Serotonin Induces Memory-Like, Rapamycin-Sensitive Hyperexcitability in Sensory Axons of *Aplysia* That Contributes to Injury Responses

Ramal M. S. Weragoda and Edgar T. Walters

Department of Integrative Biology and Pharmacology, University of Texas–Houston Medical School, Houston, Texas

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Weragoda RMS, Walters ET. Serotonin induces memory-like, rapamycin-sensitive hyperexcitability in sensory axons of *Aplysia* that contributes to injury responses. *J Neurophysiol* 98: 1231–1239, 2007. First published July 18, 2007; doi:10.1152/jn.01189.2006. The induction of long-term facilitation (LTF) of synapses of *Aplysia* sensory neurons (SNs) by serotonin (5-HT) has provided an important mechanistic model of memory, but little is known about other long-term effects of 5-HT on sensory properties. Here we show that crushing peripheral nerves results in long-term hyperexcitability (LTH) of the axons of these nociceptive SNs that requires 5-HT activity in the injured nerve. Serotonin application to a nerve segment induces local axonal (but not somal) LTH that is inhibited by 5-HT–receptor antagonists. Blockade of crush-induced axonal LTH by an antagonist, methiothepin, provides evidence for mediation of this injury response by 5-HT. This is the first demonstration in any axon of neurmodulator-induced LTH, a phenomenon potentially important for long-lasting pain. Methiothepin does not reduce axonal LTH induced by local depolarization, so 5-HT is not required for all forms of axonal LTH. Serotonin-induced axonal LTH is expressed as reduced spike threshold and increased repetitive firing, whereas depolarization-induced LTH involves only reduced threshold. Like crush- and depolarization-induced LTH, 5-HT–induced LTH is blocked by inhibiting protein synthesis. Blockade by rapamycin, which also blocks synaptic LTF, is interesting because the eukaryotic protein kinase that is the target of rapamycin (TOR) has a conserved role in promoting growth by stimulating translation of proteins required for translation. Rapamycin sensitivity suggests that localized increases in translation of proteins that promote axonal conduction and excitability at sites of nerve injury may be regulated by the same signals that increase translation of proteins that promote neuronal growth.

**INTRODUCTION**

Investigation of the effects of serotonin (5-HT) on synapses between sensory and motor neurons of *Aplysia* has revealed fundamental mechanisms of long-term synaptic plasticity (Kandel 2001) that appear to contribute to long-term memory in mammals and other invertebrates as well (e.g., Lynch 2004; Waddell and Quinn 2001). In *Aplysia*, 5-HT is usually applied to sensorimotor cocultures in repeated pulses (e.g., Bailey et al. 2004; Mauelshagen et al. 1996; Montarolo et al. 1986) that are designed to mimic patterns of noxious cutaneous stimuli used to induce both a behavioral memory, long-term sensitization of defensive withdrawal responses (Pinzker et al. 1973) and a correlated long-term facilitation (LTF) of sensorimotor synapses (Cleary et al. 1998; Frost et al. 1985). The involvement of 5-HT as a nociceptive signal is supported by observations of 5-HT release within the CNS after electrical stimulation of peripheral nerves (Marinesco and Carew 2002), whereas the importance of 5-HT for defensive sensitization in *Aplysia* is suggested by the disruption of some of the sensitizing effects of noxious stimulation by prior depletion of 5-HT from the animal (Glanzman et al. 1989).

Serotonin also has another effect on *Aplysia* sensory neurons (SNs) that would be expected to enhance defensive responses after noxious stimulation—hyperexcitability is expressed robustly in either the soma (e.g., Baxter and Byrne 1990; Bunge et al. 1997; Klein et al. 1986; Liao et al. 1999a; Walters et al. 1983) or peripheral receptive field (Billy and Walters 1989a) for short periods after local application of 5-HT. Long-term effects of 5-HT on soma excitability are uncertain; although one report described protein-synthesis–dependent, long-term hyperexcitability (LTH) of the soma after 5-HT application to dissociated SNs (Dale et al. 1987), 5-HT–induced LTH of the soma was not found in an extensive study that examined SNs both in dissociated cell culture and in excised ganglia (Liao et al. 1999a).

