Limited Contributions of Serotonin to Long-Term Hyperexcitability of Aplysia Sensory Neurons

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Liao, Xiaogang, Christine G. Brou, and Edgar T. Walters. Limited contributions of serotonin to long-term hyperexcitability of Aplysia sensory neurons. J. Neurophysiol. 82: 3223–3235, 1999. Serotonin (5-HT) has provided a useful tool to study plasticity of nociceptive sensory neurons in Aplysia. Because noxious stimulation causes release of 5-HT and long-term hyperexcitability (LTH) of sensory neuron somata and because 5-HT treatment can induce long-term synaptic facilitation of sensory neuron synapses, a plausible hypothesis is that 5-HT also induces LTH of the sensory neuron soma. Prolonged or repeated exposure of excised ganglia to 5-HT produced immediate hyperexcitability of sensory neurons that showed little desensitization, but the hyperexcitability decayed within minutes of washing out the 5-HT. Prolonged or repeated treatment of either excised ganglia or dissociated sensory neurons with various concentrations of 5-HT failed to induce significant LTH even when long-term synaptic facilitation was induced in the same preparations. Use of a high-divalent cation solution to reduce interneuron activity during 5-HT treatment failed to enable the induction of LTH in excised ganglia. Pairing 5-HT application with nerve shock failed to enhance LTH produced by nerve shock or to reveal covert LTH produced by 5-HT. The induction of LTH by nerve stimulation was enhanced rather than inhibited by treatment with methiothepin, a 5-HT antagonist reported to block various 5-HT receptors and 5-HT-induced adenyl cyclase activation. This suggests that endogenous 5-HT may have inhibitory effects on the induction of LTH by noxious stimulation. Methiothepin blocked immediate hyperexcitability produced by exogenous 5-HT and also inhibited the expression of LTH induced by nerve stimulation when applied during testing 1 day afterward. At higher concentrations, methiothepin reduced basal excitability of sensory neurons by mechanisms that may be independent of its antagonism of 5-HT receptors. Several observations suggest that early release of 5-HT and consequent cAMP synthesis in sensory neurons is not important for the induction of LTH by noxious stimulation, whereas later release of 5-HT from persistently activated modulatory neurons, with consequent elevation of cAMP synthesis, may contribute to the maintenance of LTH.

INTRODUCTION

Sensory neurons in Aplysia (Castellucci et al. 1970; Walters et al. 1983a) have played a prominent role in revealing potentially general mechanisms for the acquisition and storage of memory (for review, see Byrne et al. 1993; Krasne and Glanzman 1995; Walters 1994). This work has taken advantage of the fact that a single neuromodulator, serotonin (5-HT), can induce long-term facilitation (LTF) and growth of sensory neuron synapses (Bailey et al. 1992; Clark and Kandel 1993; Emptage and Carew 1993; Montarolo et al. 1986; Zhang et al. 1997) that closely resemble effects expressed by these cells during long-term sensitization of defensive behavior (Bailey and Chen 1983, 1988; Cleary et al. 1998; Frost et al. 1985; Walters 1987a,b). This similarity, and the observation that a 5-HT-immunoreactive interneuron that facilitates synaptic connections from Aplysia sensory neurons is strongly activated by noxious stimulation (Mackey et al. 1989), suggested that 5-HT is a major signal for inducing long-term sensory alterations after noxious stimulation. 5-HT thus has been used to mimic sensitizing stimulation in many studies of sensory neurons in excised ganglia and in cell culture preparations.

An additional effect of intense or prolonged noxious stimulation is long-term hyperexcitability (LTH) of the sensory neuron soma (Cleary et al. 1998; Miller and Walters 1998; Scholz and Byrne 1987; Walters 1987b) and peripheral receptive field (Billy and Walters 1989a). Hyperexcitability of the soma can lead to soma afterdischarge during peripheral stimulation, amplifying the synaptic output of the sensory neuron (Clatworthy and Walters 1993). The role of 5-HT in LTH has received little attention, although there is one report of LTH induction by 5-HT in dissociated sensory neurons (Dale et al. 1987). A contribution of 5-HT to LTH induction in Aplysia is also suggested by additional observations: 1) noxious peripheral stimuli like those that induce LTH (Cleary et al. 1998; Scholz and Byrne 1987; Walters 1987b) appear to release 5-HT (Glanzman et al. 1989; Levenson et al. 1999; Mackey et al. 1989), 2) exogenous 5-HT stimulates both cAMP synthesis (Abrams et al. 1984; Bernier et al. 1982; Jarrard et al. 1993; Ocorr and Byrne 1985; Ocorr et al. 1985) and protein kinase A (PKA) activity ( Muller and Carew 1998) in sensory neurons, and 3) cAMP injections can induce LTH of sensory neurons (Lewin and Walters 1999; Scholz and Byrne 1988). However, it recently has been shown that cAMP synthesis and PKA are not required for LTH induced by noxious stimulation of the body surface or by axotomy (Lewin and Walters 1999; Liao et al. 1999). At least two other contributions of 5-HT to LTH are possible. First, by rapidly increasing peripheral and central excitability, 5-HT may enhance the discharge of action potentials during prolonged or repeated noxious stimulation of a sensory neuron’s receptive field, thereby amplifying activity-dependent signals for the induction of LTH (Billy and Walters 1989a; Clatworthy and Walters 1993). Second, 5-HT might be released persistently long after peripheral trauma (Levenson et al. 1999), thereby producing LTH that is mediated continuously by short-term mechanisms in the sensory neuron.

We have found that prolonged 5-HT treatment induces little
or no LTH of sensory neurons in either excised ganglia or dissociated cell culture. 5-HT does not potentiate the induction of weak LTH by tetanic nerve stimulation, nor does a potent 5-HT antagonist prevent induction of LTH by nerve stimulation. We have also tested the ability of this 5-HT antagonist to reduce excitability when applied 1 day after noxious stimulation.

