Serotonin Mediates Learning-Induced Potentiation of Excitability

Brian D. Burrell and Christie L. Sahley

INTRODUCTION

There has been increased appreciation for the role that modulation of neuronal excitability plays during learning and memory (see reviews by Daoudal and Debanne 2003; Wu et al. 2002; Zhang and Linden 2003). Increases in excitability during learning have been observed in a number of different regions of the mammalian brain including CA1 and CA3 pyramidal cells in the hippocampus (Moyer et al. 1996, 2000; Oh et al. 2003; Stackman et al. 2002; Thompson et al. 1996), cerebellar Purkinje neurons (Shreurs et al. 1997, 1998), and pyramidal cells in the pyriform cortex (Saar et al. 1998). Learning-induced increases in excitability also have been observed in a number of invertebrate species, including sensory cells in Aplysia (Alkon et al. 1985; Antonov et al. 2001; Cleary et al. 1998), motor neurons in Lymanea (Straub and Benjamien 2001), and interneurons in Helix and the medicinal leech (Burrell et al. 2001; Gainutdinov et al. 1998).

The medicinal leech is particularly useful for studying the mechanisms that underlie learning-related neuroplastcity. Like many invertebrates, it has a well-characterized CNS in which it is possible to record from single, identifiable neurons and to monitor simultaneously learning-induced changes in behavior and the response properties of individual neurons. An additional advantage of the leech is that it is possible to focus on a single interneuron type, the S-cell, which not only integrates a variety of afferent inputs and disseminates this information throughout the CNS but also appears to be critical to certain forms of learning-related behavioral plasticity.

The S-cells form a linear network of electrically coupled interneurons that extends throughout the leech CNS (Fig. 1A). Each S-cell receives afferent input from touch, pressure, and nociceptive mechanosensory neurons as well as photoreceptive neurons (Baccus et al. 2000; Muller and Scott 1981; Peterson 1984), and action potentials that are elicited by this afferent input propagate reliably throughout the entire S-cell network. The S-cell contributes to learning-related plasticity of the leech whole-body shortening reflex, a defensive withdrawal reflex that can be initiated by either mechanical or photo stimulation and involves the near-simultaneous contraction of the entire body. Although the S-cell receives input from afferents that elicit shortening, is active during this behavior, and has direct input to at least one type of shortening motor neuron (the L motor neurons; see Fig. 1B), its activity alone is not sufficient to trigger shortening, and lesions of the S-cell network do not affect the animal’s capacity to shorten (Gardner-Medwin et al. 1973; Shaw and Kristan 1995, 1999). However, lesions of the S-cell chain completely disrupt sensitization and partially disrupt dishabituation of the leech shortening response (Burrell et al. 2003; Modney et al. 1997; Sahley et al. 1994). In addition, S-cell activity during elicited shortening is enhanced after sensitization, which may be mediated, in part, by increases in the excitability of the S-cell that also occur during sensitization (Burrell et al. 2001). This increase in excitability may due to the effects of serotonin (5-HT), a modulatory neurotransmitter that is critical for sensitization, full dishabituation, and associative learning of leech shortening (Ehrlich et al. 1992; Sahley 1994). Bath application of 5-HT directly increases S-cell excitability (Burrell et al. 2001) and the interneuron’s response to mechanosensory stimuli (Belardetti et al. 1982; Burrell et al. 2002). S-cell excitability is also enhanced by stimulation of the 5-HT-containing Retzius cells (Fig. 1B) (Burrell et al. 2001).

Although these results suggest that 5-HT contributes to sensitization-induced increases in S-cell excitability, a direct link between 5-HT- and sensitization-induced changes in S-cell excitability has not been established. Therefore the major goal of this paper is to determine the role of 5-HT in sensitization-mediated increases in S-cell excitability by blocking the effects of 5-HT.
of 5-HT on the S-cell during sensitization training in a behaviorally intact preparation. The results demonstrate that 5-HT-induced potentiation of S-cell excitability is due to activation of a metabotropic 5-HT receptor coupled to a cAMP/protein kinase A (PKA) second-messenger pathway and that blocking 5-HT’s effect on the S-cell, either at the receptor or cAMP/PKA level, prevents sensitization-induced increases in excitability. These experiments represent the first time, to our knowledge, that learning-induced changes in excitability have been directly monitored and blocked at the same time that learning was taking place.

