Published in final edited form as:

Neuroscience. 2007 August 10; 148(1): 65–81.

Neuronal Competition for Action Potential Initiation Sites in a Circuit Controlling Simple Learning

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Abstract

The spatial and temporal patterns of action potential initiations were studied in a behaving leech preparation to determine the basis of increased firing that accompanies sensitization, a form of non-associative learning requiring the S-interneurons. Little is known at the network level about mechanisms of behavioral sensitization. The S-interneurons, one in each ganglion and linked by electrical synapses with both neighbors to form a chain, are interposed between sensory and motor neurons. In sensitized preparations the strength of shortening is related to S-cell firing, which itself is the result of impulses initiating in several S-cells. Because the S-cells, as independent initiation sites, all contribute to activity in the chain, it was hypothesized that during sensitization, increased multi-site activity increased the chain's firing rate. However, it was found that during sensitization, the single site with the largest initiation rate, the S-cell in the stimulated segment, suppressed initiations in adjacent ganglia. Experiments showed this was both because (1) it received the earliest, greatest input and (2) the delayed synaptic input to the adjacent S-cells coincided with the action potential refractory period. A compartmental model of the S-cell and its inputs showed that a simple, intrinsic mechanism of inexcitability after each action potential may account for suppression of impulse initiations. Thus, a non-synaptic competition between neurons alters synaptic integration in the chain. In one mode, inputs to different sites sum independently, whereas in another, synaptic input to a single site precisely specifies the overall pattern of activity.

Keywords

interneuron; integration; sensitization; reflex; sensory cells; leech

Introduction

The neuronal changes underlying learning can occur at multiple loci even for simple forms of learning (Hochner et al., 1986; Carew and Sahley, 1986; White et al., 1993; Walters and Cohen, 1997; Cohen et al., 1997; Prescott, 1998). Such changes are usually at the level of individual synapses, both pre- and postsynaptic (Nicoll and Malenka, 1995; Roberts and Glanzman, 2003), although the intrinsic excitability of neurons may also be altered (Farley et al.,

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1983;Cleary et al., 1998;Gainutdinov et al., 1998;Burrell et al., 2001;Antonov et al., 2001;Daoudal and Debanne, 2003;Zhang and Linden, 2003;Zhang et al., 2004). Remarkably, in the leech the ablation or axotomy of a single neuron, the S-cell, in just one ganglion eliminates a form of non-associative learning, sensitization of reflexive shortening (Sahley et al., 1994;Modney et al., 1997;Burrell et al., 2003).

In some animals much is known at the level of single synapses about mechanisms underlying behavioral sensitization, but in no system is the involvement of interactions at the network level well understood. The S-cell network of the medicinal leech is a chain of interneurons, one in each ganglion, which spans the length of the animal's nerve cord (Frank et al., 1975). The S-cell in each ganglion connects with its neighbors anteriorly and posteriorly by axo-axonal electrical synapses (Muller and Carbonetto, 1979). These connections are strong and do not rectify, so that impulses rapidly and reliably propagate along the S-cell chain by way of the S-cell axon, the largest and the fastest conducting axon in the leech CNS (Gardner-Medwin et al., 1973). The S-cell network has multisegmental mechanosensory neuron inputs and motoneuron outputs (Bagnoli et al., 1975;Shaw and Kristan, Jr., 1995;Shaw and Kristan, Jr., 1999;Baccus et al., 2001;Arisi et al., 2001). S-cells fire during whole body shortening, a type of withdrawal reflex produced by cutaneous stimulation of the leech (Shaw and Kristan, Jr., 1995;Shaw and Kristan, Jr., 1999).

Sensitization of reflexive shortening is the enhanced response to a test stimulus following a strong, noxious stimulus as compared with the baseline without the strong stimulus. Although S-cell firing is not required for reflexive shortening, following a sensitizing stimulus S-cells are more active, and more reliably so, during shortening in response to the test stimulus (Modney et al., 1997;Burrell et al., 2003). Both the capacity for sensitization and the increase in S-cell activity involve 5-HT (Ehrlich et al., 1992), which increases S-cell excitability (Burrell et al., 2001).

A cutaneous stimulus causes impulses to initiate independently in multiple S-cells. Because each impulse propagates throughout the network, initiations contribute to the overall firing rate of the network (Baccus et al., 2001). But whether S cells initiate impulses at multiple sites during behavioral shortening has not been shown, nor is it known whether more initiation sites are recruited following sensitization, as has been hypothesized (Burrell et al., 2002). The experiments in this report were designed to test the hypothesis that during sensitization, S-cell firing increases in part because there are more impulse initiations along the network beyond the ganglion associated with the stimulus.

Materials and Methods

Animals and electrophysiological recordings

Leeches (*Hirudo medicinalis*, ~3 g) were obtained from a supplier (LeechesUSA, Westbury, NY), dissected and studied as described (Modney et al., 1997;Burrell et al., 2001), except that extracellular recordings were made with cuff electrodes in addition to suction electrodes. Cuff electrodes recorded S-cell action potentials extracellularly through the blood sinus within the body using two thin, Teflon-coated silver wires (75 μ m uncoated diameter; AM Systems, Carlsborg, WA) bared at the ends, which were ~ 300 μ m apart, coiled halfway around a minuten pin, and embedded in Sylgard 184 rubber (Dow Corning, Midland, MI) by curing. A sharp blade was used to carve a block of the rubber (<1 mm in diameter) containing the electrodes and the pin was removed, leaving a channel for the blood sinus including the nerve. Extracellular signals were amplified using a custom-made AC-differential amplifier with track and hold circuitry that attenuated large stimulus artifacts as necessary. High-pass filtering of the cuff electrode signal removed a slow artifact associated with muscular contractions which would have interfered with measurement of spike timing. The single S-cell soma in each

ganglion was identified by its $\sim 20 \mu\text{m}$ size, location in the central glial packet, and fast, overshooting action potential of low threshold.

Behavioral experiments

The semi-intact preparation (Fig. 1) consisted of the head and first 11 body segments of the leech, with the skin and body wall of segments 8 and 9 (s8 and s9) removed to expose connectives between ganglia for *en passant* suction electrode recording. Test stimuli to evoke whole body shortening were given on segment 5 (s5) and sensitizing shocks were given at a distance from it on segment 11 or 12 (s11 or s12) using published parameters and electrodes (Modney et al., 1997; Burrell et al., 2001; Cruz, 2006).

