SEROTONIN MEDIATES LEARNING-INDUCED POTENTIATION OF EXCITABILITY

Brian D. Burrell¹* and Christie L. Sahley²

¹Neuroscience Group, Division of Basic Biomedical Sciences, University of South Dakota School of Medicine, Vermillion, SD 57069
²Department of Biological Sciences and Graduate Neuroscience Program, Purdue University, West Lafayette, IN 47907

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*Corresponding author
Brian D. Burrell, Asst. Professor
Neuroscience Group
Division of Basic Biomedical Sciences
University of South Dakota School of Medicine
414 East Clark St.
Vermillion, SD 57069
PH: 605-677-3182
FAX: 605-677-6381
bburrell@usd.edu

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ABSTRACT

Sensitization potentiates excitability in an interneuron, the S-cell, that is critical for this form of learning in the whole-body shortening reflex of the medicinal leech. Serotonin (5HT) also increases S-cell excitability and serotonergic modulation is known to be critical for sensitization of whole-body shortening, suggesting that 5HT mediates learning-induced enhancement of S-cell excitability. In this paper, the role of 5HT in mediating sensitization-induced potentiation of S-cell excitability was examined. Potentiation of S-cell excitability by 5HT was blocked by the 5HT receptor antagonist methysergide and by intracellular injection of the G-protein inhibitor GDP-β-S, indicating that a metabotropic 5HT receptor was involved. Bath application of Rp-cAMP, an inhibitor of protein kinase A (PKA), blocked 5HT-induced potentiation of excitability, while db-cAMP, a cAMP analogue that activates PKA, mimicked the potentiating effects of 5HT on the S-cell. During sensitization of the shortening reflex in semi-intact preparations, methysergide and Rp-cAMP prevented learning-induced potentiation of S-cell excitability, as well as the increase in S-cell activity that normally occurs during sensitization. Furthermore, sensitization-induced increases in the shortening reflex did not occur in preparations treated with methysergide or Rp-cAMP. These results demonstrate that sensitization-induced enhancement of S-cell excitability is mediated by 5HT and suggests that these changes may contribute to this form of learning.
INTRODUCTION

There has been increased appreciation for the role that modulation of neuronal excitability plays during learning and memory (see reviews by Wu et al. 2002; Daoudal and Debanne, 2003; 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 (Thompson et al. 1996; Moyer et al. 1996, 2000; Stackman et al. 2002; Oh et al. 2003), 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 *Hermisenda* and *Aplysia* (Alkon et al. 1985; Cleary et al. 1998; Antonov et al. 2001), motor neurons in *Lymnaea* (Straub and Benjamin 2001) and interneurons in *Helix* and the medicinal leech (Gainutdinov et al. 1998; Burrell et al. 2001).

The medicinal leech is particularly useful for studying the mechanisms that underlie learning-related neuroplasticity. Like many invertebrates, it has a well-characterized central nervous system (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 (Muller and Scott,
1981; Peterson 1984; Baccus et al. 2000), 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 (Sahley et al. 1994; Modney et al. 1997; Burrell et al. 2003). In addition, S-cell activity during elicited shortening is enhanced following 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 modulatory effects of 5HT, 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 5HT 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 5HT-containing Retzius cells (Fig. 1B; Burrell et al. 2001).

Although these results suggest that 5HT contributes to sensitization-induced increases in S-cell excitability, a direct link between 5HT- 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 5HT in sensitization-mediated increases in S-cell excitability by blocking the effects of
5HT on the S-cell during sensitization training in a behaviorally-intact preparation. The results demonstrate that 5HT-induced potentiation of S-cell excitability is due to activation of a metabotropic 5HT receptor coupled to a cAMP/PKA second messenger pathway and that blocking 5HT’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.
MATERIALS AND METHODS

Electrophysiology

Medicinal leeches (*Hirudo medicinalis*) weighing 3 g were obtained from a commercial supplier (Leeches USA Ltd.) and maintained in pond water (0.5 g/1 L H₂O Hirudo salt (Leeches USA Ltd.)) at 18°C. Single ganglia were dissected in leech saline (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; FHC) 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 SIU5 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 packet on the ventral side of the ganglion), size (5-10 µm) and the shape of their action potential (50-60 mV amplitude, ≈ 2 msec 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 msec) and measuring the resulting change in membrane potential. Excitability was measured by recording the number of action potentials initiated during a 500 msec, 1 nA depolarizing current pulse (AP’s/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
deemed 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 msec window, 10 msec prior to action potential initiation
(Cudmore and Turrigiano, 2004). Finally, the inter-spike 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-5HT treatment measures of excitability.