Although long-term effects of 5-HT on soma excitability may be weak or difficult to bring under experimental control, an interesting possibility is that robust LTH can be induced in SN axons by 5-HT acting within peripheral nerves. Recently we discovered (Weragoda et al. 2004) that either crushing or depolarizing a peripheral nerve containing SN axons produces LTH of these axons that depends on local, rapamycin-sensitive protein synthesis, similar to that required for 5-HT–induced LTF of sensorimotor synapses (Casadio et al. 1999; Hu et al. 2006; Si et al. 2003). Moreover, SN axons might be exposed to 5-HT during trauma that causes nerve injury. This 5-HT could come from at least three sources: 1) the hemolymph, which conveys neurohumoral 5-HT after noxious stimulation (Levenson et al. 1999); 2) 5-HT–containing hemocytes attracted to an injury site (Farr et al. 1999; Ottaviani et al. 1992); and 3) serotonergic axons, which are located in the peripheral nerves (Goldstein et al. 1984; Longley and Longley 1986; McPherson and Blankenship 1992). We now show that activation of 5-HT receptors appears necessary for axonal LTH at a site of nerve injury, that local 5-HT application is sufficient to induce LTH of SN axons, and that 5-HT–induced axonal LTH requires local, rapamycin-sensitive protein synthesis.

**METHODS**

**General**

*Aplysia californica* (100–250 g; Alacrity Marine, Redondo Beach, CA) were anesthetized by injection of isotonic MgCl$_2$ solution (337 mM). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
The pedal–pleural ganglia were excised and placed in chambers with the attached posterior pedal nerve, p9, threaded through a series of connected wells (Fig. 1). Ganglia were desheathed in a 1:1 mixture of artificial seawater (ASW) and isotonic MgCl₂. To minimize sheath contraction and neuromodulator release during each experiment (Weragoda et al. 2004), the ganglia and attached nerves were bathed in low-Ca saline (1% normal [Ca²⁺]) containing (in mM) NaCl, 460; CaCl₂, 0.1; KCl, 10; MgCl₂, 66; and Tris buffer, 10 (pH 7.6). In some experiments the low-Ca saline contained only 0.02% normal [Ca²⁺] (2 μM). Excised ganglia and nerves were stored overnight at 16°C in supplemented ASW containing (in mM) NaCl, 460; CaCl₂, 11; KCl, 10; MgCl₂, 55; Na HEPES, 10; glucose, 7; MEM essential amino acids, 0.2 x normal concentration (GIBCO Laboratories Life Technology); MEM nonessential amino acids, 0.2 x normal concentration (GIBCO), and MEM vitamin solution, 0.7 x normal concentration (GIBCO); pH 7.6 (Goldsmith and Abrams 1992). Anisomycin, etamine, Rapamycin, Methiothepin mesylate, 5-hydroxytryptamine (5-HT) creatinine sulfate complex, and dopamine HCl were obtained from Sigma (St. Louis, MO). Spiperone HCl was obtained from Tocris (Ellisville, MO).

**Test stimulation**

Extracellular electrical test stimuli were applied to segments of nerve p9 while using intracellular electrodes to record action potentials (spikes) conducted to the soma of a tail SN in the pleural ganglion (Fig. 1A). The cut tips of the nerves were ≥1 cm from the test segment, which was ≥2 cm from the ganglia. In initial experiments, test stimuli were applied to a short segment between adjacent wells, with the current focused through a narrow, 2-mm-long slot covered with silicone grease (Fig. 1A). In most experiments, test stimuli were applied to a longer segment between electrodes placed on either side of the nerve within a single, 6-mm-diameter well (Fig. 1B). Axon spike thresholds were tested with ascending series of 5- and 50-ms pulses, whereas repetitive firing was tested with 1-s pulses using a test current twice the magnitude of the 50-ms threshold (approximately twice rheobase). In site-specificity experiments, proximal and distal sites were tested on each nerve (2.5–3.0 cm and 5.0–5.5 cm from the soma, respectively). Intracellular recordings were made from sensory somata at 19–21°C using standard methods (Weragoda et al. 2004). Test stimuli were applied before and 24 h after nerve crush or treatment with 5-HT or high-K saline.

**5-HT treatment**

In some experiments a nerve segment was exposed to 10 μM 5-HT in low-Ca saline continuously for 2 h (Fig. 1C). In most experiments the treated nerve segment was given five 5-min pulses of 10 μM 5-HT (~25 min total exposure) in low-Ca saline (see Emptage and Carew 1993; Liao et al. 1999a; Montaloro et al. 1986). Each 5-HT pulse was followed by 10-min washout with low-Ca saline (Fig. 1C). Nerve test wells were isolated from adjoining wells with a silicone-based vacuum grease (Dow Corning, Midland, MI).

**Axotomy**

Nerve p9 was crushed in the excised preparation using fine forceps in the middle of the test well. The forceps crush transects nearly all of the axons while sparing the surrounding sheath and (at least in vivo) permits eventual regeneration of sensory axons through the crush site (Steffensen et al. 1995). Control nerves were manipulated with forceps but not crushed.