For each preparation, 1 hr after surgery the behavioral threshold for the skin stimulus was determined for the test and for the sensitizing electrode pairs. At threshold the stimulus reliably evoked both shortening and S-cell impulses with a typical S-cell spike count of 5-6 action potentials, as reported previously. The stimulus was a single, capacity-coupled 3 ms pulse of 4 to 7 V, determined by gradually increasing stimulus strength. The test stimulus was set to 1 V higher and the sensitizing stimulus to 3 V higher than the threshold. Adjustment of the test stimulus in this way has been shown to mimic a touch stimulus applied to the skin, which preferentially activates touch (T) and pressure (P) mechanosensory neurons (Sahley et al., 1994). Behavioral training was started ~ 45 min after determining thresholds.

Test stimuli were given every 2 minutes, determined previously to be sufficient to prevent rapid habituation of the animal. Tension was checked to ensure that it stabilized prior to the next stimulation. The animal was also checked visually during the experiment to make sure that it remained behaviorally stable. The sensitizing shocks, delivered as a pair of trains, each consisting of ten 3 ms pulses at 10 Hz with a 2 min interstimulus interval (ISI), also activate the nociceptive (N) and Retzius (R) cells (Sahley et al., 1994). Sensitizing shocks began 2 minutes after the baseline shocks, followed 2 minutes later by the first of 20 test shocks given at 2-min intervals for a total of 40 min. Control leeches (nonsensitized, habituation group), generally chosen at random, were treated the same but no current was delivered through the sensitizing electrodes after the baseline shocks. The preparation was essentially an intact anterior portion of leech capable of behaviors other than shortening at any time during the experiment. Consequently, only the 25 of 36 preparations for which stable recordings of behavior and S-cell activity were obtained throughout training were analyzed. Normalized shortening responses were grouped into blocks of 4 trials and averaged.

Analysis of S-cell activity

The pattern of S-cell network activity was analyzed by (1) locating the sites of impulse initiation to particular S-cells along the chain of ganglia and (2) determining the timing of each initiation with respect to the stimulus. The published method (Baccus et al., 2001) was modified for the behaving preparation. In brief, impulses initiated in any cell of the multisegmental S-cell chain propagated rapidly and reliably to the anterior and posterior electrodes (Fig. 1). The difference in the arrival time of each impulse at the electrodes, plotted as an arrival time difference histogram (binwidth = 0.2 ms), clustered according to the site (i.e. ganglion) of initiation. The arrival time differences were converted to the relative distances between ganglia, which was measured to be ~ 5 mm in midbody segments. That the peaks corresponded to particular segments was confirmed at the end of experiments by (1) stimulating the preparation with a mechanical probe at each segment and (2) dissecting the preparation and stimulating individual S-cells intracellularly. Both methods yielded histograms with peaks that aligned with the data (See Sup. Fig. 2). The S-cell impulses were the largest recorded, and their timing at their peaks was measured and matched (spike sorting) with Axoscope (Axoscope 9; Molecular Devices Corp.). Rarely, an impulse was recorded in one electrode but not in the other, which may

represent action potentials that failed or that reflected (Baccus et al., 2001). Spikes were sorted into individual clusters of time differences (initiation sites), which were set to be at least 2.5 ms from one another (site sorting). Such clusters in the histogram were separate, usually ~5 ms apart. Some impulses occurred between clusters, but most such impulses (70%) initiated more than 200 ms after the stimulus. Their precise origin was not determined, and excluding them from analysis did not alter the overall pattern observed during the first 300 ms. The time of impulse initiation (t_{init}) was calculated as described (Baccus et al., 2001; Cruz, 2006).

Shortening began at ~200 ms and S-cell activity occurred primarily in the first 200 ms following the test stimulus. S-cell firing was sparse at the peak of the tension change, beyond 300 ms, and may represent activation beyond the initial skin stimulus. Nevertheless, activity was analyzed during the first 500 ms, with particular focus on the early part of the train.

Hyperpolarization experiments

A preparation consisting of 5 to 7 exposed ganglia with skin attached by the segmental nerves (roots) on one side and a pair of silver wires implanted in skin in the middle segment was used to study the interactions of S-cells (Fig. 5). Test stimuli were given to the segment with an S-cell impaled for recording and hyperpolarization, while S-cell activity was recorded and initiations located with suction electrodes on opposite ends of the nerve cord. In general, in the more dissected preparation the magnitude of the skin stimulus was at least 3 V higher than values used in the behavior experiments to evoke a similar number of S-cell spikes. This was because fewer sensory cells were activated, and because in 5 experiments the saline had $[Ca^{2+}]$ elevated to 8 mM to improve the stability of the microelectrode recording, which also reduced neuronal excitability. Results using elevated calcium did not differ significantly from those using normal calcium saline, and the 2 sets of data were combined in the analysis (total $n=10$). Importantly, the pattern of initiation sites did not differ markedly between the reduced and behaving preparations.

Hyperpolarizing current (0.5 – 1 nA, 500 ms duration) was passed through the recording microelectrode to reduce initiations in the impaled S-cell in response to a fixed strength test stimulus at 2 min intervals, while S-cell activity and initiation sites were measured with suction electrodes. The timing and duration of the pulse ensured that it reduced initiations without spreading to adjacent S-cells (Muller and Carbonetto, 1979). Controls without current injection were inserted in equal numbers intermittently to minimize systematic error. The injected current did not measurably affect propagation of impulses along the chain; through-conduction and conduction velocity along the chain did not change with this manipulation.

Refractory period and computational model

S-cell refractory period following action potentials was determined using an isolated nerve cord of 4 ganglia, with one S-cell in the chain impaled with a microelectrode, one suction electrode used to stimulate the S-cell axon with a 0.5 ms capacity-coupled pulse just above threshold (Fig. 8A). Each impulse elicited in the connectives was succeeded by a depolarizing 0.5 ms pulse through the intracellular microelectrode, and the current required to reach a level just above threshold was recorded. The interval between the 2 impulses, measured from the peak of one action potential to the next in the soma, was varied in a pseudo-random fashion to avoid systematic error arising from possible hysteresis.

The experiment was simulated by creating a 2-neuron S-cell model using NEURON (Hines and Carnevale, 2001). The model was created in order to study the phenomenon of suppression of impulse initiations in the S-cell chain and in particular to test the hypothesis that such suppression may be accounted for by the S-cell membrane's refractory period. Importantly, the model was generated using electrical measurements of the S-cell action potential waveform

and previously measured geometry (Baccus et al., 2001) in conjunction with the measured conduction velocity. The action potential waveform was modeled using the Hodgkin-Huxley (H-H) formalism of squid axon excitability not only for convenience but because this model has been well established and tested (Hodgkin and Huxley, 1952; Meunier and Segev, 2002). The goal was not to reproduce precisely all of the ionic conductances present in the S-cell membrane, for which no information was available, but rather to match the waveform of the action potential including the afterhyperpolarization. Thus, only the simplest possible mechanisms were modified, namely the fast sodium conductance and the delayed potassium conductance, which underlie the action potential of many invertebrate axons.