M E T H O D S

General

Aplysia californica (70–200 g) were supplied by the NIH-Aplysia Resource Facility (Miami, FL) and Alacrity Marine Biological Services (Redondo Beach, CA). Animals were housed in artificial seawater (ASW; Instant Ocean, Burlington, NC) at 15–17°C. Constant body weight was maintained on a diet of Gracilaria seaweed. Animals were dissected after injection of isotonic MgCl₂ (equivalent to ~50% of body volume) and each of the two pedal-pleural ganglia complexes (see Fig. 1B) was excised separately, put into its own dish, and desheathed in a 1:1 solution of isotonic MgCl₂ and ASW. Thus no communication from other ganglia was possible during treatment or testing. In experiments in which immediate effects of 5-HT were tested in excised ganglia, the ganglia were treated with 0.5% glutaraldehyde in a 1:1 solution of ASW and isotonic MgCl₂ for 30 s to immobilize the contractile sheath.

Drugs and solutions

5-HT ( Sigma) was applied in the bath to the final concentrations indicated. Methiothepin (ICN) first was dissolved in DMSO (at 10–20 mM) and then slowly mixed with ASW to the indicated final concentrations. The pH was adjusted to 7.4–7.5, and the final concentration of MgSO₄ was reduced greatly. Excitability testing usually was conducted in ASW containing (in mM) 460 NaCl, 11 CaCl₂, 10 KCl, 30 MgCl₂, 25 MgSO₄, and 10 Tris buffer (pH 7.6). To reduce activation of interneurons, some tests and training procedures were conducted in a high-divalent cation (“hi-di”) solution containing 2.2 times the normal concentration of Mg²⁺ and 1.25 times the normal concentration of Ca²⁺ (Trudeau and Castellucci 1992). In experiments where cells or ganglia were maintained for >24 h, the preparation was placed in culture medium containing L15 (Sigma) dissolved in an iso-osmotic saline solution [(in mM) 400 NaCl, 11 CaCl₂, 10 KCl, 27 MgCl₂, 27 MgSO₄, and 2 NaHCO₃] plus 50% hemolymph by volume. Penicillin G (Sigma, 15 U/ml) and streptomycin (Sigma, 25 μg/ml) were added. Hemolymph was collected through a needle inserted into the foot after cooling the animal to 0–2°C. The hemolymph was centrifuged to remove cells and debris, and passed through a 0.2-μm acetate syringe filter (Nalgene) for sterile filtration.

Electrophysiological tests

Standard procedures were used (Gunstream et al. 1995; Liao et al. 1999). Briefly, sensory neurons sampled from similar locations in each ventrocaudal (VC) cluster (Walters et al. 1983a) were accepted when their firing was evoked by a series of 2-s depolarizing pulses. Repetitive firing was quantified by counting the number of spikes evoked by a 1- or 2-s intracellular depolarizing pulse using 2.5 times the threshold current for initiating an action potential with a 20-ms pulse). Some of the spikes are clipped because of the limited frequency response of the recorder.

Fig. 1. Immediate hyperexcitability induced by bath application of serotonin (5-HT) to excised pleural-pedal ganglia. A: examples of immediate hyperexcitability induced by 10 μM 5-HT. Repetitive firing was evoked by intracellular delivery of a 2-s depolarizing pulse (2.5 times the threshold current for initiating an action potential with a 20-ms pulse). Some of the spikes are clipped because of the limited frequency response of the recorder. B: hyperexcitability in the presence of 5-HT and its rapid decay after washout. Sensory neurons in 1 pleural sensory cluster were tested in artificial seawater (ASW, ○), whereas sensory neurons in the contralateral cluster were tested in a high-divalent cation solution (hi-di, △). Because hi-di solution elevated spike threshold, higher currents were delivered to the hi-di group. *, significant difference from the pretests. Inset: diagram of left pleural-pedal ganglia complex, with enlarged view of pleural ventrocaudal (VC) cluster containing sensory neurons (SNs) and recording/stimulating electrode. In all but 1 study (see Fig. 3D), the VC cluster remained in the pleural ganglion.
Dissociated cell culture

Two methods were used. The first was based on those of Schacher and Proshansky (1983) and Eliot et al. (1994). Briefly, pleural and abdominal ganglia were incubated at 32–34°C for 2.5 h in 1% protease dissolved in L15 solution. Ganglia then were desheathed, and individual neurons were impaled and removed with a segment of axon attached using a glass microelectrode. Sensory neurons were transferred to a plastic petri dish coated with poly-lysine and bathed with equal amounts of L15 solution and filtered hemolymph. The cultures were maintained in an incubator at 16–18°C for 2–6 days before testing. In the second method, pleural sensory clusters were excised from pleural ganglia in a solution containing equal amounts of ASW and isotonic MgCl₂. No protease was used. Cells were dissociated by gently vibrating two micropipettes within each cluster in L15 solution. The surviving cells were transferred to a culture dish containing L15 plus 50% filtered hemolymph and maintained at 16–18°C. Culture medium was changed every other day.

Nerve stimulation

All pedal nerves were cut as far as possible from the ganglion (see Gunstream et al. 1995). Except where noted, nerve stimulation was conducted without anesthetic to permit activity-dependent plasticity. Only nerves p8 and p9 were stimulated, using a brief series of 10 (0.5-s) trains of 2-ms pulses (25 Hz) repeated at either 5-s (Walters and Byrne 1985) or 1-min intervals. Nerve shock intensity was five times the threshold for evoking synaptic activity in large unidentified neurons adjacent to the anteromedial corner of the VC cluster in the pleural ganglion. These convenient “monitor” neurons were selected because their thresholds for synaptic input from pedal nerve stimulation are similar to those of identified tail motor neurons in the pedal ganglion (which was not desheathed in these experiments). The five-times threshold nerve stimulation was two to three times that necessary to activate pleural sensory neuron axons and caused maximal synaptic input to tail motor neurons. In some experiments a single 2-ms nerve test stimulus also was delivered after testing each sensory neuron to test for an axon of that sensory neuron in the nerve. During these tests, the preparation was bathed in hi-di solution to minimize activation of modulatory neurons by the nerve test stimuli. In the nerve-crush procedure, each major pedal nerve except p1 was crushed five times at 15-min intervals, beginning at the cut end, with each crush successively closer to the ganglia.

