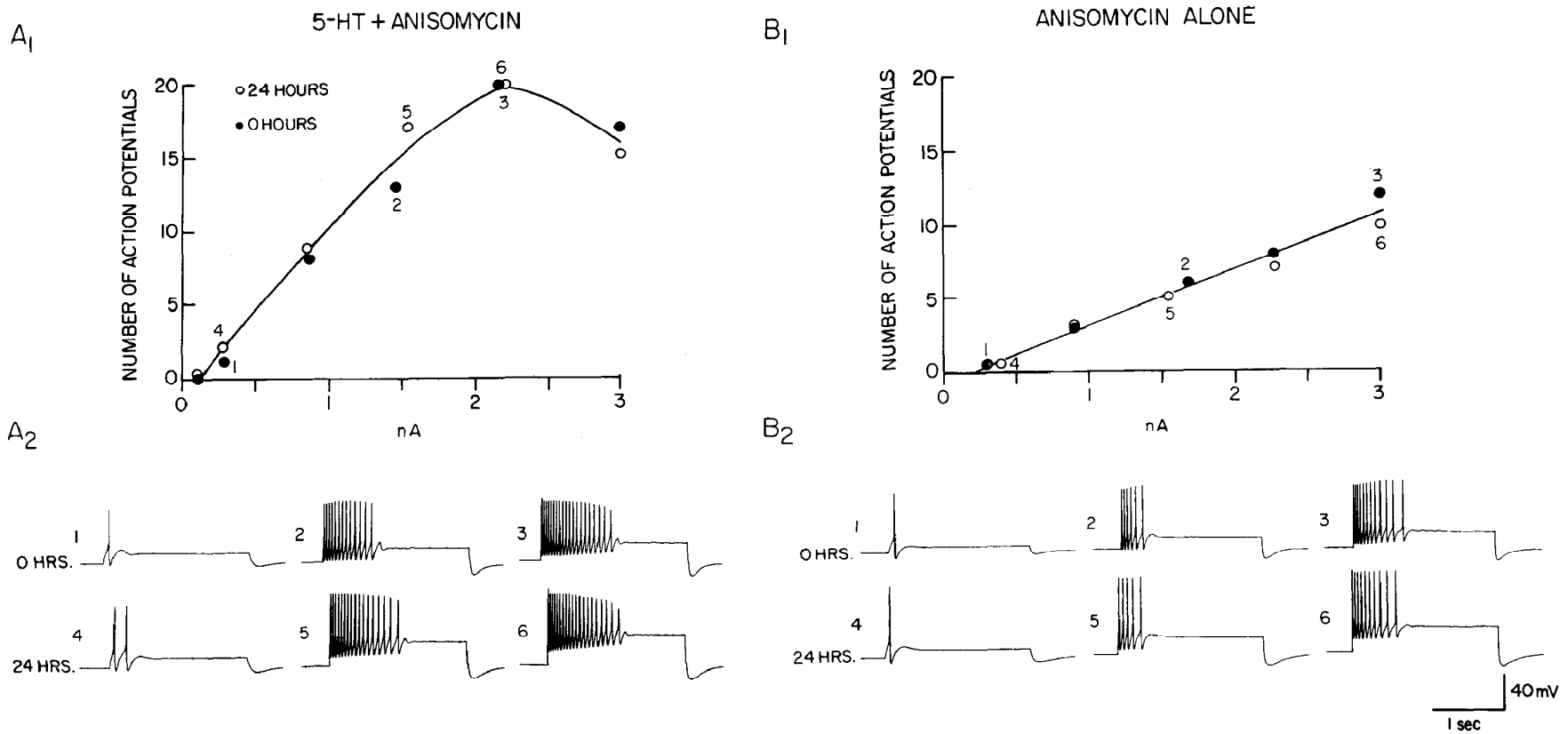

Figure 4. Long-term changes in excitability are blocked by the protein synthesis inhibitor anisomycin. A_1, In the presence of 10 μM anisomycin, 5-HT cannot evoke a long-term change in excitability. A_2, Sample records corresponding to the *numbered points* of the graph. *B*, Anisomycin alone has no effect on the excitability recorded in the presence (●) of anisomycin and 24 hr later (○).

place (Montarolo et al., 1986). One possible cause of the change in long-term excitability seen in isolated sensory neurons could be a long-lasting reduction of their net outward currents (cf. Scholz and Byrne, 1986). If this were the case, the reduction might lead to broadening of the action potential and enhanced transmitter release, which would suggest that the same biophysical mechanisms that cause the change in excitability could also contribute to synaptic facilitation. If this is the case, the excitability changes seen in sensory neurons should show the same dependency on protein synthesis as on synaptic facilitation. If, on the other hand, the excitability changes do not require protein synthesis, then it would seem unlikely that their underlying mechanisms could contribute to synaptic facilitation.