METHODS

Electrophysiology

Medicinal leeches (Hirudo medicinalis) weighing 3 g were obtained from a commercial supplier (Leeches USA) and maintained in pond water (0.5 g/1 l H2O Hirudo salt (Leeches USA) at 18°C. Single ganglia were dissected in leech saline [which contained (in mM) 110 NaCl, 5 NaOH, 4 KCl, 1.8 CaCl2, 1.5 MgCl2, and 10 HEPES] and placed in a recording chamber (chamber volume ≈ 0.75 ml) under constant perfusion (~1 ml/min). Intracellular recordings were made by impaling the cell with a sharp microelectrode made from borosilicate glass (1.0 mm OD, 0.78 mm ID; H9252) and pulled to a tip resistance of 20–30 MΩ using a Sutter P-97 puller (Sutter Instruments). Electrodes were filled with 3 M potassium acetate (KAc). Recordings were made using a BA-1S amplifier (NPI). Either a Grass S88 stimulator with SU5 stimulus isolation units (Astromed) or an STG 1004 programmable stimulator (Multichannel Systems) was used to deliver current pulses. Electrophysiological data were viewed on a digital oscilloscope and stored on a computer using a Digidata 1322A analog/digital interface and Axoscope 9.0 acquisition software (Axon Instruments).

S-cells were identified by their position in the ganglion (variable, but always in the central glial patch on the ventral side of the ganglion), size (5–10 μm), and shape of their action potential (50 to 60 mV amplitude, ≈ 2-ms spike width). Measurements of intrinsic electrical properties of the S-cell were made as follows. Input resistance was measured by injecting a hyperpolarizing current pulse (1 nA, 500 ms) and measuring the resulting change in membrane potential. Excitability was measured by recording the number of action potentials initiated during a 500 ms, 1 nA depolarizing current pulse (AP/pulse). Additional excitability measurements were made from the S-cell’s response to this stimulus. The membrane potential at which action potential initiation occurred (membrane potential threshold) was measured and defined as the point at which the velocity of the change in membrane potential equaled 20 mV/s (Bekkers and Delaney 2001). The rate of the increase in membrane potential (slope) prior to the first action potential initiation was also measured by taking the slope of the membrane potential depolarization (dV/dt) during a 5-ms window, 10 ms prior to action potential initiation (Cudmore and Turrigiano 2004). Finally, the interspike interval of the first pair of action potentials initiated during the stimulus pulse was measured (1st ISI). The resting potential of the S-cell was kept constant for both pre- and post-5-HT treatment measures of excitability.

Changes in S-cell excitability were tested prior to (pretest) a 5-min perfusion with 10 μM 5-HT (n = 9). This was followed by a 5-min wash in normal saline and then a second test of excitability (posttest). This treatment has been shown to produce robust increases in excitability that have reached a plateau by this time, although enhancement changes in excitability lasts ≥1 h (Burrell et al. 2001). The 5-HT-induced changes in excitability were compared with groups of S-cells in which the 5-HT treatment was replaced with normal saline (n = 9), 5-HT plus 100 μM methysergide (a nonspecific antagonist of metabotropic 5-HT receptors; n = 7), methysergide alone (n = 3), 5-HT plus 1 mM GDP-β-S (a membrane impermeable competitive G-protein antagonist; n = 4), GDP-β-S alone (n = 4), 5-HT plus 50 μM Rp-cAMP (a membrane permeable inhibitor of PKA; n = 5), Rp-cAMP alone (n = 5), or 50 μM dibutyryl cAMP (db-cAMP; a membrane permeable analogue of cAMP; n = 3). GDP-β-S was dissolved in filtered 3 M KAc and applied intracellularly by using the GDP-β-S solution to fill the recording electrode and then allowing it to diffuse into the interneuron. The remaining drugs were dissolved in leech saline and applied via the perfusion through the recording chamber. All drugs were obtained from Sigma (St. Louis).

Behavioral training and recordings from semi-intact preparations

Dissections were carried out in an ice-lined dissecting dish with ice-cold leech saline. Segments 1–2 were left intact and pinned dorsal to the connecting nerves. At the midpoint of each connective nerve, size (5–10 μm), and shape of their action potential (50 to 60 mV amplitude, ≈ 2-ms spike width). Measurements of intrinsic electrical properties of the S-cell were made as follows. Input resistance was measured by injecting a hyperpolarizing current pulse (1 nA, 500 ms) and measuring the resulting change in membrane potential. Excitability was measured by recording the number of action potentials initiated during a 500 ms, 1 nA depolarizing current pulse (AP/pulse). Additional excitability measurements were made from the S-cell’s response to this stimulus. The membrane potential at which action potential initiation occurred (membrane potential threshold) was measured and defined as the point at which the velocity of the change in membrane potential equaled 20 mV/s (Bekkers and Delaney 2001). The rate of the increase in membrane potential (slope) prior to the first action potential initiation was also measured by taking the slope of the membrane potential depolarization (dV/dt) during a 5-ms window, 10 ms prior to action potential initiation (Cudmore and Turrigiano 2004). Finally, the interspike interval of the first pair of action potentials initiated during the stimulus pulse was measured (1st ISI). The resting potential of the S-cell was kept constant for both pre- and post-5-HT treatment measures of excitability.

