In the CNS of the medicinal leech, *Hirudo medicinalis*, a linear network of electrically coupled neurons, the S-cells, provides an opportunity to examine these questions. The leech CNS consists of head and tail brains connected by a series of segmental ganglia, each with a single S interneuron. Each S-cell has a bifurcating axon projecting anteriorly and posteriorly into the nerve that links the ganglia (medial connective) where it forms an electrical synapse with the S-cell axon from the neighboring ganglion, similar to the axo-axonal electrical synapses observed in the CA1 pyramidal neurons (Schmitz et al. 2001), producing a chain or network of S-cells that extends throughout the leech CNS (Fig. 1). Individual S-cells are strongly coupled to one another so that action potentials arising in any one cell reliably propagate in both directions throughout the interneuron chain (Bagnoli et al. 1972). In this respect, the S-cell chain acts as a single neuron with multiple initiation sites (Baccus et al. 2001).

The S-cell network plays a critical role in nonassociative learning in the leech during the defensive withdrawal reflex, whole-body shortening. The S-cell receives excitatory synaptic input from the sensory neurons that initiate shortening (Baccus et al. 2000; Muller and Scott 1981), directly excites motor neurons that produce shortening (Gardner-Medwin et al. 1973; Magni and Pellegrino 1978), and is active during elicited shortening responses. Lesions of the S-cell chain do not affect the animal’s capacity to shorten (Sailey et al. 1994; Shaw and Kristan 1999), but cutting the axon of one S-cell or ablating an S-cell soma eliminates sensitization and disrupts dishabitation of the shortening reflex (Modney et al. 1997; Sahley et al. 1994). In addition, both the number of S-cell action potentials fired during shortening and the contribution of S-cell activity to the reflex increase during these forms of learning (Sailey et al. 1994). These changes appear to be the result of serotonergic modulation. Depletion of serotonin (5HT) from the leech CNS has the same effect on learning as lesions of the S-cell chain (Ehrlich et al. 1992) and increases in S-cell excitability observed during sensitization and dishabitation are mimicked by applying 5HT or stimulating 5HT-containing neurons (Burrell et al. 2001).

The train of S-cell action potentials produced during an elicited shortening response is the sum of staggered action potential initiations from several S interneurons within the
chain (Baccus et al. 2001). A single mechanosensory stimulus initiates impulses in several S-cells because each mechanosensory cell excites several S-cells within the chain and each region of skin within a segment is innervated by sensory neurons from several segmental ganglia. Increases in action potential initiation of the S-cell network are due to the increased contributions by individual S-cells within the chain. Action potential collisions in the S-cell chain are extremely rare, occurring to less than 1% of action potentials (Baccus et al. 2001) because the S-cell axons are the most rapidly conducting in the leech nervous system. As a result, the slower-conducting mechanosensory axons initiate activity in more distant S-cells later, when the earliest-initiated action potentials have already propagated beyond these more distant cells. Since learning-induced increases in S-cell activity are due, at least in part, to serotoninergic modulation, the effect of 5HT on the pattern of action potential initiation within the S-cell network was examined. It was important to determine whether 5HT increased the activity of the S-cell network by recruitment of additional S-cells to initiate action potentials, by causing more initiations in a fixed subset of S-cells, or by a combination of both mechanisms.

METHODS

Leeches (2–3 g) were obtained from a commercial supplier (Leeches USA, Westbury, NY) and maintained in artificial pond water (0.5 g Forty Fathoms/1 lH2O, Marine Enterprises, Baltimore, MD) in a refrigerated incubator at 18–20°C. All experiments were carried out in leech saline solution consisting of (in mM) 115 NaCl, 4 KCl, 1.8 CaCl2, and 10 Tris-maleate, at pH 7.4 (Kuffler and Potter 1964). Intracellular electrophysiological recordings were made using thin-walled, glass microelectrodes (0.75 mm ID; Frederick-Haer, Brunswick, ME) filled with 4 M potassium acetate and having a 15- to 20-MΩ resistance. Signals were amplified with a Getting 5A electrometer (Getting Instruments) and viewed on a storage oscilloscope (Tektronix). Extracellular recordings from suction electrodes were made using a Grass P15 AC preamplifier. Data were filtered (Ithaco 4302 dual 24 dB/octave filter) and converted for digital storage and future analysis using Axoscope data acquisition software with a Digidata 1200 series interface (Axon Instruments). Controlled stimulus pulses were delivered using a Grass S88 two-channel stimulator with SIU5 stimulus isolation units.