We therefore treated sensory neurons with 10 μM anisomycin for 1 hr prior to, and for the duration of, the 5-HT treatment (four 5 min applications separated by 15 min of washing). Anisomycin has previously been shown to block protein synthesis by 90–95% in *Aplysia* sensory neurons and to have no effect on cell input resistance, spike width or amplitude, or short-term synaptic facilitation (Montarolo et al., 1986). The total time of exposure to anisomycin was 3–4 hr; it was washed out after the last 5-HT application was completed. The reversal of the effects of this drug requires about 2–4 hr (Montarolo et al., 1986).

In the presence of anisomycin, 5-HT failed to cause the characteristic long-term increase in excitability (Fig. 4). Instead, it caused a slight, but not significant, decrease in the excitability of sensory neurons over 24 hr (Fig. 4*A*). The mean difference between the maximum number of spikes generated before and 24 hr after the 5-HT/anisomycin treatment was −2.6 (SEM = 4.17; $n = 12$). Furthermore, there was no significant change at any of the interpolated current levels (Fig. 5). The input resistance of the 5-HT/anisomycin-treated cells showed a slight increase overall that was not statistically significant (mean, 20 MΩ; SEM = 42 MΩ; range, −236 to +262 MΩ). Exposure for 3–4 hr to anisomycin alone (without any 5-HT treatment) also had no significant effect on the excitability of sensory neurons (Fig. 4*B*). The mean reduction in the maximum number of spikes before and 24 hr after exposure to anisomycin alone was −1.1 (SEM = 2.69; $n = 17$). As with the group treated with 5-HT and anisomycin, there were no significant changes in overall excitability at any of the interpolated current levels (Fig. 5). The cells treated with anisomycin alone also showed a slight decrease in their input resistance (mean, −22 MΩ; SEM = 32 MΩ; range, −146 to +241 MΩ) that was not statistically significant.

In contrast to the long-term changes, short-term changes in excitability caused by single applications of 5-HT did not require protein synthesis. Exposure of sensory neurons to 10 μM anisomycin for 2 hr did not block the ability of 5-HT to evoke a short-lasting increase in cell excitability (Fig. 6). Furthermore, when sensory neurons were exposed to anisomycin for 4 hr and tested 24 hr later, 5-HT could still cause short-lasting increases in cell excitability (data not shown).

Figure 7 summarizes the mean differences in the maximum number of spikes generated by the 4 groups of sensory neurons before and 24 hr after treatment. Using a 1-factor analysis of variance, we found that the 4 groups did not belong to the same population ($p < 0.01$). More detailed comparisons showed that the excitability change in the 5-HT-treated group was significantly greater than those of all the others ($p < 0.01$). Therefore, repeated applications of 5-HT can cause a long-lasting increase in sensory neuron excitability. This excitability change can occur

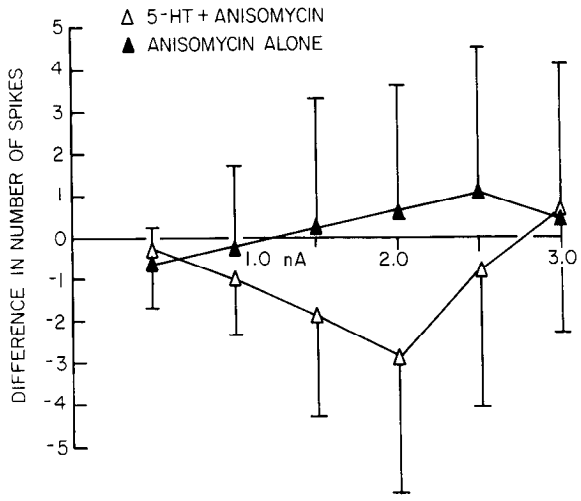

Figure 5. Neither 5-HT plus anisomycin nor anisomycin alone causes significant long-term changes in the excitability of sensory neurons at any of the interpolated current levels.

in isolated sensory neurons, can be blocked by anisomycin, and therefore requires the synthesis of new proteins during the 5-HT treatment.