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Dissections were carried out in an ice-lined dissecting dish with ice-cold leech saline. Segments 1–2 were left intact and pinned dorsal
side up in a silicone elastomer (Sylgard)-lined dish (Fig. 2). A longitudinal incision was made down the dorsal midline of segments 3–8 so that the cylinder of the leech body could be opened up, and the resulting flat sheet of skin was rotated and pinned ventral side up (this will be referred to as the body-wall portion of the preparation). A small longitudinal incision was made on the ventral side of segment 4, exposing the segmental ganglion for S-cell intracellular recordings. Segments 9–11 were left intact and pinned dorsal side up with the posterior end connected to an isometric tension transducer (model 72–4481; Harvard Apparatus) using a nylon monofilament so that whole-body shortening could be recorded and quantified. The remaining portion of the preparation, segments 12–14, consisted of the CNS only (ganglia and connective nerve), and the connective nerve posterior of the segment 14 ganglion was pulled into a suction electrode for extracellular recordings of S-cell activity. The S-cell action potential produces the largest signal in such recordings (Frank et al. 1975), so S-cell activity during elicited shortening responses could be easily monitored and recorded. The preparation was allowed to rest for 30 min in saline with 10 mM glucose before undergoing sensitization training. All training was conducted at room temperature (22–24°C).

Whole-body shortening was elicited by delivering controlled electroshocks to the skin using implanted Teflon-coated silver wire electrodes (75-μm bare diameter; A-M Systems). Each electrode consisted of a pair of silver wires in which the Teflon was removed from the portions of the wire that were in contact with the skin, and two pairs of wire electrodes were implanted in the skin. One pair was implanted at segment 4 and elicited whole-body shortening (test site) using stimulation parameters from Shaw and Kristan (1995): five stimulus pulses, 1 ms pulse duration, 10 Hz frequency. The stimulus intensity at the test site was set at just above threshold for eliciting shortening. The second set was implanted between segments 7 and 8 and was used to deliver the sensitizing stimulus (sensitizing site; 10 stimulus pulses, 1 ms pulse duration, 10 Hz frequency, intensity set at ~25% above threshold).

Immediately before training, intracellular recordings were made from the segment 4 S-cell, and its excitability properties were measured in the same manner used for the 5-HT experiments described previously. In preparations that would undergo sensitization in normal saline (n = 7), training began by making two presensitization (pretest), see Fig 4A measurements of the shortening response and the accompanying S-cell activity at an intertrial interval (ITI) of 2.5 min. This was followed by the delivery of two trains of sensitizing stimuli, again with a 2.5 min ITI. Five minutes after the last sensitizing trial, two postsensitization (posttest) measurements of the shortening reflex/S-cell activity were made (2.5 min ITI). Postsensitization measurements of S-cell excitability were made immediately after the last behavioral posttest.

To test whether sensitization-induced potentiation of S-cell excitability was mediated by 5-HT, sensitization training was carried in the presence of 100 μM methysergide (n = 6) or 50 μM Rp-cAMP (n = 5). Control experiments, in which delivery of the sensitizing stimuli was omitted, were carried out in normal saline (n = 6), 100 μM methysergide (n = 6) or 50 μM Rp-cAMP (n = 6). All drugs were bath-applied 5 min before the first pretest. Injection of membrane impermeable agents into the S-cell that might antagonize 5-HT-induced changes in excitability (e.g., GDP-β-S or PKA inhibitory peptides) was not possible using these semi-intact preparations. Mechanosensory stimuli that elicit shortening cause multiple S-cells to fire (2–5 adjacent interneurons) and increases in the S-cell network’s response due to increased stimulus intensity, 5-HT, or sensitization are distributed across multiple S-cells (Baccus et al. 2001; Burrell et al. 2002; Cruz et al. 2003). Therefore using injectable drugs to block potential serotonergic modulation of the S-cell would require finding and injecting a minimum of three separate S-cells, by which time there would have been a substantial reduction in the viability of the semi-intact preparation.