Animals were anesthetized by cooling at 4°C in artificial pond water and then transferred to a silicone-elastomer (Sylgard)-lined (Dow-Corning) dissecting dish that was surrounded by a layer of ice. All dissections were done in ice-cold leech saline. A chain of mid-body ganglia (usually ganglia 7–15) was dissected from the animal with the middle three ganglia still connected on one side to a piece of leech skin by the segmental nerve roots; this was referred to as the body-wall preparation (Fig. 2A). The body-wall preparation was pinned to a Sylgard-lined 35 × 10-mm petri dish that was filled with leech saline. In a pilot study where 5HT was applied to the skin and the resulting S-cell activity was recorded in the periphery, 5HT inhibited mechanosensory-elicited S-cell activity (data not shown). Therefore a thin Sylgard wall was placed between the skin and the chain of ganglia so that 5HT could be applied selectively to the CNS portion of the preparation, similar to earlier experiments using this type of preparation (Belardetti et al. 1982; Mar and Drapeau 1996). The bottom and sides of the Sylgard wall were lined with petroleum jelly (Vaseline) to form a waterproof seal, isolating the ganglia from the skin. A second Sylgard wall was placed on the ganglia side of the dish to reduce the overall volume of this portion of the chamber to ~800 μl. Leech saline was constantly perfused through the CNS chamber at a rate of ~2 ml/min. The skin portion of this chamber was filled with enough leech saline to just cover the skin.

A pair of Teflon-coated silver wires (uncoated diameter, 0.125 mm; AM Systems) was implanted in the skin and bared at the point of contact with the skin. These wires were implanted in middle segment (11) of the skin preparation (segments 10–12). The wires were connected to the stimulator, which delivered capacity-coupled electroshocks (1.5 ms) to the skin of an intensity sufficient to produce reflexive shortening in reduced preparations (Burrell et al. 2001). At both the anterior and posterior ends of the chain of ganglia, a length of nerve cord was drawn into a suction electrode to record evoked S-cell activity (segments 7 and 15, respectively; Fig. 2A).

The protocol for measuring the activity of individual S-cells in the network was taken from Baccus et al. (2001). First, the time required for a single S-cell action potential elicited by the suction electrode (0.5-ms stimulus pulse) at one end of the chain of ganglia to conduct to the recording suction electrode at the other end was measured (Tcond). The S-cell produced the largest signals in these recordings, permitting its activity to be readily distinguished (Bagnoli et al. 1972; Frank et al. 1975; Laverack 1969). A mechanosensory stimulus was applied to the skin and the resulting S-cell activity was recorded in both the anterior and posterior electrodes. The initiation site for each S-cell action potential was determined by measuring the difference in arrival times (Tdiff of each action potential at the two recording electrodes, Tdiff = Tant − Tpost, where Tant was the arrival time of an action potential at the anterior electrode and Tpost was the arrival time of that same action potential at the posterior electrode. Action potentials that arose in S-cells close to the anterior electrode arrived at that electrode before reaching the posterior electrode; the reverse was true for action potentials that arose closer to the posterior electrode (Fig. 2B). The values of Tdiff clustered to form peaks and the peaks corresponded to the sites of initiation in the S-cell chain (Fig. 2C) (Baccus et al. 2001). To identify the peaks for Tdiff with activity arising in particular S-cells, at least one of the activated S-cells was stimulated intracellularly in every preparation. The Tdiff of these intracellularly generated action potentials matched the Tdiff of action potentials elicited by mechanosensory stimuli. No discrepancies were observed between the two measurement techniques (data not shown).