Each S-cell consisted of a soma and an axon with the soma connected midway along the axon (Fig. 9A). To simulate the presence of the strong, nonrectifying electrical synapses between the cells, their axons were connected directly without interruption. It was assumed in this model that the gap junctions had no appreciable effect on the excitability of the S-cell, although under some circumstances they might affect the pattern of impulses in the network (Moss et al., 2005). The soma was a single compartment (isopotential) and the axon had multiple 2 μm -long compartments. The integration time step (dt) was 25 μs . The compartment size and dt were small to reduce computational error, especially at the unstable threshold. The model's geometry and parameters, many obtained from previously determined experimental and computational data (Baccus et al., 2001), are listed in Table 1a. The integration method used was backward Euler.

The standard Hodgkin-Huxley (H-H) model (Hodgkin and Huxley, 1952) was modified to emulate the action potential of the S-cell, particularly its rising phase and afterhyperpolarization, rather than resting potential and peak. Because the S-cell impulse was briefer than the squid axon impulse modeled at 6.3°C, the S-cell model was run at a higher “celsius,” which multiplied the time constants for n , m , and h by a constant. To avoid oscillations of the membrane potential following the action potential, the parameters of the H-H equations were further modified both to reproduce the duration of the S-cell's refractory period and to eliminate oscillations (Rinzel, 1990). Table 1b shows constants used in the rate equations. Small changes were made to the equations for rate constants for Na^+ activation and inactivation and K^+ activation, resulting in slight shifts or changes in slope of the voltage dependence of each rate constant. The voltage-dependence of steady-state parameters describing the fast Na^+ conductance, namely m_∞ and h_∞ , were shifted by ~ 5 mV in the depolarized direction. For the delayed K^+ conductance, n_∞ was only very slightly changed. The time constants of Na^+ (τ_m) and K^+ activation (τ_n) were reduced slightly over the -80 to 0 mV range of membrane potential. Conversely, the time constant of Na^+ inactivation (τ_h) was increased slightly over the same range.

It has been reported that 5-HT changes the excitability of the S-cell without changing the input resistance of the S-cell, measured at the soma. A 5-HT-sensitive potassium leak conductance (Destexhe et al., 1996) was added in the axonal membrane 300 μm from each side of the soma to simulate the effect of 5-HT. This type of conductance was first described in *Aplysia* sensory neurons and later modeled (Klein et al., 1982; Baxter et al., 1999). In our model, $g_{\text{k-leak}}$ was reduced from a g_{max} of 15 mS/cm^2 to 1 mS/cm^2 with 5-HT, which lowered the threshold of the S-cell (Table 1b). The experimental excitability profile of the S-cell was reproduced using current injections of similar duration as in the experiment (0.5 ms) at one end of the axon and in one S-cell soma. A second set of computational runs was made with the added $g_{\text{k-leak}}$ and using synaptic input instead of current injection. Excitatory input from a P-cell was simulated by adding synaptic processes at discrete points on the 2-cell geometry, ~ 500 - μm from the soma of each S-cell to emulate the region of synaptic input in the S-cell (Fig. 9A). The excitability profile was similar for current injection and synaptic conductances with the range of parameters used. Direct monosynaptic input to the S-cell was calculated as in the previous

model of the S-cell network using an alpha function (Baccus et al., 2001). Values of parameters used in the present model were: $G_{peak} = 0.15 - 0.40 \mu S$ and $t_{syn} = 0.1$ ms, where G_{peak} is the peak synaptic conductance and t_{syn} is the time until peak. The peak conductance of the synapse onto one S-cell (soma1 in Fig. 9A) was twice as large as for the other S-cell (soma2), to emulate the difference in synaptic strength between sensory cells and S-cells in adjacent segments and sensory cells and S-cells in the same segment (Baccus et al., 2000). Specifically, the threshold (G_{peak}) determined and set for soma2 (the “adjacent S-cell”) was one-half the value for soma1 (the “central S-cell”). Propagation of an impulse centrally along a P-cell axon between segments was determined experimentally for the P-to-Anterior Pagoda (AP) synapse to be ~ 14 ms, which was used to set the onset of synaptic input on soma2 in the model. The P-cell excites the AP neuron in a similar manner with the S-cell. The selected parameters reproduced the S-cell conduction velocity (1.2 m/s) and conduction time between ganglia (5 ms).

Statistical analysis

Data were analyzed statistically using STATISTICA (StatSoft, Tulsa, OK). Plotting was done with SigmaPlot (SPSS, Chicago, IL) and Igor Pro (WaveMetrics, Lake Oswego, OR). Behavioral and S-cell spike count data were normalized to initial levels and averaged across trial blocks. Repeated measures ANOVA was used to examine the effect of the treatment groups (sensitization vs. nonsensitization groups), time (trial blocks) and treatment group-time interaction effect. Post-hoc analyses were done to determine within-group differences. Linear regression was performed to assess the relationship between S-cell firing and shortening strength. The Pearson Product Moment Correlation so obtained was compared between the sensitized and nonsensitized groups. The nonparametric Wilcoxon Matched Pairs Test was used to analyze the number of initiations per initiation site before and after sensitization in the behaving preparation, because the data from individual S-cells were not normally distributed. A similar analysis was done in a previous report for S-cell initiations in a reduced preparation (Burrell et al., 2002). The t-test for dependent samples was used to compare the effect of hyperpolarization on impulse initiations in adjacent segments. The p value for statistical significance was set to less than 0.05. Error bars represent the Standard Error of the Mean (SEM). Values of n indicate number of preparations.

Results

Spatial pattern of S-cell impulse initiations in the behaving leech

A semi-intact behaving preparation similar to that described previously (Sahley et al., 1994) was used to determine the effect of sensitization on the spatial and temporal pattern of action potential initiations in the S-cell network during whole body shortening. Two extracellular electrodes were required to be able to identify sites and timing of impulse initiation along the nerve cord between the electrodes, which was possible in part because impulses arising in separate ganglia rarely collide (Baccus et al., 2001). Therefore an important addition to the behaving preparation was a bipolar cuff electrode applied to the blood sinus containing the connectives between ganglia 2 and 3 (g2 and g3) to be able to record impulses propagating anteriorly along the S-cell network, in addition to a suction electrode that recorded impulses *en passant* between ganglia 9 and 10 (Fig. 1). Each S-cell receives monosynaptic mechanosensory input from pressure (P) and touch (T) mechanosensory neurons whose cell bodies are in 3 ganglia. Since each sensory neuron extends fine axons out to the periphery through neighboring ganglia, each S cell receives sensory input from 5 segments ((Muller and Scott, 1981; Baccus et al., 2000), with input from more distant segments delayed because of distance and smaller diameter of the sensory fibers projecting through adjacent ganglia (Baccus et al., 2001). This means that skin stimulation at a single segment is expect to excite more distant S-cells later.