**Nerve depolarization**

Segments of nerve p9 were depolarized by rapidly withdrawing low-Ca saline from the test well (briefly exposing the nerve sheath to air) and replacing it for 2 min with high-K/low-Ca saline containing (in mM) CaCl₂, 0.1; KCl, 470; MgCl₂, 66; and Tris buffer, 10 (pH 7.6). Sham treatments consisted of identically rapid fluid exchanges using low-Ca saline (see Weragoda et al. 2004).

**FIG. 1.** Axonal testing and serotonin (5-HT) treatment. A: pedal–pleural ganglia with attached tail nerve (p9) threaded through 6-mm wells. Dual-well, short-segment (2-mm) test stimulation configuration is shown, with electrical test stimuli delivered across adjacent wells through a narrow passage covered with silicone grease to focus the test current on this 2-mm segment. Evoked spikes were recorded intracellularly in a sensory neuron (SN) soma in the pleural ganglion. 5-HT was applied concurrently to both testing wells (treated length, 14 mm) that were isolated from saline in the adjacent wells with silicone grease. 5-HT was applied to the same well (treated length, 6 mm). C: timing of axon tests and 5-HT treatments. Tests were given for each of 3–6 SNs sampled in each ganglion before (in most experiments) and 24 h after 5-HT treatment. Continuous treatment consisted of 2-h incubation in 5-HT (10 μM). In the pulsed treatment, 5-HT (10 μM) was applied in 5 pulses, each 5 min, followed by 10-min washout. Sham treatments were identical except that saline was substituted for 5-HT. D: responses monitored in the soma of a representative tail SN during axon spike threshold test and repetitive firing test. Repetitive firing is shown before (baseline) and 24 h after pulsed 5-HT treatment.
**Drug treatments**

5-HT–receptor antagonists, methiothepin and spiperone, were added to a nerve segment from 30 min before treatment until the end of treatment period (105 min total). In nerve crush experiments, methiothepin was added to the crush site from 30 min before crush until 75 min after injury (105 min total). Because translation inhibitors must penetrate the intracellular compartment, incubation with rapamycin, emetine, or anisomycin began 60 min before treatment and lasted 3 h (Weragoda et al. 2004).

**Data analysis**

Data are reported as means ± SE, with the n values indicating the numbers of ganglia tested in each condition. Three to six cells were sampled in each ganglion and averaged to yield individual data points for statistical analysis (so a study with n = 4 ganglia would include data from 12 to 24 SNs). Comparisons between treatments of paired nerve segments were made with paired t-tests. Multiple comparisons across matched ipsilateral and contralateral nerves were made using one-way ANOVA with repeated measures followed by Bonferroni’s post hoc tests. Statistically significant differences are indicated in each figure (*P < 0.05, **P < 0.01, ***P < 0.001).

**RESULTS**

**5-HT applied to a peripheral nerve induces LTH of SN axons but not somata**

To see whether 5-HT delivered to a peripheral nerve can induce LTH of the axon or soma of tail SNs, we applied 10 μM 5-HT for 2 h to a 1-cm segment of nerve p9, 1–2 cm from the pleural ganglion. After 24 h, the excitability of both the treated axon segments and SN somata was tested. During 5-HT treatment and the excitability tests the nerve and ganglia were bathed in low-Ca saline (0.02% normal, [Ca2+]i = 2 μM) to minimize sheath contraction and possible neuromodulator release. No significant difference in either spike threshold or repetitive firing properties of the soma was found between sham-treated and 5-HT–treated groups 24 h after peripheral 5-HT treatment, as monitored with intracellular test pulses 24 h after treatment (Fig. 2A; n = 8 pairs of ganglia). In contrast, when extracellular test pulses were delivered to the treated segment of nerve p9, axon spike threshold decreased (Fig. 2B; P < 0.05; n = 8), and repetitive firing increased (P < 0.01). In this initial study no baseline tests were given before 5-HT treatment, and the dual-well configuration was used for axon stimulation (Fig. 1A). We then asked whether a series of five 5-min pulses of 5-HT (10 μM) separated by 10-min washout periods would produce greater LTH of SN axons than that induced by continuous 5-HT treatment for 2 h. In this and subsequent studies we gave baseline tests before 5-HT treatment and used the single-well configuration for axon stimulation (Fig. 1B). Again, continuous 5-HT treatment induced significant long-term enhancement of repetitive firing (Fig. 2C; P < 0.001, n = 10) but the pulsed treatments, which also enhanced repetitive firing (P < 0.001, n = 8), caused a significantly greater enhancement than did the continuous treatment protocol (P < 0.01). This study also failed to reveal any significant effect of either of these 5-HT treatments to the nerve on somal repetitive firing 24 h later (baseline test vs. 24-h test for both 5-HT treatment conditions, 11.9 ± 1.7 vs. 10.7 ± 1.5 spikes, n = 18).