Changes in S-cell excitability were tested prior to (pre-test) a 5 min perfusion with 10 µM
5HT (N=9). This was followed by a 5 min wash in normal saline and then a second test of
excitability (post-test). This treatment has been shown to produce robust increases in excitability
that have reached a plateau by this time, although enhancement of excitability lasts at least 1 hr
(Burrell et al. 2001). The 5HT-induced changes in excitability were compared to groups of S-
cells in which the 5HT treatment was replaced with either normal saline (N=9), 5HT plus 100
µM methysergide (a non-specific antagonist of metabotropic 5HT receptors; N=7), methysergide
alone (N=3), 5HT plus 1 mM GDP-β-S (a membrane impermeable competitive G-protein
antagonist; N=4), GDP-β-S alone (N=4), 5HT 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 3M 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 side up in a 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 minutes 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); 5 stimulus pulses, 1 msec 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 msec 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 5HT experiments described previously. In preparations that would undergo sensitization in normal saline (N=7), training began by making two pre-sensitization (pre-test) measurements of the shortening response and the accompanying S-cell activity at an inter-trial 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 post-sensitization (post-test) measurements of the shortening reflex/S-cell activity were made (2.5 min ITI). Post-sensitization measurements of S-cell excitability were made immediately after the last behavioral post-test.

In order to test whether sensitization-induced potentiation of S-cell excitability was mediated by 5HT, 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 five minutes before the first pre-test. Injection of membrane impermeable agents into the S-cell that might antagonize 5HT-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, 5HT, 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 5HT 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 post-test excitability measurements. The averaged value from each neuron was then used for statistical analysis and graphical representation. One-way analysis of variance (ANOVA) in which the post-treatment levels had been normalized relative to the pre-treatment 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 (pre-sensitization trial 2 and post-sensitization trials 1 and 2, all normalized to the first pre-sensitization trial).
RESULTS

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

Application of 10 \( \mu \text{M} \) 5HT induced a significant increase in the number of action potentials fired by the S-cell during current injection (Fig. 3A; AP’s/pulse one-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; 1\textsuperscript{st} ISI one-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 one-way ANOVA \( F_{8,36} = 3.53, p < 0.005 \)). The 5HT-induced changes in these excitability properties were significantly different when compared to S-cells in which 5HT application was omitted (saline group; Fig. 3D; post-hoc test: AP’s/pulse \( p < 0.0005 \); 1\textsuperscript{st} ISI \( p < 0.05 \); slope \( p < 0.01 \)). No significant change was observed in the membrane potential threshold (one-way ANOVA \( F_{8,35} = 0.48 \)) or in input resistance (one-way ANOVA \( F_{8,35} = 0.50 \)). 5HT-induced increases of S-cell excitability were blocked by co-application of 100 \( \mu \text{M} \) methysergide (Fig. 3D; 5HT+methysergide). Excitability measures from the 5HT+methysergide group were significantly different compared to the 5HT group (post hoc test: AP’s/pulse \( p < 0.001 \); 1\textsuperscript{st} 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 5HT+methysergide groups, but were significantly different from the 5HT-treated group in terms of AP’s/pulse (post hoc \( p < 0.001 \)), 1\textsuperscript{st} 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-, 5HT+methysergide-, methysergide-, and the 5HT-treated groups.