**Data analysis**

Changes in S-cell excitability properties from both the 5-HT and sensitization training experiments were analyzed as follows. For each neuron, multiple measurements of a given excitability property (AP/pulse, slope, input resistance, etc.) were made and an average calculated for both the pre- and posttest excitability measurements. The averaged value from each neuron was then used for statistical analysis and graphical representation. One-way ANOVA in which the post-

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**FIG. 2.** Diagram of the semi-intact preparation used for sensitization training. The ganglion in the 4th segment was exposed so that intracellular recordings could be made from the S-cell before and after training. This portion of the preparation (segments 3–8) was rotated so that it was ventral side up during training to accommodate S-cell recordings. The rest of the preparation was pinned dorsal side up. Changes in the whole-body shortening reflex were monitored using a tension transducer attached to segments 9–11. S-cell activity during shortening was monitored using a suction electrode applied to the connective nerve posterior of the segment 14 ganglion.
treatment levels had been normalized relative to the pretreatment level was used to detect differences between the various treatment groups. Behavioral and S-cell activity data from the sensitization training experiments were analyzed using a one-way ANOVA with repeated measures (presensitization trial 2 and postssensitization trials 1 and 2, all normalized to the 1st presensitization trial).

RESULTS

5-HT activates a metabotropic receptor coupled to a cAMP/PKA pathway

Application of 10 μM 5-HT induced a significant increase in the number of action potentials fired by the S-cell during current injection [Fig. 3A; AP/pulse 1-way ANOVA, \( F(8,36) = 5.96, P < 0.0001 \)]. This was accompanied by a significant decrease in the interval between the first pair of action potentials [Fig. 3B; first ISI 1-way ANOVA, \( F(8,34) = 3.15, P < 0.01 \)] and an increase in the rate of depolarization prior to the first action potential [Fig. 3C; slope 1-way ANOVA, \( F(8,36) = 3.53, P < 0.005 \)]. The 5-HT-induced changes in these excitability properties were significantly different when compared with S-cells in which 5-HT application was omitted (saline group; Fig. 3D; post hoc test: AP/pulse, \( P < 0.0005 \); first ISI, \( P < 0.05 \); slope, \( P < 0.01 \)). No significant change was observed in the membrane potential threshold [1-way ANOVA, \( F(8,35) = 0.48 \)] or input resistance [1-way ANOVA, \( F(8,35) = 0.50 \)]. 5-HT-induced increases of S-cell excitability were blocked by co-application of 100 μM methysergide (Fig. 3D; 5HT-methysergide). Excitability measures from the 5-HT-methysergide group were significantly different compared with the 5-HT group (post hoc test: AP/pulse, \( P < 0.001 \); first ISI, \( P < 0.01 \); slope, \( P < 0.01 \)) but were not significantly different from the saline group. S-cells treated with methysergide alone were not significantly different from the saline or 5-HT-methysergide groups but were significantly different from the 5-HT-treated group in terms of AP/pulse (post hoc \( P < 0.001 \)), first ISI (\( P < 0.01 \)), and slope (\( P < 0.01 \)). There was no significant difference in membrane potential threshold or input resistance in S-cells from the saline-, 5-HT-methysergide-, methysergide-, and the 5-HT-treated groups.

Although methysergide was effective at blocking 5-HT-mediated increases in S-cell excitability, it is a relatively nonspecific 5-HT receptor antagonist and does not help in identifying the 5-HT receptor subtype(s) involved in this modulation. It is difficult to identify 5-HT receptor subtypes in invertebrates using vertebrate pharmacological tools (Tierney 2001). Therefore components of the signal transduction pathways activated by the 5-HT receptors were examined instead. This approach was used not only to characterize the cellular mechanisms mediating 5-HT modulation of the S-cell but also to provide tools with which to disrupt this process during sensitization training.

The first step was to identify whether a G-protein-coupled receptor mediated 5-HT-induced changes in S-cell excitability because there are both iono- and metabotropic 5-HT receptors (Hoyer et al. 2002). Therefore 1 mM GDP-β-S was included in the recording electrode used to monitor S-cell excitability while the interneuron was perfused with 10 μM 5-HT (5HT+GDP-β-S). GDP-β-S prevented 5-HT-induced increases in S-cell excitability (Fig. 3E). Changes in AP/pulse (post hoc \( P < 0.001 \)), first ISI (\( P < 0.05 \)), and slope (\( P < 0.05 \)) in the 5-HT+GDP-β-S group were significantly different from those treated with 5-HT alone but not significantly different from the saline group. The same result was observed in S-cells treated with GDP-β-S alone (Fig. 3E; post hoc results: AP/pulse, \( P < 0.05 \); first ISI, \( P < 0.01 \); slope, \( P < 0.01 \)). No significant difference in S-cell membrane potential threshold or input resistance was observed among the 5-HT+GDP-β-S, GDP-β-S, 5-HT, or saline groups (Fig. 3E).