In addition to counting the number of action potential initiations, the time at which each action potential arose was determined using the formula (Tant + Tpost − Tcond)/2. These data were incorporated into analysis of the rate of action potential initiation for each S-cell and for the entire S-cell network.

Nearly all action potentials recorded were initiated in S-cells from one of the three ganglia still connected to the skin (segments 10–12) in each preparation. The S-cell in the segment where the mechanosensory stimulus was applied (segment 11) is referred to here as the...
central site, while S-cells in the ganglia just anterior (segment 10) and posterior (segment 12) are termed the anterior and posterior sites, respectively. Baccus et al. (2001) observed that the more intact the preparation, the more S-cells contributed to the mechanosensory-elicted response by the network. Initially, experiments were conducted using more intact preparations (more ganglia connected to the skin), but it was prohibitively difficult to incorporate the Sylgard barrier separating the CNS from the periphery without damaging the preparation.

The effects of 5HT on the pattern of action potential initiation in the S-cell chain were tested as follows. Activity in the S-cell network was elicited by cutaneous electroshocks at 3-min intervals. For the first 6 min, activity was elicited while normal leech saline perfused through the CNS chamber. 5HT (50 μM; Sigma) dissolved in leech saline was perfused through the CNS chamber for the next 9 min followed by an 18-min posttreatment period in normal saline. Activity in the drug-treated group (n = 7) was compared with activity in a control group (n = 4) that received the same stimulation but was not treated with 5HT. Activity data from the entire S-cell network were analyzed using two-way ANOVA that examined the effects of treatment group (5HT-treated vs. control), time and the treatment group-time interaction effect. Data involving the activity of individual S-cells within the network were analyzed using the nonparametric Wilcoxon’s signed-rank test. This was done for two reasons. First, the data from individual S-cells were not normally distributed, in part because of the relatively small number of events at each S-cell during each elicited response. Second, when analyzing the effects of 5HT on frequency of S-cell action potential initiation at various intervals after skin stimulation (see Fig. 5), performing a statistical test at each interval would have increased the experimentwise error rate (the probability of accepting a significance difference where none exists). The Wilcoxon’s signed-rank provides a conservative approach that allows for a paired comparison of pretreatment versus 5HT treatment activity levels across all intervals (Sokal and Rohlf 1995a,b). All statistical analyses were performed using Statistica analysis software (Statsoft).

RESULTS

Effects of 5HT on number of action potential initiations

5HT increased the number of mechanosensory-elicted action potentials initiated by the S-cell network and the enhancement persisted throughout the subsequent post-5HT treatment period (Fig. 3). These conclusions were confirmed by 2-way ANOVA, which detected a significant effect of treatment group $[F(1,126) = 33.01, P < 0.0001]$, no effect of time $[F(2,126) = 0.33, \text{not significant or ns}]$, and a significant treatment group-time interaction effect $[F(2,126) = 3.69, P < 0.05]$. Post hoc analysis (Neuman-Keuls) confirmed that activ-
activity levels were the same in the control and 5HT-treated groups prior to the treatment stage and that activity in the 5HT group was significantly enhanced relative to the control group during the 5HT treatment stage (\( P < 0.01 \)) and post-5HT stage (\( P < 0.001 \)). In addition, activity levels during the treatment and posttreatment stage were enhanced relative to pretreatment levels in the 5HT group (\( P < 0.05 \)). These results are consistent with previously observed excitatory effects of 5HT on mechanosensory-elicited S-cell activity (Belardetti et al. 1982).

In some experiments (3 of 7), 5HT not only increased firing but also increased the number of S-cells in which impulses arose, that is, that contributed to the network-level response. In others (3 of 7), during the 5HT treatment stage there was no change in the number of S-cells that initiated action potentials, but the number of action potentials generated by the network rose. 5HT, therefore enhanced activity in the S-cell network either simply by increasing activity in S-cells that were already active or by also causing recruitment of additional S-cells into the network response. In a seventh experiment, 5HT enhanced the elicited S-cell response, but the number of S-cells contributing to the response dropped from two to one. Because this was seen only once, this preparation was excluded from further analysis of individual S-cells’ contributions to the network’s response.