**Axonal LTH is localized to the segment treated with 5-HT**

LTH of a sensory axon induced by either nerve crush or depolarization is restricted to the injured or depolarized segment (Weragoda et al. 2004). To see whether 5-HT–induced axonal LTH displays similar site specificity, we tested proximal and distal sites on nerve p9 before and after one of the tested nerve segments received 5-HT treatment and the other received sham treatment. The corresponding segments on the contralateral p9 nerve received opposite treatments, so that each 5-HT–treated site could be compared with both an ipsilateral and a contralateral sham-treated control. Again, the tests and treatments were conducted with the ganglia and nerve bathed in low-Ca saline (1% normal, [Ca2+]i = 100 μM). Planned comparisons revealed that, after 24 h, spike threshold...
decreased significantly in 5-HT–treated nerve segments relative to both ipsilateral and contralateral sham-treated segments (Fig. 3A; proximal 5-HT vs. distal sham, \(P < 0.001\); proximal 5-HT vs. proximal sham, \(P < 0.001\); distal 5-HT vs. proximal sham, \(P < 0.001\); distal 5-HT vs. distal sham, \(P < 0.001\); \(n = 4\) in each case). In addition, repetitive firing increased in nerve segments treated with 5-HT compared with both ipsilateral and contralateral sham-treated segments (Fig. 3B; proximal treated vs. distal sham, \(P < 0.001\); proximal treated vs. proximal sham, \(P < 0.01\); distal treated vs. proximal sham, \(P < 0.001\); distal treated vs. distal sham, \(P < 0.001\); \(n = 4\) in each case). These results demonstrate that axonal LTH induced by 5-HT is localized to the treated segment. Also, because action potentials evoked by testing the distal segment must travel through the proximal segment to be monitored in the soma, they indicate that 5-HT–induced LTH in a proximal axon segment does not alter afferent activity conducted from more distal axon segments. Similarly, LTH in a distal axon does not add impulses to spikes evoked by test stimuli applied to a proximal segment; i.e., LTH in one axonal segment does not appear to produce afterdischarge following the passage of impulses from other parts of the axon.

**5-HT–induced axonal LTH requires local, rapamycin-sensitive protein synthesis**

LTH of SN axons induced by nerve injury or focal depolarization, like long-term facilitation of SN synapses by 5-HT, requires local protein synthesis (Weragoda et al. 2004). To see whether 5-HT–induced axonal LTH also requires local protein synthesis, we used agents known to effectively inhibit translation of mRNAs in *Aplysia* (Casadio et al. 1999; Martin et al. 1997; Schwartz et al. 1971; Yanow et al. 1998): rapamycin, anisomycin, another general protein synthesis inhibitor, which blocks elongation site of the 60S ribosomal subunit, and emetine, a general protein synthesis inhibitor that binds to the peptide elongation site of the 60S ribosomal subunit, and anisomycin, which selectively blocks synthesis of proteins associated with growth by targeting FKBP12–rapamycin-associated protein [FRAP, also known as target of rapamycin (TOR)], emetine, a general protein synthesis inhibitor that binds to the peptide elongation site of the 60S ribosomal subunit, and anisomycin, another general protein synthesis inhibitor, which blocks peptidy transferase. By 24 h after 5-HT treatment, nerve segments that were incubated for 3 h in 20 nM rapamycin (beginning 1 h before 5-HT treatment) failed to show any 5-HT–induced decrease in spike threshold (Fig. 4; 5-HT vs. 5-HT + rapamycin, \(P < 0.01, n = 5\)) nor did they show any increase in repetitive firing (\(P < 0.01\)). Nerve segments incubated in 100 \(\mu M\) emetine also failed to display any decrease in spike threshold (Fig. 4; 5-HT vs. 5-HT + emetine, \(P < 0.001, n = 4\)) or increase in repetitive firing (\(P < 0.05\)). In addition, no hints of 5-HT–induced LTH were observed in two preparations treated with anisomycin (10 \(\mu M\)) (not shown). These results indicate that 5-HT–induced axonal LTH requires localized, rapamycin-sensitive protein synthesis.