Statistical analysis

Unless otherwise indicated, experiments employed paired comparisons (using paired t-tests) in which each animal provided a pair of mean excitability measures, one from each sensory cluster, with 6–20 cells sampled per cluster. In some experiments, comparisons with unpaired t-tests were made in single animals of numerous sampled cells from one cluster compared with those in the contralateral cluster. In these cases, the number of individual animals showing significant between-cluster differences is reported. In one study, a 1-way ANOVA followed by Dunnett’s tests was used to compare different groups tested at different times after treatment. In some studies, comparisons were made of average excitability measures from a single cluster per animal, and between-animal comparisons were made to measures from other animals tested during the same period of time using unpaired t-tests. Data that were not normally distributed were analyzed with Wilcoxon and Mann-Whitney U tests, as indicated. All tests were two tailed with the level of significance set at 0.05.

RESULTS

5-HT-induced hyperexcitability of sensory neurons decays rapidly and may involve an indirect pathway

Brief application of 5-HT often has been observed to produce immediate hyperexcitability of Aplysia sensory neurons, both in ganglion preparations (e.g., Baxter and Byrne 1990; Klein et al. 1986; Stark et al. 1996; Walters et al. 1983b) and in dissociated cell culture (Dale et al. 1987; Ghirardi et al. 1992). We asked how long hyperexcitability lasts after bathing excised ganglia for 2 h with 10 μM 5-HT. Similar exposures to 5-HT are known to induce long-term synaptic facilitation in these cells (Emptage and Carew 1993; Zhang et al. 1997) and biochemical alterations associated with long-term facilitation (e.g., Chain et al. 1999; Greenberg et al. 1987; Liu et al. 1997; Muller and Carew 1998). Our primary index of excitability was repetitive firing evoked by a 2-s pulse of depolarizing current 2.5 times the current required to reach action potential threshold with a 20-ms pulse. Tests were given before 5-HT application, in the presence of 5-HT (near the end of the 2-h treatment), and at the times after washout of the 5-HT indicated in Fig. 1 (>3 h). In each animal (n = 4), one pleural-pedal ganglia complex received the 5-HT dissolved in ASW, and the pretests and posttests were performed in ASW. The contralateral pleural-pedal ganglia complex received the 5-HT in hi-di solution, and all pretests and posttests were performed in hi-di solution (see METHODS). By greatly reducing the activation of interneurons (Trudeau and Castellucci 1992), the hi-di solution reveals effects of 5-HT on tested sensory neurons that are likely to be direct.

In the presence of 5-HT, all sensory neurons showed an increase in excitability compared with that displayed in the pretest, regardless of whether hi-di solution also was present (Fig. 1). The somewhat greater hyperexcitability in 5-HT + hi-di relative to 5-HT + ASW is a consequence of the higher action potential thresholds in hi-di solution. Because of our normalization procedure, this resulted in delivery of larger test currents to cells in the hi-di solution. When excitability was measured by injecting the same current (2 nA), into sensory neuron somata, no significant difference was found (5-HT + ASW: 23.6 ± 2.9 spikes; 5-HT + hi-di: 24.4 ± 1.60 spikes; n = 7 cells in each group).

To see how long the hyperexcitability lasted after 5-HT treatment, a one-way ANOVA with subsequent Dunnett’s tests was used to compare each test to the pretest in the ASW or hi-di groups (Fig. 1). Different populations of sensory neurons were sampled in each test. In ASW, significant hyperexcitability was observed only in the presence of 5-HT, and 10 min after washout (F₇,₉₆ = 10.28, P < 0.0001 overall, and P < 0.01 in 5-HT and P < 0.05 in the first posttest; n = 5 to 20 cells in each test). In hi-di solution, significant hyperexcitability was observed only in the presence of the 5-HT (F₇,₆₈ = 13.61, P < 0.0001 overall, and P < 0.01 in 5-HT; n = 5 to 14 cells in each test). These data indicate that hyperexcitability induced by prolonged 5-HT treatment persists <30 min after washout of 5-HT. Moreover, the lack of hyperexcitability during any posttest in hi-di solution indicates that hyperexcitability produced by a direct action of 5-HT on the sensory neurons is even shorter lasting—persisting <10 min after washout. The somewhat longer-lasting hyperexcitability observed in ASW may be the result of indirect effects of 5-HT.

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5-HT induces LTF but little or no LTH of sensory neurons in excised ganglia

Emptage and Carew (1993) showed that repeated application of 2 μM 5-HT induced 24-h LTF but not short-term facilitation of these cells’ synapses. We therefore considered the possibility that LTH might be produced by prolonged 5-HT exposure even though short-term hyperexcitability decayed rapidly (see Fig. 1). We first confirmed that both prolonged (2 h) and repeated (5 × 5 min at 20-min intervals) treatment with 5-HT (5 μM) would produce LTF of Aplysia sensory neuron synapses in excised ganglia, as previously reported (Clark and Kandel 1993; Emptage and Carew 1993; Zhang et al. 1997). We measured synaptic connections to tail motor neurons in the same cells before (day 1) and 24 h after (day 2) 5-HT treatment (Fig. 2A). Both prolonged and repeated 5-HT treatment produced significant LTF (Fig. 2B; $F_{2,28} = 5.38, P < 0.02$ overall, and $P < 0.05$ for each 5-HT-treated group compared with the untreated control group). No significant change in EPSPs from control cells occurred between days 1 and 2. We also examined soma excitability before and 24 h after the prolonged or repeated 5-HT treatments (identical to those in which EPSPs were measured). No significant difference in repetitive firing was found between either group of sensory neurons treated with 5-HT and controls treated with ASW alone (Fig. 2C), although all groups showed greater excitability on day 2 than day 1 (cf. Gunstream et al. 1995). No significant differences between 5-HT-treated sensory neurons and controls were found in spike threshold, spike amplitude, or resting potential (not shown).