Interestingly, while 5HT significantly increased the number of S-cell action potential initiations at the network level, the excitatory effect differed across individual S-cells within the network (Fig. 4). At the central site, which was the most active, the number of initiations during 5HT treatment did not increase in a statistically significant manner (Wilcoxon’s signed-rank test \( z = 0.84, \) ns). A greater 5HT-induced increase in the number of initiations was observed at the anterior site, but again this enhancement was not statistically significant (\( z = 1.89, \) ns). However, the posterior site S-cell (post + 1), which produced the fewest action potentials of all the activated sites, was significantly enhanced following 5HT treatment (\( z = 2.20, P < 0.05 \)).

Effects of 5HT on frequency of action potential initiation

A more detailed analysis of the 5HT-induced changes in S-cell activity was conducted by examining the frequency of action potential initiations in the entire S-cell chain and in individual interneurons within the network. Frequency was determined with respect to time after delivery of the mechanosensory stimuli. Specifically, for each elicited response, the number of action potential initiations in a 10-ms period was calculated and converted to frequency in hertz. The pattern of activity observed by Baccus et al. (2001) is similar to the pattern of activity observed by Baccus et al. (2001). The effects of 5HT on the frequency of action potential initiations, or instantaneous frequency, by the S-cell network varied with the interval following stimulation of the skin. At the shortest intervals (<30 ms), the instantaneous frequency of the network during 5HT treatment was not different from that before treatment (~60–100 Hz), but was much higher (~100–175 Hz) at intermediate intervals (30–80 ms; Fig. 5A). At longer intervals
The instantaneous frequency during the 5HT treatment stage was higher than the corresponding pretreatment frequency, but the relative enhancement was not as great as in the preceding intervals. The observed changes represented a statistically significant increase in the rate of action potential initiation in the S-cell network across all intervals (Wilcoxon’s signed-rank test $z = 2.79, P < 0.01$).

When the frequency of action potential initiation was examined in the individual S-cells that contributed to the network response, the effects of 5HT were found to vary with the site of initiation. As expected from our earlier study (Baccus et al. 2001), the pattern of initiations at the central site strongly resembled the pattern of the entire S-cell network (Fig. 5, A and B) and had the highest instantaneous frequency of any of the S-cells that contributed to the network-level response. 5HT-induced increases in the central site initiation rate were observed 30–60 ms after skin stimulus and accounted for nearly all of the increase in instantaneous frequency at the network level observed at those times. However, the overall instantaneous frequency increase at all intervals after skin stimulation was not statistically significant at the central site ($z = 1.31, P = 0.19$). Activity at the anterior site accounted for much of the later activity observed at the S-cell network level (Fig. 5, A and C). Although 5HT increased the rate of impulse initiations and thus contributed to an increase in the network instantaneous frequency at 70–80 ms after skin stimulation, the overall increase in instantaneous frequency was not significant ($z = 1.36, P = 0.17$). The posterior site made the weakest contribution to the pretreatment S-cell network response and had the strongest 5HT-induced enhancement (Fig. 5, A and D). Although the posterior site’s initiation rate was not as high as the central site’s or that of the whole S-cell network, the posterior site did contribute to the network level instantaneous frequency 30–80 ms after skin stimulation, and the overall instantaneous frequency of the site was significantly enhanced during the 5HT treatment stage ($z = 2.89, P < 0.01$).

**Effects of 5HT on the activity pattern within the S-cell network**

A consequence of action potential initiations occurring at different times in multiple S-cells along the chain is that different parts of the S-cell network experience different patterns of activity (Baccus et al. 2001). This happens because when two action potentials arise at different points along the chain, for example first at the central and then at the anterior S-cells, the interspike interval in the anterior direction will be less than the interval in the posterior direction. The greatest number of action potential initiations occurred in the central site, which was closest to the mechanosensory stimulus, followed in magnitude by the anterior and then posterior sites (Figs. 4 and 5). This bias toward anterior site initiations occurred in spite of the fact that the anterior and posterior site S-cells were equidistant from the skin stimulus. Evidence for a similar bias was presented in Baccus et al. (2001). The result of this anterior bias on activity in different parts of the S-cell network is as predicted: the interspike intervals of action potentials recorded at the anterior end of the chain are less than at the posterior end (Fig. 6), although this trend is not statistically significant.