**5-HT–receptor antagonists block serotonin-induced axonal LTH**

To begin to test the specificity of the 5-HT effects and screen for drugs that can be used to test the contributions of 5-HT to injury-induced LTH, we delivered pulses of 5-HT in the presence of one of two 5-HT–receptor antagonists previously shown to be effective in *Aplysia*. Spiperone blocks a phospholipase C/protein kinase C (PLC/PKC)–mediated effect of 5-HT on SN synapses in *Aplysia* (Dumitriu et al. 2006). Methiothepin is an inhibitor of various 5-HT receptors in *Aplysia*, including adenylyl cyclase–coupled receptors (Cohen et al. 2003) and may also antagonize some PLC-coupled 5-HT receptors (Dumitriu et al. 2006). Nerve segments were incubated with 100 \(\mu M\) methiothepin or 100 \(\mu M\) spiperone from 30 min before until the end of 5-HT treatment. The decrease in axonal spike threshold produced by 5-HT treatment was blocked by both antagonists (Fig. 5) (5-HT vs. 5-HT + methiothepin, \(P < 0.05, n = 7\); 5-HT vs. 5-HT + spiperone, \(P < 0.01, n = 9\)). Although methiothepin blocked the 5-HT–induced increase in repetitive firing (5-HT vs. 5-HT + methiothepin, \(P < 0.001, n = 7\)), the decrease in repetitive firing observed with spiperone was not statistically significant (\(n = 9\); Fig. 5). The blockade by either methiothepin or spiperone of at least some of the manifestations of 5-HT–induced LTH

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**FIG. 3.** Restriction of 5-HT–induced axonal LTH to the treated nerve segment. For each SN tested, electrical stimuli were delivered to proximal and distal test segments before and 24 h after pulsed treatment with 5-HT (10 \(\mu M\)) to one of the segments. For simplicity, the well containing the ganglia (which would be to the left) has been omitted from each diagram. Only the 5-HT–treated segments displayed a long-term decrease in threshold (A) or a long-term increase in repetitive firing (B). Asterisks indicate significant differences from both ipsilateral and contralateral sham-treated controls (ANOVA/Bonferroni tests; see RESULTS).
suggestions that this form of LTH may involve more than one type
of 5-HT receptor and multiple signaling pathways (see DISCUSSION and Dumitriu et al. 2006).

Although methiothepin is an effective 5-HT antagonist in Aplysia, it can also antagonize some dopamine effects (Cohen et al. 2003), raising the possibility that dopamine receptors might be involved directly or indirectly in 5-HT–induced LTH. However, application of dopamine (10 μM) in the same pulsed protocol used for 5-HT–induced LTH failed to produce any evidence of LTH, as monitored by both action potential threshold and repetitive firing 24 h later (Fig. 5; n = 3). This observation indicates that any antagonistic effect of methiothepin on injury-induced LTH (see following text) is unlikely to be a consequence of blocking dopamine receptors.

Blocking 5-HT receptors prevents LTH induced by crush but not by depolarization of the nerve

We demonstrated previously that either crush injury or local depolarization of a peripheral nerve segment in Aplysia induces localized LTH of sensory axons (Weragoda et al. 2004). One hypothesis to explain these results is that both nerve injury and depolarization cause local release of 5-HT, which then induces axonal LTH. As a test of this hypothesis we examined the effects on LTH of applying a 5-HT–receptor antagonist before either nerve crush or depolarization produced by elevation of extracellular K⁺. Methiothepin (100 μM) was used because of its effectiveness in blocking both the long-term decrease in spike threshold and increase in repetitive firing induced by 5-HT treatment (Fig. 5).

Nerve p9 was crushed with fine forceps, which transected most of the axons while leaving the sensory sheath intact (Steffensen et al. 1995). Pretreatment with methiothepin prevented the long-term decrease in spike threshold caused by nerve crush (Fig. 6, crush vs. crush + methiothepin, P < 0.01, n = 7) as well as the increase in repetitive firing (P < 0.05). Application of methiothepin to uncrushed nerves

FIG. 4. 5-HT–induced axonal LTH of Aplysia SNs requires local protein synthesis and is rapamycin sensitive. Three independent studies were performed to compare the effects of 5-HT to sham treatment or to cotreatment with each of the 2 indicated inhibitors. For clarity and ease of comparison, the 5-HT results are combined into a single column and results from all 3 experiments are displayed together, although statistical comparisons were performed separately (see text). Pulsed 5-HT (10 μM) treatment reduced axonal spike threshold and increased repetitive firing compared with responses of sham-treated segments. Application to the same well used for electrical testing and 5-HT treatment (see Fig. 1B) of rapamycin (20 nM) or emetine (100 μM) before and during 5-HT application resulted in significant inhibition of the effects of 5-HT on spike threshold and repetitive firing. Asterisks indicate significant differences in paired experiments evaluated with paired t-test (see text).

FIG. 5. 5-HT receptor antagonists block 5-HT induction of axonal LTH. Three independent experiments were performed to compare the effects of 5-HT to sham treatment or to cotreatment with each of the 2 indicated inhibitors, as well as to compare dopamine treatment to sham treatment. For simplicity of presentation, the 5-HT results are combined into a single column, and results from all 3 studies are displayed together, although statistical comparisons were performed separately (see text). Cotreatment of 5-HT with methiothepin (100 μM) or spiperone (100 μM) significantly inhibited the decrease in spike threshold, whereas methiothepin, but not spiperone, significantly inhibited the increase in repetitive firing induced by 5-HT. Dopamine (DA, 10 μM) failed to induce axonal LTH, as monitored by spike threshold and repetitive firing. Asterisks indicate significant differences in paired experiments with 5-HT, evaluated with paired t-test. All solutions contained 1% normal [Ca²⁺].
had no significant effect on spike threshold or repetitive firing compared with sham treatment of contralateral segments (n = 4).