Discussion

Behavioral significance

The long-lasting changes in the excitability of sensory neurons described here were caused by repeated applications of 5-HT to isolated sensory cells in culture. If, in the intact animal, repeated exposure to sensitizing stimuli also caused similar long-term excitability changes in the response of sensory neurons to mechanical stimulation, this could have significant behavioral consequences. Specifically, the excitability change in the sensory neurons would contribute to the long-lasting synaptic facilitation of connections to follower cells, the motoneurons and interneurons of the gill withdrawal reflex. This would result in an increase in both the mono- and polysynaptic components of motoneuron excitation during gill withdrawal and would lead to enhanced withdrawal.

Possible mechanisms

The long-lasting change in excitability could be seen even in sensory neurons grown alone in culture in the absence of the postsynaptic target cell, the motoneuron. Thus, the presence of the motoneuron was not required for the excitability changes, which must have been caused by a direct effect of 5-HT on the sensory neurons. In addition to the effect of an increase in transmitter mobilization (Hochner et al., 1986a, b), the cause of short-term synaptic facilitation is, in part, the closure of a distinct class of K^+ channel—the S channel (Klein et al., 1982; Siegelbaum et al., 1982). This in turn causes spike broadening and enhanced transmitter release. The same mechanism appears to contribute to an increase in the excitability of sensory neurons and counteracts the accommodation produced by long-duration stimuli (Klein et al., 1986).

The long-lasting change in excitability in sensory neurons could, like the short-term change, be caused by a reduction in outward currents (Scholz and Byrne, 1986, 1987). Sensory neu-

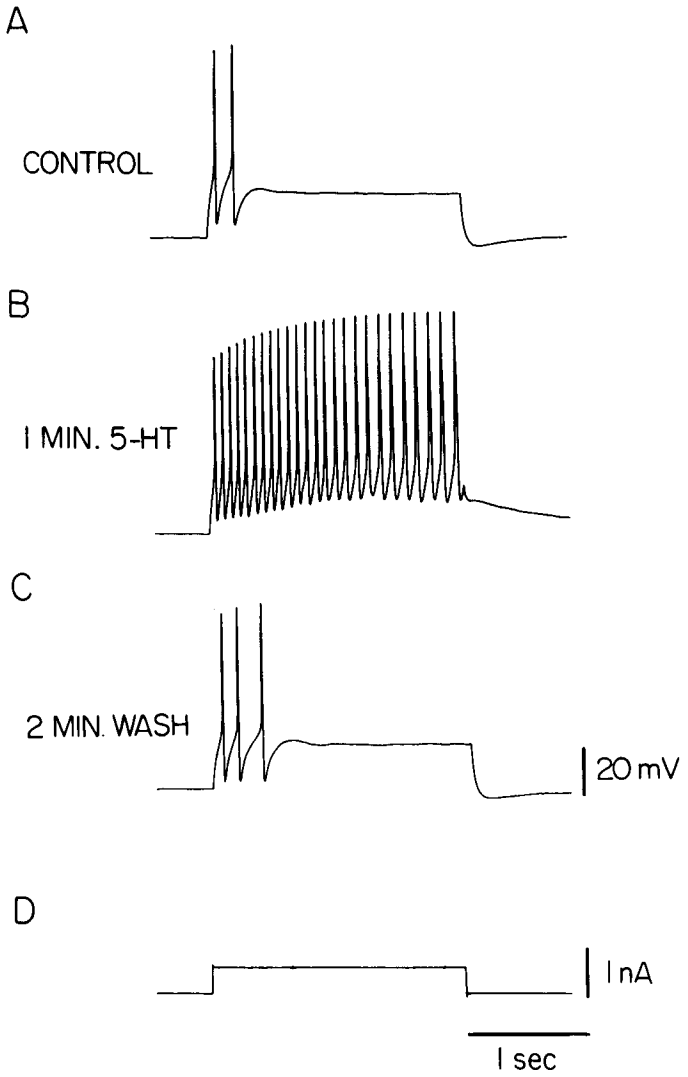

Figure 6. Short-term changes in excitability do not require protein synthesis. *A,* Recording from an isolated sensory neuron that has been exposed to 10 μM anisomycin for 2 hr. The injection of depolarizing current (*bottom trace*) evokes 2 action potentials. *B,* One minute after the application of 1 μM 5-HT, the same current pulse evokes a continuous train of pulses. Note how the afterpotentials following the depolarizing pulse have been altered by the 5-HT. *C,* After 2 min of washing, the same current pulse evokes only 3 action potentials. The membrane potential of the cell and the amplitude of the injected current pulse shown in *D* are the same as in *A–C.*