Next, the involvement of a cAMP/PKA second-messenger cascade was examined. 5-HT-induced increases in S-cell excitability properties were blocked when 50 μM Rp-cAMP was included in the perfusate (Fig. 3F; 5HT+Rp-cAMP). Changes in AP/pulse (post hoc \( P < 0.005 \)), first ISI (\( P < 0.05 \)), and slope (\( P < 0.01 \)) in the 5-HT+Rp-cAMP group were significantly different from those treated with 5-HT alone but not significantly different from the saline group. The same result was observed in S-cells treated with Rp-cAMP alone (Fig. 3F), indicating that the drug did not affect S-cell excitability directly (post hoc results: AP/pulse, \( P < 0.0005 \); first ISI, \( P < 0.05 \); slope, \( P < 0.01 \)). No significant difference in S-cell membrane potential threshold or input resistance was observed among the 5-HT+Rp-cAMP, Rp-cAMP, 5-HT, or saline groups (Fig. 3F). If 5-HT modulation of excitability does require the activity of a cAMP/PKA second-messenger system, then increasing the intracellular level of cAMP should mimic the effects of 5-HT on this interneuron. A 5-min perfusion of the S-cell with 50 μM db-cAMP produced increases in AP/pulse and slope and decreases in first ISI that were statistically indistinguishable from those of 5-HT (Fig. 3F) but statistically different from the saline group (post hoc results: AP/pulse, \( P < 0.0001 \); first ISI, \( P < 0.05 \); slope, \( P < 0.05 \)). No significant difference in S-cell membrane potential threshold or input resistance was observed between the db-cAMP, 5-HT, or saline groups (Fig. 3F). Together, the results from the Rp-cAMP and db-cAMP experiments demonstrate that 5-HT-induced increases in S-cell excitability require activation of a cAMP/PKA second-messenger cascade.

Sensitization is blocked by treatments that inhibit 5-HT modulation of excitability

To examine whether sensitization-induced increases in S-cell excitability are the result of serotonergic modulation, sensitization training was carried out under conditions that block potentiation of excitability by 5-HT. Specifically, semi-intact preparations were treated with either 100 μM methysergide or 50 μM Rp-cAMP during sensitization training. In addition, the effects of these treatments on sensitization of whole-body shortening and the accompanying increase in S-cell activity during shortening were examined.

The intensity of the whole-body shortening reflex more than doubled after delivery of the sensitizing stimuli (Fig. 4, B and C) in preparations that underwent sensitization training in normal saline (sens/saline group). S-cell activity during shortening also increased relative to presensitization levels (Fig. 4, B and D). No change in either the shortening reflex or S-cell activity was observed in preparations from sensitization control experiments in which delivery of the sensitizing stimuli was...
Sensitization-induced increases in both the shortening reflex and S-cell activity were blocked in preparations treated with either methysergide (sens/methy group) or Rp-cAMP (sens/Rp-cAMP group; Fig. 4, C and D).

Sensitization-induced changes in whole-body shortening were confirmed using two-way ANOVA with repeated measures [treatment effect, \(F(5,29) = 4.00, P < 0.01\); trial effect, \(F(2,58) = 0.11\); interaction effect, \(F(10,58) = 3.20, P < 0.005\)]. Post hoc analysis confirmed that the postsensitization shortening in the sens/saline group was significantly greater compared with shortening in all the sensitization control groups (con/saline, \(P < 0.001\); con/methy, \(P < 0.005\); con/Rp-cAMP, \(P < 0.0005\)). Furthermore, shortening in the sens/saline group was significantly greater than in preparations that underwent sensitization training in the presence of either methysergide (sens/methy \(P < 0.01\)) or Rp-cAMP (sens/Rp-cAMP, \(P < 0.01\)). The level of shortening in the sens/methy and sens/Rp-cAMP groups was not significantly different from any of the sensitization control groups (con/saline, con/methy, and con/Rp-cAMP). Similarly, sensitization-induced increases in S-cell activity during the shortening response were confirmed statistically [treatment effect, \(F(5,29) = 3.02, P < 0.05\); trial effect, \(F(2,58) = 7.91, P < 0.001\); interaction effect, \(F(10,58) = 2.25, P < 0.05\)]. Post hoc analysis showed that postsensitization S-cell activity in the sens/saline group was significantly greater than activity in the preparations sensitized in the presence of methysergide (sens/methy, \(P < 0.01\)) or Rp-cAMP (sens/Rp-cAMP, \(P < 0.05\)) or any of the sensitization control groups (con/saline, \(P < 0.001\); con/methy, \(P < 0.01\); con/Rp-cAMP, \(P < 0.05\)). Post-sensitization S-cell activity was not significantly different between any of the sensitization control groups or between these groups and the sens/methy or sens/Rp-cAMP groups.