Behaving preparation

Shortening and S-cell activity in response to test stimuli were measured for 18 animals that received sensitizing shocks and compared with 7 animals that were not sensitized (Fig. 2A). Consecutive single trials were grouped into blocks of 4 trials, represented by filled (sensitization group) and open (non-sensitization group) circles. Each trial was normalized to the average of 3 trials obtained initially, the baseline level, which was designated 100%, marked as “b” on the x-axis and indicated by the dotted line in Fig. 2A and B. Baseline tension measurements between animals in the sensitization and nonsensitization (control) groups did not differ significantly (Sensitized: 3.2 ± 0.9 grams; Control: 3.9 ± 1.4 grams; unpaired t-test, $p=0.634$). However, responses to the test stimulus were significantly greater for leeches in the sensitization group as compared to leeches in the control group, 100% versus 40% of baseline values, respectively. Repeated measures ANOVA revealed a significant group effect [$F(1, 23) = 8.8$, $p<0.01$] and a significant group x block interaction [$F(4, 92) = 3.5$, $p<0.05$], which indicated that performance for leeches in the 2 groups differed across trial blocks. The Newman-Keuls post hoc analyses showed that trial block 1 in the sensitization group was significantly elevated as compared to all of the other trial blocks ($p<0.05$), with an appreciable decrement in responding by trial block 2. However, after the first trial block in the sensitization group, shortening was maintained at a level higher than corresponding trial blocks in the nonsensitized group.

Shortening responses in the nonsensitized group did not show a significant decline across trial blocks, as the responses reached a fairly low level by trial block 1. A similar pattern was seen when the trial blocks were binned in groups of 3 or 5 trials (data not shown). The decline in responding has generally been attributed to habituation, a mechanism also thought to arise in the central nervous system (CNS), independent of sensitization and separate from sensory adaptation and muscle fatigue. One test of habituation is that it reverses after delivery of sensitizing-type shocks at the end of habituation training (dishabituation). Therefore, in some experiments a dishabituating stimulus was given at the end of 20 trials. The responses in these animals returned to near baseline levels as determined by comparing the mean normalized responses in the last trial block (B5) with responses following delivery of dishabituating shocks. Just before dishabituation, the mean response in 9 animals was $40.3 \pm 4.4\%$; after the dishabituating stimuli the mean response rose back to near baseline at $87.3 \pm 21.9\%$. These responses were significantly different (paired t-test; $p<0.05$) and showed that dishabituation was independent of sensory adaptation or muscle fatigue and that the decline in responding was primarily the effect of habituation. In addition, in 4 preparations in which only 2 post-baseline test stimuli were given, at 30 and 45 min, no decrement in responding was observed. The mean response at 30 minutes post-baseline was $85.7 \pm 9.4\%$ and at 45 minutes it was $88.4 \pm 14.3\%$ while the response on average in the last trial block (B5) for animals in the habituation (nonsensitized) group was $30.8 \pm 4.8\%$ (unpaired t-test showed significant difference between each of the 2 times points against B5; $p<0.001$). Thus, the preparation did not deteriorate. A similar pattern of responding was obtained in previous reports (Burrell et al., 2001; Burrell et al., 2003).

S-cell firing was recorded during shortening and displayed as mean normalized spike counts across the same trial blocks in the sensitized and non-sensitized groups (Fig. 2B). Each spike count was normalized to the average of 3 baseline counts, which was designated as 1.0. S-cell firing in the sensitized animals increased across trial blocks, as revealed by the ANOVA (Repeated measures; group effect, $F(1, 23) = 13.8$, $p<0.01$; block effect, $F(4, 92) = 15.4$, $p<0.01$; the group x block interaction did not reach significance). To avoid the effect of habituation, the 2 groups were analyzed during the early part of the training protocol (Baseline, B1 and B2), which showed a significant group x block interaction (Repeated measures ANOVA, $F(2, 46)=8.6$, $p<0.001$). Thus, with focus on early trial blocks, S-cell firing for the

sensitized and nonsensitized animals differed, clearly showing an effect of sensitization. The Pearson Product Moment Correlation ($r=0.3$) revealed that the number of S-cell spikes rose in direct proportion to the increase in strength of shortening in the sensitization group. A two-sided t-test showed that the relationship between number of spikes and the strength of shortening was statistically significant ($p<0.0001$). No such relationship was found in the nonsensitization group. Moreover, the r^2 values for both groups were compared and showed a statistically significant difference ($z=5.15$, $p<0.0001$). These data were consistent with the findings of Sahley et al. (1994), which showed a positive correlation between the number of S-cell spikes and the strength of shortening for individual trials only in sensitized animals. Thus, impulse initiation sites were analyzed for the first trial block and compared with the baseline response as described in the next part of this section.

Sensitization and the location of impulse initiations

To determine the spatial and temporal pattern of S-cell initiations along the chain during sensitization, S-cell activity was analyzed only during the first trial block of the sensitization group, to avoid the effect of habituation, and corresponding initiation sites determined as described in the Methods. The timing of initiation of each impulse was calculated with respect to the onset of the test stimulus. The results for one animal presented in Fig. 3 show the cumulative S-cell responses over 3 baseline trials and over 3 trials immediately following delivery of the sensitizing shocks. The histograms in A and B compare the initiation sites between baseline and post-sensitization trials. The numerical values of the arrival time differences of impulses, clustered into peaks, were labelled c, a1, a2, p1, p2 for the ganglion associated with the central segment receiving the stimulus and for the successive anterior and posterior ganglia, respectively. The PST histograms in Fig 3C-D show the total network activity; the underlying initiation times in the separate S-cells, expressed as rates, are shown in Sup. Fig 3. Mean firing rate for each 20 ms time bin after the test stimulus was calculated as the number of spikes in 3 trials divided by the number of trials and by the binwidth. Initiations in adjacent S-cells were delayed with respect to those in the central S-cell, as also previously observed (Baccus et al., 2001). The delays were consistent with the structure of the sensory-to-S-cell input (Sup. Fig.1). Sensory axons conducting impulses from skin of the central segment elicit synaptic potentials first in the central ganglion and, once sensory impulses propagate to the next ganglia, elicit impulses in those ganglia later. In particular, the conduction delay between ganglia for P-cells is greater than 10 ms (Wallace et al., 1977; Elliott and Muller, 1983). An S-cell action potential arising in response to P-cell input to the central ganglion will in the same 10 msec propagate at least 2 ganglia farther, with a delay between ganglia of less than 5 ms (Muller and Carbonetto, 1979). The delays do not entirely explain the particular distribution that was measured, as will be considered below.