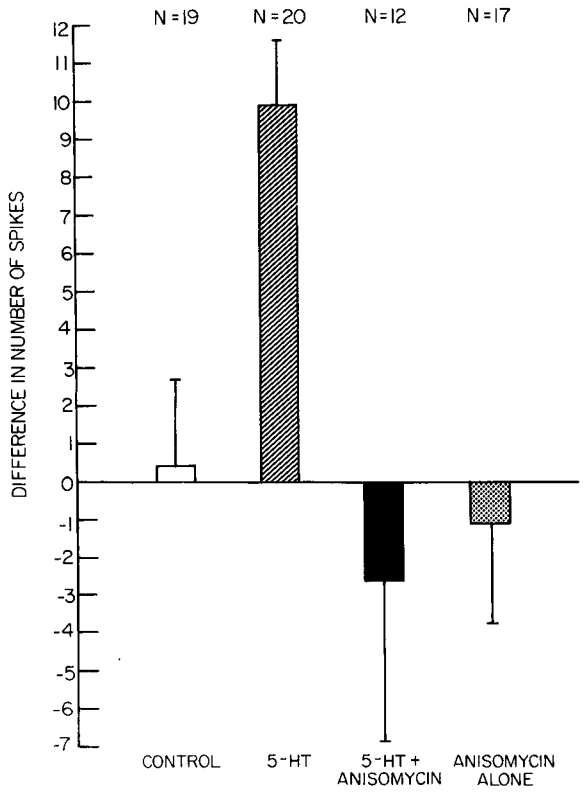

Figure 7. Summary bar chart showing the mean difference in the maximum number of spikes before and 24 hr after the 4 treatments. *Bars* represent 1 SEM.

rons possess at least 4 types of K^+ current: the S current, the A current, the delayed rectifier, and the Ca^{2+}-dependent K^+ current. In principle, any of these currents could be modified to give rise to the long-term excitability changes described here. One obvious candidate is the S current, since it is known to be modulated by 5-HT and can cause short-lasting changes in excitability. Indeed, preliminary evidence suggests that repeated 5-HT applications can close S K^+ channels in a long-lasting way (N. Dale et al., unpublished observations; see also Scholz and Byrne, 1986, 1987). But other K^+ currents could also have a contributory role.

Closure of K^+ channels during the long-term excitability change might cause a modest increase in the resting input resistance of sensory neurons, as occurs during the short-term changes in excitability (Klein et al., 1986). While there was a suggestion that the input resistance of the 5-HT-treated neurons increased with respect to other cells, this was not found to be statistically significant. These experiments were performed by comparing the resting input resistances of neurons penetrated on 2 separate days. Given the variable nature of intracellular impalements, the quality of penetration of a neuron will vary over 2 d. Determinations of the input resistance at the cell resting potential, made with hyperpolarizing currents, measure leakage currents through the cell membrane, which largely depend on the quality of impalement. The variability in cell impalement would therefore tend to obscure modest changes in input resistance measured in this way. In contrast, the excitability of a cell depends not only on the resting leakage but on additional conductances recruited by the depolarizing current pulses used to evoke the spikes; thus it might depend less on the variability of quality in cell impalements. This might explain why there were clear-cut changes in neuronal excitability, while the change in input resistance was less significant.

Dependence on protein synthesis

As with synaptic facilitation, the long-term excitability changes described here require protein synthesis during the short period of 5-HT treatment. This suggests that the biophysical mechanisms underlying the excitability change could contribute to long-term synaptic facilitation. It also implies that the expression of gene products, required for modulating ion channels in the membrane active in the long-term process, is not required in the short-term process. Obviously, the genome will control many neuronal properties, such as size, geometry, receptor, and ion channel expression. However, this evidence suggests that

the number of active ion channels in the membrane may be under the control of the genome, and can be modified in a long-lasting way in response to external stimuli.

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