The lack of sensitization in methysergide- or Rp-cAMP-treated groups was not due to any detrimental effect of these drugs on the preparations during training. Shortening and S-cell responses were stable during sensitization control experiments in these preparations and were statistically indistinguishable from the con/saline group (Fig. 4, C and D). In addition, the lack of sensitization in the sens/methy or sens/Rp-cAMP groups was not due to any differences in the level of the whole-body shortening response at the start of training. There was no significant difference in the presensitization trial 1 shortening reflex from the sens/saline, sens/methy, and sens/Rp-cAMP groups [1-way ANOVA, \(F(2,16) = 1.20\)]. Furthermore, the lack of sensitization was not due to any deficits in the

FIG. 3. 5-HT increases excitability via a metabotropic 5-HT receptor that activates a cAMP/protein kinase A (PKA) second-messenger pathway. Changes in excitability properties before and after application of 10 \(\mu M\) 5-HT: action potentials (APs/pulse (A), 1st interspike interval (ISI) in the train (B), and slope of depolarization (dV/dT) prior to the 1st AP in the train (C)). D: average percent change (±SE) in excitability properties in 5-HT-treated (black bars) and saline control (white bars) S-cells (same data used in E and F). Co-application of methysergide blocked 5-HT’s effects on excitability (dark gray-bars) but did not alter excitability itself (light gray-filled bars). E: co-application of GDP-β-S blocked 5-HT’s effects (fine right-hatched bars) but had no effect by itself (coarse right-hatched bars). F: co-application of Rp-cAMP block 5-HT’s effects (fine left-hatched bars) but had no effect by itself (coarse left-hatched bars). Application of db-cAMP (cross-hatched bars) mimicked the effects of 5-HT.
ability of the sensitizing stimuli to activate the S-cell, which is critical for the induction of sensitization (Burrell et al. 2003). The number of S-cell action potentials elicited during the two sensitizing stimulation trials was 20.4 ± 1.5 and 19 ± 3.44 for the sens/saline group, 21 ± 2.43 and 18.8 ± 3.68 for the sens/methy group, and 21 ± 4.13 and 15.3 ± 3.78 for the sens/Rp-cAMP group (average presensitization S-cell response during shortening was 7.71 ± 1.54 action potentials). One-way ANOVA with repeated measures showed no significant effect of treatment group \( [F(2,13) = 0.06] \) and no significant interaction effect \( [F(2,16) = 1.97] \). There was a significant effect for the repeated measures \( [F(1,13) = 8.73, P < 0.05] \) that was the result of the first sensitizing stimulus consistently eliciting more S-cell action potentials than the second sensitizing stimulation in all three treatment groups.

**Sensitization alters S-cell frequency distribution**

Changes in the frequency distribution of S-cell activity as a result of sensitization were also analyzed. The average frequency from the two pre- and postsensitization trials was calculated at 100 ms intervals following onset of the test stimulus and then averaged across all preparations in the sens/saline group. The frequency of S-cell activity from the postsensitization trials was enhanced relative to the presensitization levels becoming greater at later poststimulus intervals (Fig. 5). This sensitization effect was statistically significant based on ANOVA \( [F(1,36) = 10.94, P < 0.005] \). Not surprisingly, there was also a significant effect of the poststimulus interval as a result of the frequency decreasing at each time interval \( [F(1,36) = 5.35, P < 0.001] \). There was no interaction effect \( [F(5,36) = 0.27] \). The pre- versus postsensitization change in overall S-cell frequency elicited during shortening is shown in the Fig. 5, inset.