For this and other experiments, initiations in adjacent S-cells did not overlap in time, similar to previous reports (Baccus et al., 2001). Moreover, Baccus et al. (2001) showed that simultaneous initiations in adjacent S-cells rarely occur, and as a consequence, S-cell impulses rarely collide.

Of all 18 preparations studied, the number of initiations in the central S-cell following sensitization increased in 8, remained the same in 6, and decreased in 4 when measured during the first 150 ms post-stimulus, the time in which most if not all of the S-cell spikes initiated in the central ganglion. The pattern of S-cell network firing typically varied from one trial to the next. For example, it was common to observe initiations in S-cells anterior to the central S-cell but not posterior to it in one trial, but in the next trial the opposite was observed. Because of this trial-by-trial variability, the overall pattern of S-cell initiations on average did not differ between baseline and sensitization trials, and between experimental and control animals, as shown in Fig. 4 and described below.

Work with reduced preparations suggested that treatment with serotonin, which mimics the effects of sensitization (Burrell et al., 2001), was accompanied by an increase in the number of S-cell initiation sites (Burrell et al., 2002). Figure 4A shows a comparison of the number of initiation sites before and after sensitizing stimuli for all 18 sensitized preparations. For each preparation the numbers of initiations in individual S-cells during 3 baseline trials were summed, as were the numbers during 3 consecutive post-sensitization trials (trial block 1), and these were used for the overall averages. The nonparametric Wilcoxon Matched Pairs Test showed that the number of initiations increased significantly only in the central S-cell ($p < 0.05$, $n = 18$). Initiations in the S-cell immediately adjacent and posterior to the central S-cell (p1) also appeared to rise, but the increase was not statistically significant. Fig. 5B shows the total number of initiation sites in each trial for the sensitized and for the nonsensitized preparations. To compare these data with those in A, the counts were averaged across blocks of 3 consecutive trials each, beginning with the baseline. Repeated measures ANOVA revealed no significant difference in the number of initiation sites between groups and between trial blocks in each group (sensitized, $n = 18$; non-sensitized, $n = 7$).

Together, the data confirm that in the sensitized preparations S-cell activity was generally enhanced in response to test stimuli, but the increase could not be attributed to a significant increase in impulse initiation sites along the S-cell chain as hypothesized. The S-cell in the central, stimulated segment fired first and most; typically its initiation rate increased during the early part of the train. Such an effect could in part be the result of interaction between S-cells in the chain.

Suppression of impulse initiations in neighboring S-cells

Because the spread of impulse initiations along the S-cell network following sensitization was more limited than expected, we evaluated mechanisms by which action potential initiations in S-cells neighboring the central S-cell might be suppressed. For example, the initial burst of impulses from the central S-cell could render neighboring S-cells refractory to impulse initiations if action potential initiations in the central S-cell suppressed initiations in adjacent S-cells. To test this hypothesis, a reduced preparation was used, similar to that of Baccus et al. (2001), consisting of a chain of 5 ganglia with skin connected on one side by the roots for sensory stimulation (Fig. 5A). With the S-cell in the central ganglion impaled and test shocks given on the skin in the central segment, impulse initiations were recorded from suction electrodes at both ends of the nerve cord while hyperpolarizing current was injected into the S-cell soma to reduce impulse initiations there. The idea was that if impulse initiations in the central S-cell suppressed initiations in adjacent S-cells, then reducing initiations in the central S-cell would augment initiations in S-cells in adjacent ganglia.

In 10 preparations the sites of S-cell initiations were determined when the central S-cell was hyperpolarized and an equal number when it was not, for each preparation (Fig. 5). (The example in Sup. Fig. 4A,B compared 9 trials obtained under each condition.) When the central S-cell was hyperpolarized, impulse initiations in an adjacent S-cell were increased while those in the central S-cell were reduced (Sup. Fig. 4). Initiations in adjacent S-cells occurred in the early part of the train, during the first 200 ms following the test stimulus, with few initiations arising late in the train. The increase in initiations in an adjacent S-cell was not due to an anode break response when the hyperpolarizing pulse was shut off; most of the spikes arose early in the train during a 500-ms current pulse. In 6 of 10 experiments, a previously inactive site initiated an impulse (for example, p1 in Sup. Fig. 4B).

The current pulses injected into the S-cell in these experiments produced a hyperpolarization that presumably spread to each adjacent S-cell attenuated to $\sim 10\%$ of its initial value, as previously reported (Muller and Scott, 1981). This would oppose any enhancement of initiations brought about by suppressing initiations in the central S-cell. The degree to which

the hyperpolarizing current raised the threshold for action potentials in adjacent S-cells was not assessed. It was also possible that the injected current passed into the sensory neurons themselves, although this was unlikely and would have been limited to the central ganglion (Muller and Scott, 1981). In any case, impulse initiations should decrease rather than increase with the spread of hyperpolarizing current to S-cells ~5mm away in adjacent ganglia, resulting in an underestimate of the effect of suppression.

To determine the overall effect of reducing impulse initiations in the central S-cell on initiations in adjacent S-cells, the numbers of initiations in adjacent S-cells in each of 10 experiments were summed (Fig 5B). Because of the cable properties of the S-cell membrane, the hyperpolarization in the adjacent S-cell as a result of current spread from its neighbor is delayed by about 150 ms (Muller and Carbonetto, 1979). Therefore, to avoid the inhibitory effect of the hyperpolarizing pulse, only impulses within the first 150 ms were counted. In addition, only S-cells immediately adjacent to the central S-cell, namely a1 and p1, were considered in the analysis. Impulses initiated in these S-cells, considered as “adjacent ganglia,” were combined because during a single trial block, test stimuli could elicit impulses in either ganglion, as mentioned in the previous section. In all experiments, the hyperpolarizing current significantly reduced initiations in the central S-cell (t-test for dependent samples; $p < 0.001$). This reduction was associated with a significant increase in the number of initiations in adjacent ganglia (Fig. 5B; two-tailed t-test for dependent samples; $p < 0.05$, $n = 10$). These results support the hypothesis that initiations in the central S-cell suppress initiations in neighboring S-cells.