Given previous indications that 5-HT or other sensitization-related modulators would induce LTH in pleural sensory neurons (e.g., Dale et al. 1987; Scholz and Byrne 1987), the failure to observe LTH after prolonged or repeated application of 5-HT was unexpected. Therefore we conducted a series of studies in which several factors that might influence the induction of LTH by 5-HT were varied. We omitted the pretests and compared the excitability of cells in untreated ganglia to that of cells in contralateral ganglia (from the same animals) that had been treated with 5-HT. Again, no significant difference was found in the excitability of sensory neurons in control ganglia and those in contralateral ganglia given prolonged or repeated 5-HT treatment when the treatment and tests were performed in ASW (Fig. 3A). We wondered whether the lack of apparent LTH might have resulted from concurrent release of inhibitory neuromodulators during testing and/or application of 5-HT. However, when activity of modulatory interneurons was reduced by bathing with hi-di solution during testing (Fig. 3B) or continuously during 5-HT treatment, overnight culture, and testing (Fig. 3C), we still failed to find significant LTH. Another way to reduce the potential influences of extrinsic neuromodulators released from interneurons is to treat and test clusters of sensory neuron somata after excision from the ganglia (see Fig. 1, inset). This isolates the sensory neuron somata from all other neuronal somata and most of the neuropil. When we gave prolonged or repeated 5-HT treatment to excised sensory neuron clusters bathed in ASW, we found no significant LTH 24 h later (Fig. 3D). Because the soma excision procedure allows axonal injury signals to reach the soma before testing, these results also suggest that 5-HT treatment does not enhance the ability of slow axonal injury signals to induce LTH (see Gunstream et al. 1995).

No significant LTH was induced by concentrations of 5-HT that typically have been used in these preparations (Fig. 2) (see Emptage and Carew 1993; Zhang et al. 1997). However, we

![Figure 2](http://jn.physiology.org/)

**FIG. 2.** 5-HT treatment induces long-term synaptic facilitation (LTF) but no long-term hyperexcitability (LTH) of sensory neurons. A: examples of repetitive firing (bottom) and synaptic connections from pleural sensory neurons to pedal motor neurons (top) tested before and 24 h after repeated application of 5-HT (5 μM) to excised pleural-pedal ganglion preparations. B: significant increase in excitatory postsynaptic potential (EPSP) 24 h after treatment with either a single 2-h pulse of 5-HT or 5 × 5-min pulses of 5-HT at 20-min intervals (both 5 μM). Comparisons are with EPSPs in sensory neurons treated with ASW. Change in EPSP on day 2 (d2) is expressed as a percentage of the EPSP evoked by the same sensory neuron in the pretest on day 1 (d1). C: change in excitability 24 h after 5-HT treatment is not significant (n.s.) after either prolonged or repeated treatment with 5 μM 5-HT. Change in excitability is expressed as the repetitive firing (spike number) on day 2 minus the firing evoked on day 1.
were curious to see if LTH might emerge if we used very high concentrations of 5-HT, such as have occasionally been used in studies of Aplysia synapses (e.g., Fitzgerald and Carew 1991). For example, we wondered whether a high concentration of 5-HT might be able to compensate for the lack of excitability were found within several animals when analyzed individually, with a very high concentration of 5-HT. *, statistically significant changes in excitability were found within several animals when analyzed individually, even though the differences across the entire group of animals were not significant.

5-HT fails to induce LTH of sensory neurons in dissociated cell culture

The only previous study of 5-HT-induced LTH in Aplysia sensory neurons treated isolated sensory neurons growing in dissociated cell culture with four 5-min pulses of 1 μM 5-HT (Dale et al. 1987). We first attempted to extend this finding by seeing if a single 2-h pulse of 5-HT also would induce LTH. Repetitive firing was measured with a series of 2-s depolarizing pulses (1, 2, 3, 4, and 5 nA) injected into the cell soma before and 24 h after a single 2-h pulse of 5 μM 5-HT. The pretest and 5-HT application were given 4–7 days after dissociation. Figure 4 shows both the mean responses to the 1-nA pulse and the means of the maximal responses observed during the entire series of pulses (1–5 nA). Maximal firing was often evoked by currents lower than the 5-nA maximum (see also Fig. 5), presumably because the largest pulses depolarized the sensory neuron to levels where Na⁺ channels became inactivated. Excitability in both the 5-HT-treated and control cells was higher 24 h after the pretest as predicted by previous demonstrations that excitability progressively increases for ~1 wk after dissociation (Liao et al. 1999). However, no significant differences were found between the excitability of the 5-HT-treated sensory neurons and the ASW controls at any current level in either the pretest or posttest, regardless of which day the 5-HT treatment was given.

Similar results were obtained using a procedure in which dissociated sensory neurons were tested 24 h after 5-HT treatment in the absence of a pretest (not shown). Sensory neurons (n = 50) treated with either 5 or 10 μM 5-HT (for 1.5–3 h) 2–4 days after dissociation responded with 12.7 ± 3.7 (SE) spikes in the 1-nA test, whereas corresponding control cells (n = 55) responded with 11.6 ± 2.6 spikes (P = 0.6, not significant). No differences were observed with any of the other test currents. Sensory neurons from only one of the six animals used in this treated and control sensory neurons. Two isolated ganglion preparations and one excised cluster preparation displayed lower excitability in the 5-HT-treated sensory neurons, and in the latter case the decreased excitability was significant (6.1 ± 1.0 vs. 10.0 ± 1.1 spikes, n = 18 cells in each group, P < 0.02, treatment with 5 × 5 min applications of 5-HT). Two isolated ganglion preparations and one excised cluster preparation displayed significantly greater excitability in sensory neurons treated with 2-h applications of 5-HT than in contralateral control sensory neurons (respectively: 11.5 ± 1.2 vs. 7.2 ± 0.7 spikes, n = 12 and 10 cells, P < 0.01; 14.2 ± 2.2 vs. 4.6 ± 1.4 spikes, n = 6 and 7 cells, P < 0.01; 7.0 ± 1.1 vs. 4.4 ± 0.5 spikes, n = 18 cells in each group, P < 0.05). These animals were all from the same source (Alacrity Marine Biological Services) and examined during the winter of 1994–1995, so the dramatic variability cannot be explained by differences in supplier or season. We did not pursue these studies further because the physiological significance of such high 5-HT concentrations seems doubtful. 100 μM 5-HT is approximately 100 times the threshold for producing immediate hyperexcitability of the sensory neurons or facilitation of their synaptic connections in excised ganglia (Empgate et al. 1996), and much higher than that for many other effects. For example, it is 100,000 times the threshold for modulating cardiac and buccal muscle in Aplysia (see Levenson et al. 1999 for review).
A set of experiments showed any trend for 5-HT-induced LTH (26.3 vs. 15.6 spikes at 1 nA, n = 6 and 7 cells, respectively, P = 0.14). However, cells from the other animals, including two treated identically (5 μM 5-HT given 4 days after dissociation), showed no hint of a 5-HT effect.