Although methysergide was effective at blocking 5HT-mediated increases in S-cell excitability, it is a relatively non-specific 5HT receptor antagonist and does not help in
identifying the 5HT receptor subtype(s) involved in this modulation. It is difficult to identify 5HT receptor subtypes in invertebrates using vertebrate pharmacological tools (Tierney, 2001). Therefore components of the signal transduction pathways activated by the 5HT receptors were examined instead. This approach was used not only to characterize the cellular mechanisms mediating 5HT 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 5HT-induced changes in S-cell excitability since there are both ionotropic and metabotropic 5HT 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 5HT (5HT+GDP-β-S). GDP-β-S prevented 5HT-induced increases in S-cell excitability (Fig. 3E). Changes in AP’s/pulse (post-hoc p < 0.001), 1st ISI (p < 0.05) and slope (p < 0.05) in the 5HT+GDP-β-S group were significantly different from those treated with 5HT 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’s/pulse p < 0.05; 1st ISI p < 0.01; slope p < 0.01). No significant difference in S-cell membrane potential threshold or input resistance was observed between the 5HT+GDP-β-S, GDP-β-S, 5HT or saline groups (Fig. 3E).

Next, the involvement of a cAMP/PKA second messenger cascade was examined. 5HT-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’s/pulse (post-hoc p < 0.005), 1st ISI (p < 0.05) and slope (p < 0.01) in the 5HT+Rp-cAMP group were significantly different from those treated with 5HT 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’s/pulse p < 0.0005; 1\textsuperscript{st} ISI p < 0.05; slope p < 0.01). No significant difference in S-cell membrane potential threshold or input resistance was observed between the 5HT+Rp-cAMP, Rp-cAMP, 5HT or saline groups (Fig. 3F). If 5HT 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 5HT on this interneuron. A 5 min perfusion of the S-cell with 50 \mu M db-cAMP produced increases in AP’s/pulse, slope and decreases in 1\textsuperscript{st} ISI that were statistically indistinguishable from those of 5HT (Fig. 3F), but statistically different from the saline group (post hoc results: AP’s/pulse p < 0.001; 1\textsuperscript{st} 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, 5HT or saline groups (Fig. 3F). Together, the results from the Rp-cAMP and db-cAMP experiments demonstrate that 5HT-induced increases in S-cell excitability require activation of a cAMP/PKA second messenger cascade.

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

In order to examine whether sensitization-induced increases in S-cell excitability are the result of serotonergic modulation, sensitization training was carried out under conditions which block potentiation of excitability by 5HT. Specifically, semi-intact preparations were treated with either 100 \mu M methysergide or 50 \mu M 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 following delivery of the sensitizing stimuli (Fig. 4B & C) in preparations that underwent sensitization training in normal saline (Sens/Saline group). S-cell activity during shortening also increased relative to
pre-sensitization levels (Fig. 4B & 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 omitted (Fig. 4C & D; Con/Saline group). 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. 4C & 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 post-sensitization shortening in the Sens/Saline group was significantly greater compared to 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 post-sensitization 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. 4C & 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 pre-sensitization trial 1 shortening reflex from the Sens/Saline, Sens/Methy and Sens/Rp-cAMP groups (one-way ANOVA $F_{2,16} = 1.20$). Furthermore, the lack of sensitization was not due to any deficits in the 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 pre-sensitization 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 ($F_{1,13} = 8.73$, $p < 0.05$) which was the result of the first sensitizing stimulus consistently eliciting more S-cell action potentials than the second sensitizing stimulus 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 post-sensitization trials was calculated
at 100 msec 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 post-sensitization trials was enhanced relative to the pre-sensitization trials with differences between the average activity levels becoming greater at later post-stimulus 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 post-stimulus 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 post-sensitization change in overall S-cell frequency elicited during shortening is shown in the Fig. 5 inset.