S-cell impulse refractory period and suppression of action potential initiations

To further assess this activity-dependent inhibitory effect of one S-cell upon its neighbors, the shortest interval between initiation of an impulse in the central S-cell and the initiation of the next impulse in an adjacent S-cell was determined (Fig. 6, inset). In this analysis, the interval between arrival of spikes originating from the central S-cell and arising in either the immediate anterior or posterior adjacent S-cells was measured. A total of 21 trials from 5 preparations used in the preceding experiment were pooled. The trials were from control conditions (S-cell not hyperpolarized) and were selected because there were initiations in adjacent S-cells within the train. Measurements were made up to 300 ms following the stimulus. A total of 31 interspike intervals (ISI) in 5 ms time bins was measured (Fig. 6). The greatest number of ISIs was in the range of 10-30 ms. No impulses were initiated in an adjacent S-cell within less than 10 ms after the arrival of an impulse initiated in the central S-cell. This is a measure of refractory period and is comparable to a similar distribution and refractory period measured in the central S-cell itself (Sup. Fig. 5), which had a shorter minimum interval (8.5 ms), presumably because of stronger synaptic input, but similar peak.

It is apparent from the data in the preceding section that after a cutaneous stimulus, impulse initiations in an S-cell do not depend only on excitatory input from sensory neurons, but also on an inhibitory influence associated with the firing of other S-cells. S-cells neighboring a stimulated segment receive approximately one-half the excitatory sensory synaptic input of that to the central S-cell (Baccus et al., 2000). The mechanism of suppression may therefore be effective in keeping the neighboring S-cells below threshold for initiating an action potential.

One mechanism by which the action potential in one S-cell might suppress that in another would be by synaptic inhibition. But inhibitory postsynaptic potentials (i.p.s.p.s) are not ordinarily observed from recordings in the soma or in axons of the S-cell. If such an influence exists it would have to be indirect by way of inhibition of the sensory neurons that provide excitatory input onto the S-cell. Another, simple mechanism for suppression could be a combined consequence of the refractory period of the S-cell impulse when it reaches the adjacent S-cells and the delayed synaptic input from sensory axons projecting between ganglia. In this case the interaction between S-cells would not be synaptic, but would be a consequence

of biophysical properties and network geometry. A step in evaluating this possibility is to model and measure the relative refractory period of impulses in the S-cell.

S-cell refractory period: Model and Experiment

To test the possibility that the suppression of impulse initiations was a consequence of the S-cell's refractory period, i.e. its membrane's intrinsic inexcitability following an action potential, the refractory period of the S-cell was modeled as described in Methods and determined experimentally. It has been difficult to obtain whole-cell patch clamp recordings of the S-cell, which has not been successfully isolated in culture medium, as other cells in the leech have (Nicholls, 1987). Nevertheless, the S-cell membrane has been assumed to contain sodium and potassium channels responsible for generating an action potential typical of other invertebrate neurons, with properties that were similar to that of the well-characterized and modeled squid giant axon (Baccus et al., 2001).

A qualitative description of the process at the macroscopic level was used. For the model certain parameters of the standard HH model were adjusted to reproduce the experimentally measured S-cell action potential waveform (range of resting potentials, duration, and non-oscillating afterhyperpolarization) and S-cell geometry together with conduction velocity. In short, the rate constants of the standard H-H model that described the activation and inactivation parameters of the fast, inactivating sodium channels and the slow, non-inactivating potassium channels were modified (see Table 1 for list of parameters and constants).

In Fig. 7, the modified H-H model (gray traces) was compared with the standard H-H model (black traces) in terms of the action potential waveform (A) and the underlying kinetics of the h (B) and g_k (C) variables. There was little change in the shape of the action potential (A), except for a small increase in the slope of the upstroke, which was appropriate for the fast action potential of the S-cell, a representative example of which is shown in red. Although the values of resting potential and peak are different between the recording and the model, the values in the model are within the range of observed recordings (see below); it was the rising phase and the afterpotential that were modeled. Another minor difference between the modified and standard H-H models was observed during repolarization when the membrane potential approached a slightly less negative equilibrium potential, which was closer in appearance to the experimentally recorded S-cell impulse waveform, shown in red in the inset. The S-cell action potential half-width and duration of the afterhyperpolarization were 1.2-ms and 10-ms, respectively; for the model, it was 1.2-msec and 11-msec, respectively. The duration of the afterhyperpolarization was measured from the point at which the membrane potential reached its most negative level to the point at which it first returned to the resting level. The resting potential of the model, at -63 mV, was within the -50 mV to -65 mV range of resting potentials measured from the S-cell.

Changes in h and g_k influence the events occurring at the end of the impulse as the membrane potential returns to resting levels. The inset graphs in Fig. 7 are magnified views of the respective waveforms starting at the 12 ms time point to highlight these relevant changes. The overall effects of the modifications are the elimination of the oscillatory behavior and the more gradual return of the membrane potential to resting level (Fig. 7A, inset). These effects are produced primarily by adjusting the h and g_k variables. The h variable of the H-H model is responsible for the inactivation of the fast sodium channel. Depolarization during the impulse quickly reduces the fraction of sodium channels available for activation, which then slowly recover upon repolarization (Fig. 7B). The modified H-H model shows slower but steadier recovery from inactivation at the end of the impulse (Fig. 7B, inset). The kinetics of g_k , the conductance of the potassium channel responsible for repolarization, change actively during the return of the membrane potential to resting level (Fig. 7C), but without oscillation (Fig. 7C, inset). This lingering effect of increased g_k enhances the recovery of sodium channels from

inactivation, but by slowing the return of the membrane potential to resting level, it makes the membrane less excitable for a period of time.

The S-cell's refractory period for firing an impulse was determined experimentally as shown in Fig. 8. The first action potential, the "conditioning" impulse generated by the suction electrode, was recorded in the soma of the S-cell and was followed by an injection of depolarizing current (0.5 ms pulse) into the soma just above threshold for evoking a single action potential (Fig. 8B), as described in Methods. The shorter the interval between the 2 impulses, the greater the amount of injected current required to evoke the second action potential.

The amount of injected current for each interval between impulses was plotted to show an excitability profile for the S-cell (Fig. 8C, filled circles). The amount of current was normalized to the baseline level, which was the average of points at long inter-impulse intervals (~18-25 ms). The experimental data points were approximated by a single exponential function (nonlinear regression fit, $R = 0.98$). The S-cell membrane appeared to be relatively refractory to a second impulse at intervals of ~12-ms and shorter, until ~8-ms at which it approached its absolute refractory period. The experiment was simulated with the compartmental model of the S-cell and the points obtained (Fig. 8C, open circles) showed excellent correspondence with the experimental results.