Our failure to induce LTH in dissociated sensory neurons with a prolonged pulse of 5-HT led us to reexamine the long-term effects on sensory neuron excitability of repeated pulses of 5-HT. We used the same culture methods and stimulation protocols described by Dale et al. (1987); in particular, four 5-min pulses of 1 μM 5-HT delivered at 20-min intervals. Excitability was tested with 40-, 2-, 3-, 4-, and 5-nA current pulses before and 24 h after 5-HT treatment. Only the results of the 1- to 3-nA tests are presented because higher currents often caused clear decreases in spike amplitude and frequency, presumably because of Na channel inactivation. Figure 5 summarizes the results from 54 different sensory neurons dissociated from the pleural ganglia of five different animals. Like Dale et al. (1987), we found that excitability was greater 24 h after 5-HT treatment than during the pretest on day 1. However, control cells that were treated identically but without exposure to 5-HT showed a similar increase in excitability. No significant differences were found between these groups when comparisons were made between the difference in number of spikes evoked in the pretest on day 1 and the posttest on day 2 (Fig. 5). No differences in the pattern of results was seen among animals that were taken at different times of the year (January 1998 and May and June 1999). Likewise, no obvious differences were seen between cells from animals supplied by the NIH-Aplysia Resource facility (n = 17 cells) and those from animals supplied by Alacrity Marine Biological Services (n = 37 cells). Taken together, these results indicate that 5-HT, at concentrations of 1–10 μM, does not effectively induce LTH in dissociated sensory neurons.

5-HT fails to enhance LTH induced by tetanic nerve stimulation

Although we did not find conditions under which application of 5-HT reliably induces LTH, it seemed likely that 5-HT would interact cooperatively with stimuli that do induce LTH. Repeated in vitro stimulation involving high-frequency (“tetanic”) stimulation of Aplysia pedal nerves is reported to cause a modest depression of net outward current 24 h later (Noel et al. 1991), indicating that tetanic nerve stimulation can cause LTH. Studies in this laboratory (see Fig. 6) showed that relatively weak LTH can be induced by applying a brief series of 10 (0.5-s) trains of 2-ms pulses (25 Hz) to pedal nerves p8 and p9 at 5-s intervals. The LTH generated by this procedure is expressed most prominently in sensory neurons the axons of which are activated by the tetanic nerve stimulation (Brou and Walters, unpublished observations). Therefore we asked whether application of exogenous 5-HT would enhance LTH in sensory neurons activated by tetanic nerve stimulation.

The excitability of sensory neurons was tested shortly after tetanic stimulation of nerves p8 and p9 and then 24 h later.
Tetanic stimulation was delivered in either the presence or absence of 10 μM 5-HT, applied 5 min before the tetanus and left in the chamber for 2 h. To identify sensory neurons with axons in the tetanized nerves, after the soma of each cell was tested, a single, 2-ms, supramaximal shock was delivered to nerves p8 and p9 to see if an axon spike was evoked. This shock was five times the threshold for synaptic input to selected neurons (of unknown function) near the VC cluster (see Methods). All tests were conducted in hi-di solution to reduce activation of modulatory neurons by the nerve test stimuli. To facilitate comparisons among the groups, variation in background excitability was minimized by expressing repetitive firing in each sensory neuron as the difference between the median test response and the median pretest response. Because some of the data were skewed, the summary results are presented as medians and nonparametric statistics were used. Pre-and posttreatment comparisons within the same cells were performed with Wilcoxon tests with the number of cells indicated on the figure. Comparisons between clusters were performed with Wilcoxon tests if opposite clusters within the same animals received the two treatments, or were performed with Mann-Whitney U tests if comparisons were made between animals. As described in the preceding text (see Figs. 1–3), treatment with 5-HT in ASW for 2 h caused a large increase in repetitive firing during the 5-HT exposure (P < 0.01 in each of 3 clusters, n = 8–14 cells tested in each cluster), but failed to induce LTH in any of the clusters (data not shown). Exposure to ASW alone for an equal period of time caused no immediate or long-term change in excitability (Fig. 6A). High-frequency, tetanic stimulation of nerves p8 and p9 while the preparation was bathed in ASW caused no change in excitability in tests given 10–30 min later (Fig. 6A). Twenty-four hours after tetanic stimulation, the tetanized sensory neurons displayed significantly more repetitive firing than they had during the pretest (Fig. 6A, P < 0.05). Sensory neurons exposed to 10 μM 5-HT during and after tetanic stimulation also displayed LTH (Fig. 6A, P < 0.05). Selected comparisons showed that there was no difference between the groups tetanized in the presence and absence of 5-HT (Wilcoxon test, n = 5 pairs of clusters), whereas both tetanized groups were more excitable than the control group (Mann-Whitney U tests for between-animal comparisons, P < 0.05 in each case). Although 5-HT did not enhance tetanus-induced LTH, the 5-HT did cause a large increase in excitability while it was present (Fig. 6A, P < 0.0001).

The lack of enhancement of tetanus-induced LTH by 5-HT might be attributed to the activation during pairing of inhibitory systems that oppose the effects of 5-HT on LTH induction. We began to test this possibility by tetanizing the nerves and applying 5-HT while the preparation was bathed in hi-di solution to minimize activation of modulatory interneurons. Although hi-di solution may interfere with some potential mechanisms of synergism, it would not be expected to block direct actions of 5-HT on the sensory neuron. The hi-di solution failed to prevent robust hyperexcitability from occurring during exposure to exogenous 5-HT (Fig. 6B, P < 0.01). Hi-di did, however, prevent LTH induction by tetanic stimulation alone. Similarly, when delivered in hi-di, 5-HT paired with tetanic stimulation failed to produce any LTH. Taken together, these data suggest that 5-HT does not enhance the ability of tetanic stimulation to induce LTH and, conversely, that sensory neuron activation does not enhance a covert capacity of 5-HT to induce LTH. These results also suggest that tetanus-induced LTH of sensory neurons requires activation of interneurons by the tetanic stimulation.