In contrast to its blockade of crush-induced LTH, pretreatment of the nerve segment with 100 μM methiothepin did not significantly interfere with long-term depression of axonal spike threshold after 2-min depolarization of the axons with high-K/low-Ca saline (Fig. 6, n = 4). As shown previously (Weragoda et al. 2004), we found that the LTH induced by this high-K/low-Ca saline (which depolarizes SN membranes to about 0 mV under conditions in which observable synaptic transmission and sheath contraction are blocked) was manifest as a decrease in spike threshold (P < 0.05 compared with baseline 24 h earlier and P < 0.001 compared with sham-treated controls) but not a significant increase in repetitive firing 24 h after treatment (Fig. 6). These findings suggest that a critical step in the induction of axonal LTH by nerve crush is the activation of methiothepin-sensitive 5-HT receptors. They also suggest, however, that 2-min depolarization of the nerve segment without injury (in low-Ca saline) can induce one manifestation of LTH—a long-term decrease in axonal spike threshold—without activating methiothepin-sensitive 5-HT receptors.

**DISCUSSION**

**5-HT contributes to LTH of sensory axons**

Although most investigations of 5-HT–induced plasticity in *Aplysia* have focused on synapses (reviewed by Bailey et al. 2004; Kandel 2001; Reissner et al. 2006; Roberts and Glanzman 2003), 5-HT also causes short-term hyperexcitability in other compartments of the SN, including the soma (e.g., Baxter and Byrne 1990; Bunge et al. 1997; Dale et al. 1988; Klein et al. 1986; Liao et al. 1999a) and peripheral receptive field (Billy and Walters 1989b). In addition, 5-HT causes short- and intermediate-term hyperexcitability of the SN axon (Gunstream et al. 1996; LJ Klaassen and ET Walters, unpublished observations). SN synapses also exhibit long-term alterations induced by 5-HT (Emptage and Carew 1993; Martin et al. 1997; Montarolo et al. 1986; Sherff and Carew 1999). We have now demonstrated long-term alterations of axonal excitability by focal application of 5-HT, which to our knowledge is the first demonstration of the induction of long-term axonal hyperexcitability by a chemical neuromodulator in any species. One day after either a single 2-h application of 5-HT or five 5-min pulses of 5-HT, axonal spike threshold was decreased and repetitive firing increased. This LTH, like axonal LTH induced by either nerve crush or depolarization (Weragoda et al. 2004), is localized to the treated or injured site (thus contributing to site-specific rather than general sensitization; Walters 1987, 1994). We do not know whether 5-HT acts directly on the sensory axons or indirectly, e.g., by triggering release of factors from glia. Although indirect effects are possible (e.g., involving cell adhesion molecules; any indirect effects are unlikely to depend on conventional Ca\(^{2+}\)-dependent secretion because LTH induction occurred in saline containing \(\leq 1\%\) of the normal concentration of Ca\(^{2+}\)) (see Weragoda et al. 2004).

Our block of crush-induced axonal LTH by pretreatment of the nerve segment with the 5-HT–receptor antagonist methiothepin is consistent with the possibility that 5-HT released during injury is a major contributor to the induction of axonal LTH. Endogenous 5-HT can be brought close to sensory axons after peripheral injury in at least three ways. First, noxious stimulation of the body surface causes global elevation of 5-HT in the hemolymph (Levenson et al. 1999). Although humorally transported 5-HT might produce a general increase in axonal excitability after nerve injury, the localization of LTH to a region <2 mm from the site of injury (Weragoda et al. 2004) indicates that a diffuse elevation of 5-HT in the hemolymph does not account for the localized, crush-induced LTH we report here. Second, some of the inflammatory-like hemocytes in *Aplysia* (Farr et al. 1999) and other molluscs (Ottaviani et al. 1992) contain 5-HT, and hemocytes in *Aplysia* accumulate at a site of nerve injury (Clatworthy and Grose 1999; Farr et al. 1999). Under our in vitro conditions, however, any 5-HT released from neuroendocrine cells or hemocytes was minimized by omitting hemolymph and hemocytes from the medium. Third, many serotonergic neurons in *Aplysia* have axons in peripheral nerves, including nerve p9 (Goldstein et al. 1984; Longley and Longley 1986; McPherson and Blankenship 1992). Within tens of minutes after nerve transection (Longley and Longley 1986) or nerve crush (RMS Weragoda, LJ Klaassen, and ET Walters, unpublished observations) varicosities develop in serotonergic axons near an injury site. Proof that neurotransmitter is released from injury-induced axonal varicosities or endbulbs is lacking in any system, but indirect evidence both from our observations of the effects of methiothepin and from transected mammalian axons is consistent with release of neurotransmitters from axonal endings at sites of nerve injury (e.g., Devor and Janig 1981; Zochodne et al. 2001).