Modeled effects of delayed sensory input and serotonin on S-cell initiations

A compartmental model (Fig. 9A) of the S-cell was devised consisting of 2 neurons whose axons were connected without interruption to simulate the strong, nonrectifying electrical synapse between S-cells and sensory input from a P-cell with both early (soma1) and delayed (soma2) synaptic input onto the two S-cells. The simulation emulated the experiment by injecting depolarizing current with the same pulse duration (0.5 ms) and by determining the threshold for initiating an impulse at each interval, producing an excitability profile that closely resembled the experimental profile (Fig. 8C). It was predicted that initiation of a second impulse in an adjacent S-cell in response to the delayed synaptic input would fail when that input arrived when the S-cell membrane was relatively refractory. In the example given, it was at ~9 msec, or the difference between the arrival time of the delayed synaptic input from a sensory neuron to soma2 due to impulse propagation delay (~14 msec) and the arrival time of the first impulse from soma1 to soma2 (~5 msec). The simulation (Figure 9B) confirmed that, in the context of the S-cell network's geometry and a basal threshold level, a second impulse failed if evoked in response to an input at ~9 msec or earlier following the first impulse. In this simulation, synaptic input onto each S-cell was used instead of injected current to mimic the physiological source of excitation and its location, i.e. the synapses were placed at a distance from the soma (see Methods). In addition, the relative strength of P-to-S-cell excitatory input between central and adjacent ganglia was emulated; synaptic input to soma2 was one-half that to soma1. The predictions of the model were consistent with the observation that a ~10 ms delay was required for an impulse successfully to initiate in adjacent S-cells if it followed an impulse arising from the central S-cell (Fig. 6). Thus, the model, which was based on the action potential waveform of the S-cell including its after-hyperpolarization, has demonstrated the feasibility of the S-cell membrane's refractory period as a mechanism that underlies the suppression of impulse initiations observed experimentally.

Serotonin (5-HT) was found to be necessary for the expression of sensitization of the shortening reflex in the leech and to increase the excitability of the S-cell (Ehrlich et al., 1992; Burrell et al., 2001; Burrell et al., 2002). Our present results showed a significant increase in the number of initiations in the central ganglion but not in any other ganglion. The present model was extended to include the influence of 5-HT on the pattern of initiations shaped by the effect of the S-cell's refractory period. The precise membrane mechanism of 5-HT in the S-cell was not

known; however, a mechanism in *Aplysia* sensory neurons had been well studied. Thus, the effect of 5-HT was modeled after a potassium leak channel (k_{leak}) in *Aplysia* whose gating was sensitive to neuromodulators (Klein et al., 1982; Baxter et al., 1999). The channel was added onto the membrane $\sim 300 \mu\text{m}$ from the soma to simulate conductances in the region where the S-cell made synaptic connections (see Table 1). Synaptic input was also used in this simulation, with the same relative strength as above. Under baseline conditions, the channel had a high conductance ($g_{\text{max}} = 15 \text{ mS/cm}^2$) and the S-cell membrane excitability profile was similar to that in the first simulation (compare Fig. 10B and C, baseline mechanism). When the effect of 5-HT was simulated, the potassium leak conductance was reduced 15-fold to a g_{max} of 1 mS/cm^2 . For the neighboring S-cells, with an equal number of stimulations, slightly more impulses were initiated in the presence of the 5-HT mechanism, and the refractory period was shifted to shorter intervals, from 14 ms in the baseline to 13 ms in the 5-HT mechanism. Experimentally, the test stimuli produced several impulses in the S-cell in the central ganglion, so a given level of sensory input caused that S-cell to generate more and faster impulses in response to the same level of input; it thereby continued to dominate the firing of the network, since it received the earliest and strongest input. Thus, although serotonin evidently increases the excitability of many S-cells, the increased initiations in the central S-cell appear to suppress initiations in adjacent S-cells.

Discussion

Impulse initiations in the S-cell during sensitization

Sensitization increased the overall activity in the S-cell network in the behaving preparation, but unexpectedly the spatial pattern of initiations in the various S-cells did not change markedly. The total increase in S-cell activity during sensitization was achieved primarily by increased activity in the central S-cell, which suppressed firing of neighboring S-cells, which received delayed excitation.

In neurons and networks having multiple initiation sites, it is important to determine whether such sites of initiation are independent and under what conditions they interact. When sites are independent their initiations simply sum (Baccus et al., 2001). The contribution of each cell may be diluted so that it is only weakly correlated with the total output. But particularly in the sensitized state the S-cell chain does not operate in a mode of integration; rather, it functions in a mode in which a single location controls the activity at all sites. Thus, a single site generates a large signal relative to that of other sites and its activity correlates with the total firing of the network. It is not known whether the transition between these 2 modes of firing in the network relates simply to the level of network activity, to synaptic input, or to a combination of these.

Suppression of initiations occurs in the network of leech heart interneurons (HN cells) where separate initiation sites in a *single* neuron interact. Similarly to the S-cell network, initiation sites in the HN cells extend across segmental ganglia, albeit only in the anterior region (Calabrese, 1980). In this system, control of initiations by a dominant site coordinates the rhythmic activity of the leech heart, similarly to the function of the pacemaker in vertebrate heart. In crayfish mechanoreceptor interneurons, initiation sites in multiple ganglia receiving separate sensory input, produce impulses mostly in the stimulated ganglion with few impulses arising from the next ganglion (Calabrese and Kennedy, 1974). Integration of inputs occurs at separate locations, so postsynaptic inhibition can selectively affect specific points of excitation. Initiation sites that interact may be important for signal processing, as in dendrites of retinal ganglion cells sensitive to the direction of movement (Oesch et al., 2005). In snails, voltage sensitive dyes have revealed the presence of multiple initiation sites in single neurons (Zecevic, 1996).

Proposed mechanism for a form of neuronal competition or mutual inhibition

Suppression of spike initiations in S-interneurons by spikes originating in another ganglion resembles a form of lateral inhibition in which individual S-cells interact. This inhibition is not synaptic, but becomes evident with delayed or weaker synaptic input to S-cells in ganglia adjacent to that in which the impulses initiate. No inhibitory synaptic potentials have been reported in the S-cell, which itself uses glutamate as an excitatory transmitter (Crisp and Muller, 2006) and receives glutamatergic input from sensory cells (Baccus et al., 2000). The absolute and relative refractory periods obtained in our experiments, ~ 8 ms and ~ 12 ms respectively, agreed with values for the S-cell (7.3 ms and 15.4 ms, respectively) obtained independently using extracellular stimulation of a chain of ganglia (Bagnoli et al., 1972). Conduction delays are uniform along the S-cell chain from segment to segment, as are delays for sensory axons, so the effects of initiations in the central ganglion are similar in both neighboring ganglia.