Potent 5-HT antagonist fails to block induction of LTH

In a systematic comparison of 5-HT antagonists that have been used to characterize 5-HT receptors in mammals, Cohen et al. (1997) found that 20 μM methiothepin completely blocks adenyl cyclase activation by 5 μM 5-HT in membranes prepared from pleural sensory clusters. Methiothepin also is reported to inhibit two cloned 5-HT receptors in Aplysia that are coupled to phospholipase C (Li et al. 1995) and a cloned 5-HT receptor that inhibits adenyl cyclase (Angers et al. 1998). We asked whether inhibiting these 5-HT receptors with methiothepin would interfere with the induction of LTH by
repeated nerve stimulation in excised ganglia preparations. In these experiments, we omitted the pretests and tested all sensory neurons in ASW without shocking the nerves during the 24-h tests to look for sensory neuron axons. In early experiments, we used an unpaired design (between-animal comparisons) to demonstrate first that tetanic nerve shock induces LTH of sensory neurons. Two stimulus protocols were used (10 0.5-s, 25 Hz trains of suprathreshold 0.2-ms pulses delivered to pedal nerves p8 and p9 at 5-s intervals, and 10 of the same trains at 1-min intervals). No difference was found in the LTH produced by each protocol, so the results were pooled. As shown with somewhat different testing procedures (see Fig. 5A) (Noel et al. 1991), repeated nerve shock in ASW caused significant 24-h LTH of sensory neurons compared with the excitability of sensory neurons from unshocked control preparations that were tested during the same period (Fig. 7A, left, $P < 0.05$). Using a paired design, we later examined the effect of 50–75 $\mu$M methiothepin on LTH induced by tetanic nerve shock. This concentration range significantly reduces immediate hyperexcitability produced by 5-HT treatment (see next section). Nerve shock in the presence of either 50 or 75 $\mu$M methiothepin failed to reduce the LTH produced by nerve shock (Fig. 7A, right). Indeed, LTH was greater in the methiothepin-treated sensory neurons than in the contralateral cells exposed to the effects of shock alone in four of five preparations.

We also used an unpaired design to examine excitability

24 h after delivering five crushes to each of the major pedal nerves (except p1). In the absence of anesthetic, each nerve crush, like strong nerve shock, causes brief, high-frequency activation of the sensory neurons and widespread activation of other neurons in the ganglia (unpublished observations). The repeated nerve crush in ASW caused significant 24-h LTH of sensory neurons compared with sensory neurons tested in preparations with uncushioned pedal nerves (Fig. 7B, left, $P < 0.005$). Subsequently, we used a paired design to see if the presence of 50 $\mu$M methiothepin during nerve crush would influence LTH induction. In all four preparations, sensory neurons exposed to the effects of nerve crush in the presence of methiothepin were more excitable 24 h later compared with contralateral sensory neurons exposed to the effects of nerve crush without methiothepin, and the overall effect was significant (Fig. 7B, right, $P < 0.01$). Therefore LTH induced by either shocking or crushing pedal nerves under unanesthetized conditions is not blocked by the presence of a potent 5-HT antagonist during nerve stimulation. Indeed the enhancement by methiothepin of LTH induced by both forms of nerve stimulation suggests that endogenous 5-HT might have inhibitory influences on LTH induction in these preparations.

**Methiothepin inhibits expression of LTH**

The development of LTH in *Aplysia* sensory neurons sometimes can be blocked by inhibiting spike activity for prolonged periods between nerve injury or noxious stimulation and subsequent testing (Gasull et al. 1997). This suggested that neuromodulators such as 5-HT might be released continually long after the initial trauma, a possibility supported by the recent finding that 5-HT is elevated in hemolymph 24 h after strong shock to the body surface (Levenson et al. 1999). Because of methiothepin’s potency in blocking 5-HT receptors, we thought this agent might provide evidence about potential contributions of continuing 5-HT release to the expression of LTH during tests 24 h after noxious stimulation.

We first confirmed that methiothepin reduces sensory neuron hyperexcitability produced by bath application of 5-HT (see also Cohen et al. 1997). In every preparation tested ($n = 6$ animals), significant reduction of hyperexcitability was observed in clusters exposed to 5-HT plus methiothepin compared with those exposed to 5-HT alone. Figure 8A, (left) shows results ($P < 0.05$) from a representative experiment in which sensory neurons in one cluster were tested in 5 $\mu$M 5-HT and 50 $\mu$M methiothepin, whereas sensory neurons in the contralateral cluster were tested in 5-HT alone. Decreasing the 5-HT concentration and increasing the methiothepin concentration increased the apparent inhibition by methiothepin (Fig. 8A, right). We then examined the effect of applying methiothepin during testing 24 h after noxious stimulation. Figure 8B shows repetitive firing evoked 24 h after all major pedal nerves (except nerve p1) had been crushed five times at 15 min intervals in ASW. Cells tested in 20 $\mu$M methiothepin responded with significantly fewer spikes than contralateral cells tested without methiothepin ($P < 0.05$).

We found that 20 $\mu$M methiothepin applied to unstimulated ganglia had no obvious effect on basal excitability (Fig. 8C). However, higher concentrations of methiothepin (50–100 $\mu$M) significantly reduced basal excitability in unstimulated ganglia ($n = 5$, data not shown). This reduction of basal excitability by

![FIG. 7. Methiothepin fails to inhibit induction of LTH by noxious nerve shock.](http://jn.physiology.org/ by 10.220.33.2 on April 9, 2017)
higher concentrations of methiothepin might have been caused by decreasing the effects of background release of 5-HT in the ganglia. Alternatively, methiothepin might directly depress sensory neuron excitability independent of its blockade of 5-HT receptors. Indeed, excitability of dissociated sensory neurons grown in cell culture (in the absence of 5-HT) is reliably depressed by methiothepin at concentrations of ≥5 μM (Liao and Walters, unpublished observations). Although sensory neurons in ganglia (which showed no effect from 20 μM methiothepin) are much less sensitive to the depressive effects of methiothepin than are dissociated sensory neurons (Liao and Walters, unpublished observations), the ability of methiothepin to depress excitability of dissociated sensory neurons in the absence of 5-HT suggests that some of methiothepin’s immediate effects on sensory neuron excitability in ganglia are not a consequence of the drug’s blockade of 5-HT receptors. This is particularly true when higher concentrations of methiothepin are used, which always seemed to decrease excitability. For example, in three preparations tested 4–24 h after repeatedly shocking nerve p9, 50 μM methiothepin significantly decreased repetitive firing. In addition, 50–100 μM methiothepin significantly reduced repetitive firing 24 h after intense bilateral pinching of the intact animal (n = 4 preparations). These inhibitory effects might involve direct actions on sensory neuron excitability as well as blockade of 5-HT receptors.