The 5HT treatment increased the firing frequency and shortened the interspike intervals recorded both anteriorly ($\chi^2 = 61.70, P < 0.0001$) and posteriorly ($\chi^2 = 79.38, P < 0.0001$). However, the interspike intervals were disproportionately shortened in the posterior region relative to the pretreatment stage so that the anterior and posterior became more nearly equal. These results are consistent with the increased contribution of the posterior S-cell to the network level response during the 5HT-treatment stage (Figs. 4 and 5). Furthermore, the results demonstrate that 5HT, in addition to increasing the rate of action potential initiation, modulates the relative patterns of activity that occur in the anterior and posterior regions of the S-cell network.

**DISCUSSION**

**Modulation of an electrically coupled neural network**

Networks of electrically coupled neurons have distinctive computational properties. Input can be received from multiple sources by one or many cells within the network to produce a variety of activity patterns by the network as a whole (Brivanlou et al. 1998). The network, in turn, can alter the activity of groups of neurons widely distributed throughout the CNS in a coordinated or synchronous manner (Beierlein et al. 2000; Galarreta and Hestrin 1999; Gibson et al. 1999; Peinado 2001).

![Graph A](image1.png)

**FIG. 6.** 5HT modifies the pattern of S-cell activity experienced by different parts of the S-cell network. Data presented show the distributions of interspike intervals (ISIs) recorded at the posterior (post) and anterior (ant) suction electrodes before and during 5HT treatment. A: prior to 5HT treatment there was a tendency for action potential initiations to proceed anteriorly along the S-cell chain. B: during 5HT treatment, the pattern of activity was more uniformly distributed, as a result of the increased contribution of posterior initiation sites, and activity became skewed toward shorter ISIs at both the anterior and posterior electrodes.
While it is well established that modulation of individual neurons can change the output of a neural circuit, such modulation typically involves circuits of various types of neurons with distinctly different response properties and connections (Harris-Warrick and Marder 1991). In contrast, neurons that comprise electrically coupled networks typically are similar in terms of bioelectrical properties, morphology, neurotransmitters used, the type of input received, and the types of neurons they drive (Brivanlou et al. 1998; Galarreta and Hestrin 1999; Meister et al. 1995).

In the S-cell network, mechanosensory stimuli that elicit whole-body shortening activate multiple S-cells within the network in an asynchronous manner that prevents action potentials from colliding (Baccus et al. 2001). This allows impulse initiations in each S-cell to contribute to the output at the network level. Electrical coupling between S-cells allows action potentials to propagate reliably throughout the network. In this respect, the S-cell chain resembles a single neuron with multiple action potential initiation sites that contribute to the overall activity pattern of the cell, similar to neurons found in a variety of invertebrate and vertebrate species (Antic et al. 2000; Calabrese 1980; Chen et al. 1997; Kriebel et al. 1969; Martina et al. 2000; Meyrand et al. 1992; O’Shea 1975; Vedel and Moulins 1978; Zecevic 1996). In one study, 5HT was found to modulate the activation pattern of multiple initiation sites with in a single motoneuron (Meyrand et al. 1992). Addition of 5HT to the leech CNS produced an increase in the number and frequency of action potentials elicited in the S-cell chain by mechanosensory stimuli. The effects of 5HT were more pronounced for S interneurons near but outside the stimulated segment. In general, the more impulse initiations that occurred in an activated S-cell prior to 5HT treatment, the less the effect of 5HT. Thus a statistically significant enhancement in the number and frequency of action potential initiations occurred only at the posterior site.