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

In agreement with earlier observations (Burrell et al. 2001), S-cell excitability increased following sensitization training and the pattern of sensitization-induced increases in excitability properties was identical to those produced by 5HT in this study. Sensitization produced an increase in AP’s/pulse (Fig. 6A, D; one-way ANOVA $F_{5,15} = 5.52, p < 0.005$), a decrease in the 1st ISI (Fig. 7B, D; $F_{5,15} = 4.48, p < 0.05$) and an increase in slope (Fig. 6C, D; $F_{5,15} = 4.72, p < 0.01$) relative to S-cells from non-sensitized 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 5HT-induced enhancement of excitability, namely methysergide and Rp-cAMP (Fig. 6D), demonstrating that enhanced S-cell excitability during sensitization was mediated by 5HT. Post-hoc analyses confirmed that sensitization-induced changes in excitability properties were significantly different in the S-cells in the Sens/Saline group compared to those in the Sens/Methy (AP’s/pulse $p < 0.001$; 1st ISI $p < 0.005$; slope $p < 0.005$) or the Sens/Rp-cAMP (AP’s/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 than the Con/Saline (AP’s/pulse p < 0.005; 1st ISI p < 0.005; slope p < 0.001), Con/Methy (AP’s/pulse p < 0.005; 1st ISI p < 0.01; slope p < 0.005), and Con/Rp-cAMP (AP’s/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 5HT induced similar increases in excitability in the S-cell and extends those findings by demonstrating that 5HT mediates sensitization-induced potentiation of S-cell excitability. 5HT-induced potentiation of S-cell excitability is mediated by a metabotropic 5HT receptor that up-regulates 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 5HT-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 5HT binding (methysergide) or prevented cAMP activation of PKA (Rp-cAMP), demonstrating that learning-induced potentiation of S-cell excitability is a 5HT-dependent process.

Interestingly, treatments that blocked 5HT-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 since the drugs used to block serotonergic modulation of the S-cell were bath-applied (see Materials and Methods) and may have prevented 5HT- or cAMP/PKA-dependent changes in other cells that contribute to sensitization. For example, 5HT 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, 5HT 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 5HT 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 (Sahley et al. 1994; Modney et al. 1997; Burrell et al. 2003). 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 5HT-containing Retzius cells throughout the leech CNS since 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 non-sensitized shortening reflex. In addition, results presented here and from previous experiments (Sahley et al. 1994; Burrell et al. 2001) 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 5HT- 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 5HT-induced increases in S-cell excitability is not known, but it likely involves the modulation of multiple membrane conductances (see reviews by Zhang and Linden 2003; Frick and Johnston 2005). 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 1\(^{st}\) 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 following learning in both vertebrates and invertebrates (Alkon et al., 1985; Thompson et al. 1996, Moyer et al. 1996, 2000; Shreurs et al. 1997, 1998; Cleary et al. 1998; Gainutdinov et al. 1998; Saar et al. 1998; Antonov et al. 2001; Burrell et al. 2001; Straub and Benjamin 2001; Stackman et al. 2002; Oh et al. 2003) 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) (Bliss and Lomo, 1973; Andersen et al. 1980; 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; Morozov et al. 2003; Le Ray et al. 2004; Nolan et al. 2005), 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 5HT modulation of S-cell excitability interacts with either form of synaptic plasticity.

The results presented here show a relationship between learning- and 5HT-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 potentiation of 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 (Moita et al. 2003; Daly et al. 2004). Such recruitment of a cell into a neural circuit may represent a general mechanism by which modulation of excitability reconfigures neural networks during learning.
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Reference List


54. **Thompson LT, Moyer JR, Jr. and Disterhoft JF.** Transient changes in excitability of rabbit CA3 neurons with a time course appropriate to support memory consolidation. *J Neurophysiol* 76: 1836-1849, 1996.


FIGURE LEGENDS

Figure 1. The S-cell network and organization of the whole-body shortening neural circuit. (A) The leech CNS consists of a chain of 21 ganglia (one per body segment) plus fused ganglia in the head and tail regions (not shown) that are linked by a connective nerve, and each ganglion contains a single S interneuron that projects an axon into the anterior and posterior connective nerves. At the midpoint of each connective, these axons form an S-to-S electrical synapse, resulting in a chain or network that extends throughout the leech CNS. (B) Whole-body shortening can be initiated by mechanosensory stimuli that activate touch (T), pressure (P) and sometimes nociceptive (N) neurons, which have synaptic input onto local motor neurons (Nicholls and Purves 1970), i.e. those that are in either the same segment or in the segment adjacent to where the stimuli are applied (L=longitudinal motoneuron, DE and VE = dorsal and ventral excitor motor neurons). More distant motorneurons must be activated by an unknown neuron or neurons that are responsible for triggering whole-body shortening (these triggering elements presumably receive input from the T-, P-, and N-cells). The S-cell is also activated during shortening by the mechanosensory neurons and has synaptic output onto the L-motor neurons and the 5HT-containing Retzius cell (Wang 1999). The Retzius cell, in turn, has an excitatory (+) modulatory input onto the S-cell (Burrell et al. 2001). Connections marked by an asterisk (*) are polysynaptic.