**DISCUSSION**

5-HT produces immediate but not long-term hyperexcitability of sensory neurons

Pleural sensory neurons in *Aplysia* function, at least in part, as nociceptors (Clatworthy and Walters 1993; Walters and Cohen 1997; Walters et al. 1983a). It is thus interesting that 5-HT application immediately increases the excitability of peripheral terminals both of these sensory neurons (Billy and Walters 1989b) and of nociceptors in mammals (Taiwo and Levine 1992). As has now been observed in many studies of sensory neurons in *Aplysia* ganglia (e.g., Baxter and Byrne 1990; Dale et al. 1987; Klein et al. 1986; Stark et al. 1996; Walters et al. 1983b) and dissociated cell culture (Dale et al. 1987; Ghirardi et al. 1992), we found that 5-HT also produces an immediate increase in excitability of pleural sensory neuron somata. Robust hyperexcitability was expressed in the presence of 5-HT, even after nearly 2-h exposure (Fig. 1), so this effect shows little apparent desensitization. After washout of the 5-HT, the hyperexcitability decayed within ~30 min. Similarly, Stark and colleagues (1996) found that hyperexcitability decayed within ~2 min after a 6-min pulse of 5-HT. In our study, the decay of hyperexcitability after 5-HT treatment is reminiscent of the transient early synaptic facilitation produced by repeated pulses of 5-HT in excised ganglia (Mauelshagen et al. 1996).

Unlike 5-HT-induced synaptic facilitation in excised ganglia, which reappears within a day after decay of the short-term form (Empetage and Carew 1993; Mauelshagen et al. 1996), significant LTH did not emerge after 5-HT treatment, even though the 5-HT produced significant LTF of sensory neuron synapses measured at the same time (Fig. 2). Repeated 5-min pulses of 5-HT and a single 2-h pulse of 5-HT proved equally ineffective at inducing LTH in excised ganglia. Only with a very high concentration (100 μM) of 5-HT did we see LTH induction in some preparations, but this concentration also caused long-term reduction of excitability in other preparations, so that the overall effect was not statistically significant (Fig. 3E). These complex results at high 5-HT concentrations are consistent with other findings of both facilitatory and
inhibitory effects of 5-HT applied to ganglia (Fitzgerald and Carew 1991). They suggest that, even at lower concentrations where the effects may not be as apparent, exogenous 5-HT has diverse actions on different types of modulatory neurons that influence sensory neuron excitability.

The complications of 5-HT’s potential effects on modulatory neurons can be avoided by applying 5-HT exclusively to sensory neurons, which is achieved in dissociated cell culture. We found no significant induction of LTH when 5-HT was applied in a single 2-h pulse to dissociated sensory neurons after several days in culture. We also failed to find LTH when 5-HT was applied in four repeated pulses using the methods reported by Dale et al. (1987). The difference in our results and those of Dale and colleagues might reflect unknown differences in experimental conditions, such as differences in hemolymph (a critical component of the culture medium) taken from different animals. Another possibility is that because Dale and colleagues did not know the excitability of dissociated sensory neurons progressively increases with time in culture (Liao et al. 1999), their apparent 5-HT effect may have resulted from slight differences between groups of sensory neurons in their time of testing or from chance differences in their slowly developing responses to dissociation and culture. Although 5-HT might be able to induce LTH under conditions that we have not examined, we conclude that 5-HT treatment is usually not sufficient to induce significant LTH in Aplysia sensory neurons in either excised ganglia or dissociated cell culture preparations.

Our failure to induce LTH with 5-HT treatment is also somewhat surprising in view of the report of Muller and Carew (1998) that prolonged or repeated treatment of ganglia with 10 μM 5-HT causes a significant increase in PKA activity 20 h later (see also Greenberg et al. 1987). A persistent increase in PKA activity would be expected to enhance sensory neuron excitability by depressing soma K⁺ conductances (e.g., Baxter and Byrne 1990; Goldsmith and Abrams 1992; Klein et al. 1986; Siegelbaum et al. 1982). One possible explanation is that small procedural differences affect the duration of long-term PKA activation by 5-HT; for example, Hegde and colleagues (1997) did not find evidence for 24-h PKA activation after similar 5-HT treatment. Another possibility is that 5-HT simultaneously can induce opposing modulatory processes that block the hyperexcitability that would otherwise be expressed during increased PKA activity. Finally, direct and indirect effects of 5-HT on PKA activity might be occluded partially by a persistent increase in PKA activity that follows nerve injury (Liao et al. 1999), produced in these cases by dissection.

Are 5-HT and 5-HT-stimulated cAMP synthesis important for induction of LTH by noxious stimuli?

Although 5-HT alone was generally unable to induce LTH, several observations suggested that 5-HT would nonetheless have some involvement in the induction of LTH. Noxious stimulation releases 5-HT into the hemolymph (Levenson et al. 1999) and produces LTH of pleural sensory neurons (Clatworthy and Walters 1993; Klein et al. 1986), and increase transmitter release from sensory neurons onto modulatory interneurons (reviewed by Byrne and Kandel 1996). Conversely, sensory neuron spike activity evoked by nerve stimulation should enhance cAMP synthesis and long-term alterations dependent on cAMP synthesis (e.g., Abrams and Kandel 1988; Ocorr et al. 1985; Scholz and Byrne 1988). Therefore we were surprised to find that pairing 5-HT with tetanic nerve stimulation did not enhance the ability of the tetanic stimulation to induce LTH or to enhance a covert capacity of 5-HT to induce LTH. This suggested that 5-HT makes little contribution to the induction of LTH by noxious stimulation. Alternatively, application of exogenous 5-HT may produce parallel inhibitory effects that obscure synergistic interactions of 5-HT and noxious stimulation that would normally occur during the induction of LTH.