**Sensitization-induced enhancement of S-cell excitability is 5-HT dependent**

In agreement with earlier observations (Burrell et al. 2001), S-cell excitability increased after sensitization training, and the pattern of sensitization-induced increases in excitability properties was identical to those produced by 5-HT in this study. Sensitization produced an increase in AP/pulse \( [F(5,15) = 5.52, P < 0.005] \), a decrease in...
the first ISI [Fig. 6, B and D; $F(5,15) = 4.48, P < 0.05$], and an increase in slope [Fig. 6, C and D; $F(5,15) = 4.72, P < 0.01$] relative to S-cells from nonsensitized preparations, but there was no change in membrane potential threshold [$F(5,15) = 0.29$] or input resistance [$F(5,15) = 1.56$]. Furthermore, sensitization-induced potentiation of excitability was blocked by treatments that prevented 5-HT-induced enhancement of excitability, namely methysergide and Rp-cAMP (Fig. 6D), demonstrating that enhanced S-cell excitability during sensitization was mediated by 5-HT. Post hoc analyses confirmed that sensitization-induced changes in excitability properties were significantly different in the S-cells in the sens/saline group compared with those in the sens/methy (AP/pulse, $P < 0.001$; 1st ISI, $P < 0.005$; slope, $P < 0.01$) groups. Excitability changes in the sens/saline group were also significantly different from the con/saline (AP/pulse, $P < 0.005$; 1st ISI, $P < 0.005$; slope, $P < 0.001$), con/methy (AP/pulse, $P < 0.005$; 1st ISI, $P < 0.01$; slope, $P < 0.005$), and con/Rp-cAMP (AP/pulse, $P < 0.01$; 1st ISI, $P < 0.05$; slope, $P < 0.05$) groups. There were no significant differences in the excitability properties between any of the sensitization control groups or between the sens/methy, sens/Rp-cAMP and any of the control groups (Fig. 6D).

**Discussion**

These results confirm previous work (Burrell et al. 2001) in which both sensitization training and 5-HT induced similar increases in excitability in the S-cell and extends those findings by demonstrating that 5-HT mediates sensitization-induced potentiation of S-cell excitability. 5-HT-induced potentiation of S-cell excitability is mediated by a metabotropic 5-HT receptor that upregulates cAMP production and activates PKA, although additional signaling pathways may be involved as well. The fact that serotonergic modulation of the S-cell requires a cAMP/PKA second messenger cascade is consistent with earlier findings showing that 5-HT-mediated increases in the S-cell response to mechanosensory stimuli utilize a cAMP/PKA pathway (Belardetti et al. 1982). Sensitization-induced enhancement of S-cell excitability was blocked by treatments that either antagonized 5-HT binding (methysergide) or prevented cAMP activation of PKA (Rp-cAMP), demonstrating that learning-induced potentiation of S-cell excitability is a 5-HT-dependent process.

Interestingly, treatments that blocked 5-HT-mediated potentiation of S-cell excitability during sensitization training prevented sensitization-induced increases in S-cell activity and whole-body shortening. These results suggest that increased S-cell excitability contributes to sensitization of the shortening reflex but cannot be considered conclusive because the drugs used to block serotonergic modulation of the S-cell were bath-applied (see methods) and may have prevented 5-HT- or cAMP/PKA-dependent changes in other cells that contribute to sensitization. For example, 5-HT decreases afterhyperpolarization (AHP) in T and P mechanosensory neurons through a cAMP-dependent process (Catarsi and Brunelli 1991; Catarsi et al. 1995). Decreased AHP in these cells could enhance afferent input to the S-cell (or other neurons that contribute to shortening) by reversing branch point propagation failure of action potentials (Mar and Drapeau 1996) or inducing action potential reflection at these branch points (Baccus et al. 2000, 2001). In addition, 5-HT modulation of the S-to-S electrical synapses may increase the instantaneous frequency of action potentials as they propagate through the S-cell network (Moss et al. 2005), which could also contribute to increases in the rate of S-cell activity. It is not known if 5-HT modulation of the S-to-S electrical synapses is also a PKA-dependent process.