The observation that methiothepin prevented the induction of axonal LTH by nerve crush in 1% normal [Ca\(^{2+}\)] is interesting because this level of extracellular Ca\(^{2+}\) blocks most signs of fast synaptic transmission and neuromodulation within the CNS of *Aplysia* (Weragoda et al. 2004). The apparent release of 5-HT in 1% normal [Ca\(^{2+}\)] might be explained by 1) inhibitory effects of methiothepin persisting during a critical period after washout of the methiothepin solution and return of normal extracellular [Ca\(^{2+}\)]; 2) a secretory process in *Aplysia* nerve that can be triggered by the Ca\(^{2+}\) influx that occurs under this reduced concentration gradient; 3) secretion of 5-HT in response to Ca\(^{2+}\) released from intracellular stores (e.g., Ishibashi et al. 2007); or 4) secretion of 5-HT by Ca\(^{2+}\)-independent, voltage-dependent mechanisms that have been described in some neurons (e.g., Knight et al. 1989; Zhang and Zhou 2002).

**Axonal 5-HT treatment fails to induce LTH of the SN soma**

We found that application of 5-HT to a nerve segment failed to induce LTH in the SN soma, even though it induced axonal LTH, and even though the inhibition of crush-induced axonal LTH by methiothepin indicates that local 5-HT is a major signal for injury-induced axonal LTH. Somal LTH is induced by nerve crush (Walters et al. 1991) and by various agents thought to be stimulated by 5-HT, including cyclic adenosine monophosphate (cAMP) (Lewin and Walters 1999; Scholz and Byrne 1988), extracellular signal-related kinase (ERK) (Sung et al. 2001), PKC (Manseau et al. 1998), and TGFβ1 (Chin et
al. 1999; Farr et al. 1999) when applied to excised ganglia-nerve preparations. This difference suggests either that 5-HT receptors are not linked to these signaling pathways in the SN axon, or that these signaling pathways in the axon are not linked to somal LTH mechanisms.

Interestingly, global application of 5-HT either to ganglia-nerve preparations or to isolated SNs also fails to induce reliable somal LTH (Liao et al. 1999a), despite the fact that 5-HT application produces short-term somal hyperexcitability in these same preparations. This might be explained by a lack of coupling between 5-HT receptors on the soma (Barbas et al. 2005; Zhang et al. 1991) and somal LTH mechanisms. Moreover, because synapses and peripheral axons are also exposed to 5-HT in these preparations, somal hyperexcitability mechanisms may not be linked effectively to signals that are activated by 5-HT at synapses and then translocated to the nucleus to support synaptic LTF (e.g., importins; Thompson et al. 2004) or to the centripetal signaling systems from axon to soma that induce somal LTH after nerve injury (Gunstream et al. 1995; Sung et al. 2004; Walters et al. 1991). On the other hand, LTH of somata can be induced in dissociated SNs if postsynaptic neurons are present, showing that 5-HT receptors can be coupled to somal LTH mechanisms under some conditions (Glanzman 2006). Because ganglia-nerve preparations contain postsynaptic motor neurons, failure to observe 5-HT–induced somal LTH in these preparations might result from bath-applied 5-HT causing concomitant inhibitory effects within ganglia that prevent the induction of somal LTH.

Implications about cellular signals that induce LTH and the functions of LTH

The ability of either of the 5-HT antagonists, methiothepin and spiperone, to prevent 5-HT–induced axonal LTH suggests that this form of LTH may involve both adenylyl cyclase–coupled receptors and PLC-coupled receptors (Cohen et al. 2003; Dumitriu et al. 2006), although the types of 5-HT receptors on peripheral axons of SNs remain to be identified. Interestingly, the cAMP–protein kinase A pathway in peripheral nerves is known to contribute to the maintenance but not to the induction of somal LTH (Liao et al. 1999b; see also Bedi et al. 1998). Further studies are needed to define the receptors and signaling pathways that induce and maintain axonal LTH after focal 5-HT treatment or nerve injury.