Figure 2. Diagram of the semi-intact preparation used for sensitization training. The ganglion in the fourth 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.

**Figure 3.** 5HT increases excitability via a metabotropic 5HT receptor that activates a cAMP/PKA second messenger pathway. Changes in excitability properties before and after application of 10 µM 5HT: (A) AP’s/pulse; (B) 1st ISI in the train; (C) slope of depolarization (dV/dT) prior to the first action potential in the train. (D) Average percent change (±standard error) in excitability properties in 5HT-treated (black-filled bars) and saline control (open bars) S-cells (same data used in E and F). Co-application of methysergide blocked 5HT’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 5HT’s effects (fine right-hatched bars), but had no effect by itself (coarse right-hatched bars). (F) Co-application of Rp-cAMP block 5HT’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 5HT.

**Figure 4.** Methysergide and Rp-cAMP block sensitization-induced increases the shortening reflex and S-cell activity. (A) Time line of training protocol. (B) Sensitization training increased the intensity of the elicited shortening response (top traces) and the S-
cell activity during shortening (bottom traces). Average (±standard error) increase in the whole-body shortening reflex (C) and S-cell activity (D) in the sensitized (filled circles) versus control (open circles) preparations trained in normal saline. Both methysergide (filled triangles) and Rp-cAMP (filled squares) blocked sensitization-induced increases in behavior and S-cell activity. Neither methysergide nor Rp-cAMP had any effect on shortening or S-cell activity when applied to preparations that did not undergo sensitization training (open triangles and squares, respectively).

**Figure 5.** Sensitization increases the rate of S-cell activity. Frequency distribution of S-cell activity prior to (open bars) and after (filled bars) sensitization. For each preparation, the average S-cell frequency from both pre- and post-sensitization tests was calculated for each interval and then those numbers were used to calculate the average and standard error shown in the graph. **Inset** Average (±standard error) overall S-cell frequency during elicited shortening.

**Figure 6.** Sensitization increases S-cell excitability and those changes are blocked by treatments that antagonized 5HT’s effect on this interneuron. Changes in excitability properties before and after application of sensitization training: (A) AP’s/pulse; (B) 1st ISI in the train; (C) slope of depolarization (dV/dT) prior to the first action potential in the train. (D) As with 5HT, S-cells from preparations that had undergone sensitization training in normal saline (Sens/Saline; filled bars) exhibited an increase in AP’s/pulse and slope, a decrease in 1st ISI, and no change in membrane potential threshold or input resistance. No significant change in these excitability properties was observed in S-cells from the control
(no sensitizing stimuli delivered) preparations in normal saline (Con/Saline; open bars). Sensitization-induced increases in excitability were not observed in preparations trained in methysergide (Sens/Methys; fine hatched bars) or Rp-cAMP (Sens/Rp-cAMP; fine cross-hatched bars). No changes in excitability were observed in control preparations treated with either methysergide (Con/Methys; coarse hatched bars) or Rp-cAMP (Con/Rp-cAMP; coarse cross-hatched bars)
Figure 1
Figure 3

A. Pre 5HT and Post 5HT activity shown with voltage traces.

B. Similar to A, but with a different setup.

C. Another setup similar to A and B.

D. Graph showing percent changes in response properties.

E. Graph showing percent changes in response properties with different treatments.

F. Graph showing percent changes in response properties with different treatments.
Figure 4

A. 1st Pre-test 2nd Pre-test Sensitizing Stimuli 1st Post-test 2nd Post-test

B. Shortening Response

C. Shortening Response (% Baseline)

D. S-cell Activity (% Baseline)
Figure 5