The S-cell appears to have two roles during sensitization. First, an intact S-cell network is necessary during the delivery of the sensitizing stimuli based on experiments in which sensitization was prevented when the S-cell chain had been lesioned (Burrell et al. 2003; Modney et al. 1997; Sahley et al. 1994). The precise role of the S-cell during this induction stage of sensitization is not known, but as the present results show,
sensitizing stimuli strongly activate the S-cell. This may lead to the stimulation of 5-HT-containing Retzius cells throughout the leech CNS because these cells receive synaptic input from the S-cell (Wang 1999). Second, the S-cell itself may contribute to producing the sensitized response, i.e., increased shortening. Sahley et al. (1994) observed a strong correlation between the level of S-cell activity and the intensity of the sensitized shortening response, whereas no such correlation was detected in the nonsensitized shortening reflex. In addition, results presented here and from previous experiments (Burrell et al. 2001; Sahley et al. 1994) show that S-cell excitability and activity during shortening are enhanced during sensitization. The S-cell may also be incorporated into other behaviors as a result of sensitization. Debski and Friesen (1986) observed an increase in S-cell activity during sensitization of leech swimming behavior, which is also 5-HT- and cAMP-dependent (Zaccardi et al. 2004), and suggested that increased S-cell activity might contribute to this behavior during sensitization even though this interneuron is not part of the swimming neural circuit. However, the necessity of the S-cell in the “expression” of the sensitization for shortening or swimming has not been conclusively demonstrated.

The biophysical basis for 5-HT-induced increases in S-cell excitability is not known, but it likely involves the modulation of multiple membrane conductances (see reviews by Frick and Johnston 2005; Zhang and Linden 2003). The observed increase in slope suggests an enhancement of voltage-gated Na\(^+\) currents or a reduction of voltage-gated K\(^+\) currents. The increase in the AP's/pulse (and possibly the decrease in 1st ISI) suggests modulation of currents that regulate the repeated firing of action potentials by a neuron, such as those mediated by Ca\(^{2+}\)-activated K\(^+\) channels or persistent Na\(^+\) channels (Sah and Faber 2002; Wu et al. 2004).

Potentiation of excitability is an important mechanism for encoding and storing information during learning and memory. Increased excitability has been observed after learning in both vertebrates and invertebrates (Alkon et al. 1985; Antonov et al. 2001; Burrell et al. 2001; Cleary et al. 1998; Gainutdinov et al. 1998; Moyer et al. 1996, 2000; Oh et al. 2003; Saar et al. 1998; Shreurs et al. 1997, 1998; Stackman et al. 2002; Straub and Benjamin 2001; Thompson et al. 1996) and dysfunctions in the modulation of excitability may contribute to age-related deficits in learning and memory (Moyer et al. 2000; Wu et al. 2002). In addition, modulation of excitability interacts with activity-dependent forms of neuroplasticity that may contribute to learning and memory, such as long-term potentiation (LTP) or long-term depression (LTD) (Andersen et al. 1980; Bliss and Lomo 1973; Daoudal et al. 2002; Frick et al. 2004). Modulation of excitability may also act to change the threshold for induction of LTP or LTD (Cohen et al. 1999; Le Ray et al. 2004; Morozov et al. 2003; Nolan et al. 2004), a process often referred to as metaplasticity (Abraham and Bear 1996). LTP and LTD are observed in synapses formed by the mechanosensory T- and P-cells onto the S-cell (Burrell and Sahley 2004), but it is not known if 5-HT modulation of S-cell excitability interacts with either form of synaptic plasticity.

The results presented here show a relationship between learning- and 5-HT-induced potentiation of excitability in the S-cell. To our knowledge, these experiments represent the first time that learning-induced modulation of excitability has been directly monitored and blocked at the same time that learning-induced changes in behavior were taking place. These results suggest, albeit not conclusively, that increased S-cell excitability contributes to sensitization of the shortening reflex. It is hypothesized that S-cell excitability during learning contributes to increases in the rate of S-cell activity during the shortening response and that this increase in activity allows the interneuron to contribute to the reflex in a way that is does not under basal conditions (Lisman 1997). The S-cell would be effectively “recruited” into the shortening neural circuit as a result of learning, a process that has been observed in both vertebrates and invertebrates (Daly et al. 2004; Moita et al. 2003). Such recruitment of a cell into a neural circuit may represent a general mechanism by which modulation of excitability re-configures neural networks during learning.

ACKNOWLEDGMENTS

The authors thank Drs. Brenda L. Moss and Kevin M. Crisp for helpful discussions during the preparation of this paper and Rodney McPhail at the Purdue University Department of Biological Sciences for the Fig. 2 drawing.

GRANTS

This work was supported by National Institute of Neurological Disorders and Stroke Grant R01-NS-34927 to C. L. Sahley with Kenneth J. Muller, National Institute of Research Resources Grant P20 RR-015567 to B. D. Burrell, and a subproject of National Institute of Research Resources Grant P20 RR-015567 to B. D. Burrell that is designated as a Center of Biomedical Research Excellence.

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