Serotonin is not essential for all forms of axonal LTH in Aplysia SNs. Depolarizing a nerve segment for 2 min is also sufficient to induce long-term depression of axonal threshold (Wegadorga et al. 2004). However, depolarization-induced LTH was not blocked by methiothepin (Fig. 6), and it differs from both 5-HT–induced and injury-induced LTH in not increasing repetitive firing. Block of crush-induced axonal LTH by methiothepin suggests that LTH induced by our focal nerve crush procedure does not depend on signals generated by the transient axonal depolarization expected during axotomy (e.g., Spira et al. 1993). Differences between 5-HT–induced and depolarization-induced LTH suggest that at least two forms of axonal LTH exist, involving somewhat different alterations of axonal conductances and different signaling pathways.

Our demonstration that LTH induction by nerve crush is blocked by methiothepin suggests that a major function of 5-HT–induced axonal LTH is to enhance sensory signaling near sites of axonal injury. Long-lasting depression of axonal spike threshold should help to maintain sensory input from damaged regions both acutely (e.g., by enhancing conduction across segments of sensory axons depolarized by injury) and over longer periods (e.g., by overcoming conduction block caused by impedance mismatch between small regenerating neurites and larger surviving axonal segments; see Weragoda et al. 2004). When a site of injury-induced neuromodulator release is close to SN axons within peripheral receptive fields, the resulting enhancement of repetitive firing in these axonal segments may amplify the effectiveness of sensory receptor potentials, a sensitizing effect that, in mammals, could contribute to inflammatory pain and primary hyperalgesia (Billy and Walters 1989a; Clatworthy and Walters 1993; Dulin et al. 1995; Walters 1987). Interestingly, 5-HT immediately increases the excitability of mammalian C fibers, likely contributing to hyperalgesia during inflammation and nerve injury (e.g., Moulem et al. 2005; Sommer 2004). It is not yet known whether 5-HT produces long-term changes in the excitability of mammalian axons.

Rapamycin-sensitive protein synthesis is involved in long-term alterations distant from the neuronal soma

We have shown that LTH of SN axons induced by 5-HT treatment, like axonal LTH induced by nerve injury or local depolarization (Weragoda et al. 2004), and 5-HT–induced LTF of SN synapses (Martin et al. 1997; Sherff and Carew 1999), depends on local protein synthesis. Because peripheral nerve is a complex tissue containing many cell types, the local protein synthesis required for axonal LTH might be in either I) the sensory axons themselves (see Grabham et al. 2005; Martin et al. 1997) or 2) other cells, such as glia. We know, however, that 5-HT–induced axonal LTH does not require local gene transcription in glia or other cells because it is not inhibited by local application of a transcription inhibitor, actinomycin D, to the nerve segment (Klaassen and Walters, unpublished observations). Moreover, evidence that protein synthesis occurs in molluscan axons has come from demonstrations of translation of mRNAs in severed neurites of Aplysia SNs (Martin et al. 1997; Moccia et al. 2003) and in identified axons in Lymnaea (Van Minnen et al. 1997), as well as the discovery of ribosomes and polyribosomes in the squid giant axon (Sotelo et al. 1999).

It is interesting that both synaptic LTF (Casadio et al. 1999; Hu et al. 2006; Si et al. 2003) and axonal LTH (see also Weragoda et al. 2004) in Aplysia are rapamycin sensitive. Rapamycin selectively inhibits a highly conserved eukaryotic protein kinase, TOR, which enhances many growth-related processes, in part, by increasing the translation of translational machinery, such as ribosomal proteins and elongation factors (reviewed by Wullschleger et al. 2006). TOR is inhibited by most stresses, but activated by growth-inducing stresses, such as mechanostress in muscle cells (see Reiling and Sabatini 2006). This suggests that TOR activation may contribute to neuronal growth associated with LTF and LTH. In the case of synapse-specific LTF of synapses in Aplysia (Casadio et al. 1999; Kim et al. 2003; Si et al. 2003) and long-term synaptic plasticity in other phyla (e.g., Beaumont et al. 2001; Ostroff et al. 2002; Schratt et al. 2004), local TOR activation is likely to promote growth of new synapses by enhancing protein synthe-
sis at synaptic sites that may be distant from the soma. In the case of axonal injury, which usually occurs quite distant from sites of translation in the soma, TOR is likely to promote regenerative growth of axons by increasing the local synthesis of growth-related proteins at the injury site (e.g., Verma et al. 2005; Zheng et al. 2001). Injury-induced axonal LTH is unlikely to be linked directly to growth. However, because of geometric factors (narrow diameter, extensive branching, and abundance of varicosities; e.g., Steffensen et al. 1995), regenerating axons are necessarily prone to conduction block (see Debanne 2004). Thus adaptive, localized increases in translation of proteins that promote axonal conduction and excitability at sites of nerve injury may be regulated by the same signals that increase translation of proteins that promote axonal growth.

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REFERENCES