It is likely that several cellular mechanisms contributed to enhancement of activity in the S-cell network. First, 5HT increases S-cell excitability directly, increasing the number of action potentials generated by a fixed input and lowering the threshold for action potential initiation (Burrell et al. 2001). This would make the S-cell network more responsive to afferent input. Second, 5HT might enhance afferent input to the S-cell network by increasing the amount of neurotransmitter released by the sensory cell terminals, as it appears to do in Aplysia (Hawkins et al. 1993). Third, 5HT can relieve action potential conduction block at central branch points in leech sensory cells (Mar and Drapeau 1996). Relief of conduction block can enhance synaptic transmission to the S-cell (Baccus et al. 2000; Muller and Scott 1981). There is also an intermediate state in the P-cells, reflection, where action potential propagation is delayed at the branch point so that in addition to propagating through the branch point, it reverses direction, activating a portion of sensory cell’s synapses twice and causing facilitation (Baccus 1998; Baccus et al. 2000). Although it has yet to be determined, it is predicted that 5HT could enhance synaptic transmission from P sensory cells to the S-cell by relieving conduction block, thereby causing reflection (Baccus et al. 2000). In a compartmental model that mathematically simulated P-cell synaptic input onto the S-cell chain, Baccus et al. (2001) showed that conversion from the blocked to the reflected state, as might be expected in the presence of 5HT, increased the number of action potential initiations.

Functional consequences of modulation of the S-cell network

The results demonstrate the integrative consequences to the output of a network of electrically coupled neurons with spatially distributed inputs. In effect, the sensory inputs provide a set of delay lines that permit sensory activation of more distant S-cells at later times. Peak activity at the central S-cell may be close to its maximum output, and if the sensory input coincides with the peak activity, this might limit 5HT’s enhancement of activity. If the other S-cells are farther from saturating levels of firing at the time of their (delayed) peak input, they should show a greater enhancement of initiations during 5HT treatment, and they do. Thus the presence of additional S-cells contributing to the network-level response allows for a more extended burst of activity at a higher frequency than would be produced by a single S-cell. The observed increases in S-cell network activity suggest a mechanism for how this interneuron contributes to the changes in behavior during learning, a process that is thought to be mediated by 5HT (Burrell et al. 2001). The greater burst of high-frequency activity in the S-cell during an evoked response may enhance synaptic transmission by the S-cell, increasing firing in some postsynaptic neurons and initiating it in others where the synapses usually have a high failure rate (Lisman 1997). Increases in S-cell firing rate may enhance release of the neuropeptide myomodulin, a neuromodulator that is present in the S-cell and known to increase excitability in the Retzius cells (the main 5HT-containing neurons in the leech CNS) (Keating and Sahley 1996; Vilim et al. 1996, 2000; Wang et al. 1999). Perhaps it is these effects that bring the S-cell to a threshold it reaches, following sensitization, when it makes a significant and critical contribution to the shortening reflex.

Serotonergic modulation of the S-cell chain also alters the pattern of activity experienced by different parts of the network. Normally, the pattern of action potential initiations is biased in the anterior direction with more initiations occurring in S-cells anterior to the skin stimulus location than in posterior cells (see current results and Baccus et al. 2001). As a result, the anterior portion of the chain experiences the elicited train of action potentials at higher instantaneous frequency than the posterior portion. 5HT changes the pattern of action potential initiations so that posterior S-cells make a significantly stronger contribution to elicited activity in the network. Consequently, both anterior and posterior portions of the S-cell network experience the elicited train of action potentials at approximately equivalent instantaneous frequencies. It is not known if this more uniform distribution of activity throughout the network contributes to learning-related behavioral changes in the shortening reflex.

In conclusion, the results demonstrate that modulatory neurotransmitters can alter the activity of a network of electrically coupled interneurons by differentially modifying the contributions that individual neurons make to the network level response. This change in network activity may be the result of a combination of changes in the level of presynaptic input to the network and in excitability of the component neurons within the network. It shall be important to determine which cellular mechanisms underlie modulation of the S-cell network and
how such modulation affects the S-cell’s role within the larger neural circuit that mediates whole body shortening. This will in turn help in understanding more generally the mechanisms of neuromodulation in other networks of electrically coupled neurons that contribute to processes such as neural plasticity and sensory processing.

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