As an independent test of contributions of 5-HT to the induction of nociceptive LTH, we used a potent 5-HT antagonist, methiothepin, which blocks several 5-HT receptor types in Aplysia (Angers et al. 1998; Cohen et al. 1997; Li et al. 1995). Methiothepin was applied during noxious stimulation, produced either by shocking or crushing exposed nerves in an excised ganglia preparation. In neither case did methiothepin interfere with induction of LTH (Fig. 7). Indeed, methiothepin caused a significant increase in sensory neuron excitability 24 h after repeated nerve crush, and a similar tendency was observed after tetanic nerve shock. The same pattern has been seen after methiothepin was applied to the CNS during extensive prodding of the body in a semi-intact preparation (X. Liao and E. T. Walters, unpublished observations). Because methiothepin potently blocks adenylyl cyclase activation in Aplysia CNS by 5-HT (Cohen et al. 1997) and also blocks other 5-HT receptors in this animal (Angers et al. 1998; Li et al. 1995), the failure of methiothepin to interfere with LTH induction adds to our evidence that 5-HT is not important for induction of LTH in Aplysia sensory neurons. In fact, it suggests that 5-HT inhibits LTH induction (despite causing short-term hyperexcitability). These conclusions, however, depend on methiothepin being specific for 5-HT receptors. It recently has been found that methiothepin antagonizes dopamine-induced cAMP synthesis in addition to 5-HT-induced cAMP synthesis (J. E. Cohen and T. W. Abrams, personal communication). Thus the enhancement of LTH induction in the presence of methiothepin might be due to a blockade of inhibitory receptors (e.g., dopamine or possibly FMRFamide) (Belardetti et al. 1987; Billy and Walters 1989b; Mackey et al. 1987; Montarolo et al. 1988) or to a direct depression of the excitability of inhibitory interneurons co-activated by nerve stimulation.

Because relatively low concentrations of methiothepin completely block the activation of adenylyl cyclase in sensory neuron membranes by 5-HT (Cohen et al. 1997) and by dopamine (J. E. Cohen and T. W. Abrams, personal communication), it seems likely that methiothepin reduced cAMP synthesis caused by intense nerve stimulation (Fig. 6). The failure of this probable reduction in cAMP synthesis to inhibit LTH induction provides important support for the conclusion that
the cAMP/PKA pathway is not required for induction of LTH by either noxious pinching of the body surface (Lewin and Walters 1999) or axotomy (Liao et al. 1999). Given that the 5-HT-cAMP-PKA pathway is critical for the induction of LTF by noxious stimulation (for review, see Byrne et al. 1993), these data suggest that separate signal transduction pathways may be responsible for inducing LTH of the sensory neuron soma and for inducing LTF of the sensory neuron synapses. Recent evidence suggests that a nitric oxide–cGMP-dependent protein kinase pathway is critical for induction of LTH by noxious stimulation (Lewin and Walters 1999).

Despite the pharmacological complications involved in interpreting methiothepin’s effects, the simplest interpretation of the failure of methiothepin to block LTH induction is that 5-HT and 5-HT-induced cAMP synthesis are not required for LTH induction. Combined with our observations that 5-HT treatment is not sufficient to induce LTH and that 5-HT fails to enhance LTH induction by nerve stimulation, we conclude that 5-HT release does not induce LTH in *Aplysia* sensory neurons during noxious stimulation.

**Does nociceptive sensitization involve persistent release of 5-HT?**

Intense or repeated noxious stimulation of *Aplysia* produces two, related types of long-term “sensitization” that traditionally have been the province of separate disciplines. First, long-lasting behavioral sensitization—enhancement of defensive behaviors such as gill, siphon, and tail withdrawal (Cleary et al. 1998; Frost et al. 1985; Pinsker et al. 1973; Walters 1987a,b) largely has been examined by investigators of learning and memory. Second, long-lasting sensitization (enhanced sensitivity) of sensory neurons largely has been studied by pain physiologists in mammalian models but is displayed clearly in *Aplysia* sensory neurons (which function as nociceptors) after noxious stimulation (Billy and Walters 1989b, Miller and Walters 1998; Walters 1987b). In *Aplysia*, both types of sensitization have been assumed to represent persistent, intrinsic changes in nociceptive sensory neurons triggered by relatively transient exposure to 5-HT and other signals (reviewed by Walters 1994; Walters and Ambron 1995). In the dissociated sensory neuron cultures in which much of this research has been conducted, it would not be possible for persistently released extrinsic signals to play a role in maintaining long-term sensory alterations. However, in more intact preparations, noxious stimulation might cause a long-lasting increase in activity of modulatory neurons that could help to maintain long-term sensitization of both sensory neurons and defensive behavior. We found that the presence of methiothepin during excitability testing 24 h after noxious stimulation significantly reduced the expression of LTH (Fig. 8). Because 5-HT is probably degraded rapidly (e.g., Levenson et al. 1999), this finding suggests that part of the 24-h memory of noxious stimulation is mediated by continuing release of 5-HT.

The interpretation of methiothepin’s actions are complicated, however, because the drug has nonspecific effects, including blockade of dopamine receptors (J. E. Cohen and T. W. Abrams, personal communication) and direct depression of sensory neuron excitability (Liao and Walters, unpublished observations). With this reservation in mind, the possibility that methiothepin antagonizes persisting 5-HT release is of considerable interest in view of the finding that 5-HT levels in *Aplysia* hemolymph are elevated 24 h after noxious shock to the body surface (Levenson et al. 1999), and the observation that expression of LTH induced by nerve crush depends partly on persisting PKA activity (Liao et al. 1999). Moreover, long-lasting blockade of spike activity in excised ganglia after nerve crush (using hi-di solution or tetrodotoxin) can reduce subsequently tested LTH (Gasull et al. 1997). Together, these three types of observation suggest that 1-day memory of noxious stimulation involves a continuing defensive arousal mediated by persistent activity of 5-HT-containing interneurons, and by consequence, ongoing activation of PKA in sensory neurons. This mechanism should complement the long-lasting activation of PKA caused by degradation of regulatory subunits, which has been observed after prolonged 5-HT application (Chain et al. 1999; Greenberg et al. 1987). Although persistent activity of modulatory neurons may be one nociceptive memory mechanism, the fact that long-term changes in sensory neuron excitability after peripheral trauma are expressed under conditions in which interneuronal activity and neuromodulator release are blocked (Miller and Walters 1998; E. T. Walters, C. G. Brou, and M. Ungless, unpublished observations) shows that some components or phases of nociceptive memory are also stored as lasting changes intrinsic to the sensory neurons.

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