Our model of S-cell refractoriness was based on relatively simple modifications of the Hodgkin-Huxley (H-H) model, primarily in the inactivation (h) variable of the fast sodium conductance and the activation (n) variable of the delayed potassium conductance (Hodgkin and Huxley, 1952). In the absence of voltage clamp data on S-cell membrane conductances, the S-cell action potential was modeled using the simplest possible mechanisms that involved basic sodium and potassium conductances of many invertebrate and vertebrate axons. Our approach has mimicked the effects of the membrane mechanism that underlies the refractory period for the S-cell action potential. Importantly, it has shown that S-cell membrane refractoriness is a plausible mechanism for suppression of initiations at neighboring sites in the network. Membrane mechanisms equivalent to alterations in the kinetics of the regenerative sodium and delayed potassium conductances may account for nonsynaptic competition between the S-cells.

Because 5-HT was known to mimic the effects of sensitization of the S-cell network, sensitization was modeled by addition of a 5-HT-sensitive potassium leak conductance. This conductance was discovered in *Aplysia* sensory neurons and has been implicated in the mechanism that enhances synaptic transmission after sensitization of the gill withdrawal reflex (Klein et al., 1982; Siegelbaum et al., 1982; Baxter et al., 1999). Consistent with the model, S-cell excitability is increased in the presence of 5-HT, although input resistance measured at the soma is not (Burrell et al., 2001), perhaps because conductances electrically distant from the S-cell soma decrease. Our data showed that the refractory period of the S-cell imposed an upper limit (~ 100 Hz) to the firing frequency of the network, which was confirmed by the model. In the presence of 5HT, the model predicted an enhancement of firing frequency because the refractory period was shortened. With the same duration of synaptic input, the central S-cell would initiate more impulses because of increased firing frequency, which was observed experimentally in the sensitized state.

Physiological and behavioral significance

A competition for initiation sites resembling inhibition between strongly electrically coupled neurons depending upon delayed synaptic input has not been demonstrated in other systems, although there is increasing evidence for the importance of axo-axonal electrical synapses in diverse systems, including those such as the mammalian hippocampus involved in memory processing and storage (Schmitz et al., 2001; Spruston, 2001). Inhibitory interactions have long been known to enhance both spatial and temporal discrimination of sensory stimuli (Hartline and Ratliff, 1957; Ratliff et al., 1967).

The S-cell network might function as a type of nonlinear filter, where high frequency input (i.e. initiations) tends to exclude input from other ganglia, whereas low frequency input sums linearly with low frequency input from other ganglia. A single S-cell with enhanced initiations

suppresses contributions from other S-cells, but if all cells generate less firing then activity is integrated across S-cells in the network.

The pattern of S-cell initiations during sensitization may enhance activity in the L-motoneurons, which excite all longitudinal muscle, thereby strongly contributing to shortening (Stuart, 1970). The sensory- and S-cells all excite the L-motoneurons (Muller, 1979). Although the S-cell differs from other giant axons because it is not itself able to generate significant shortening of the animal (Gardner-Medwin et al., 1973), input to the L-motoneuron from the S-cell coincident with sensory-cell input may enhance the motoneuron's response, similar to an effect observed in the crayfish escape circuit (Edwards et al., 1998). Thereby, the S-cell may strengthen shortening during sensitization.

Coincidence detection may also work at the level of sensory-to-S-cell synapses, which exhibit both long-term potentiation (LTP) and depression (LTD) and involve NMDA receptors (Burrell and Sahley, 2004). It is not known whether transient refractoriness in neighboring S-cells due to impulse initiations in the central S-cell would impede LTP or cause depression in those synapses in favor of LTP in the central S-cell synapses, as with spike-timing dependent plasticity (Dan and Poo, 2004; Constantine-Paton, 2006). Activity in the brain, shaped by inhibitory interactions and intrinsic inexcitability, such as reported here, may contribute fundamentally to mechanisms by which the brain processes information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Supported by NIH grant R01-NS34927. We thank Dr. Brian Burrell for help with the behavioral preparation, Drs. Alec Urazaev, Emmanuel Ngu, Kevin Crisp, Yuanli Duan, Ellen Barrett, Lynne Fieber, Stephen Roper, and Wolfgang Nonner for helpful discussions, and Dr. Stephen Baccus for valuable suggestions and critical reading of an earlier version of the manuscript. Present address of G. Cruz is Room 225, Monell Chemical Senses Center, 3500 Market Street, Philadelphia, PA 19104

References

- Antonov I, Antonova I, Kandel ER, Hawkins RD. The contribution of activity-dependent synaptic plasticity to classical conditioning in Aplysia. *J Neurosci* 2001;21:6413–6422. [PubMed: 11487665]
- Arisi I, Zoccolan D, Torre V. Distributed motor pattern underlying whole-body shortening in the medicinal leech. *J Neurophysiol* 2001;86:2475–2488. [PubMed: 11698536]
- Baccus SA, Burrell BD, Sahley CL, Muller KJ. Action potential reflection and failure at axon branch points cause stepwise changes in EPSPs in an interneuron essential for learning. *J Neurophysiol* 2000;83:1693–1700. [PubMed: 10712489]
- Baccus SA, Sahley CL, Muller KJ. Multiple sites of action potential initiation increase neuronal firing rate. *J Neurophysiol* 2001;86:1226–1236. [PubMed: 11535672]
- Bagnoli P, Brunelli M, Magni F. A fast conducting pathway in the central nervous system of the leech *Hirudo medicinalis*. *Arch ital Biol* 1972;110:35–51. [PubMed: 5050121]
- Bagnoli P, Brunelli M, Magni F, Pellegrino M. The neuron of the fast conducting system in *Hirudo medicinalis*: identification and synaptic connections with primary afferent neurons. *Arch ital Biol* 1975;113:21–43. [PubMed: 1156079]
- Baxter DA, Canavier CC, Clark JW Jr, Byrne JH. Computational model of the serotonergic modulation of sensory neurons in Aplysia. *J Neurophysiol* 1999;82:2914–2935. [PubMed: 10601429]
- Burrell BD, Sahley CL. Multiple forms of long-term potentiation and long-term depression converge on a single interneuron in the leech CNS. *J Neurosci* 2004;24:4011–4019. [PubMed: 15102916]

- Burrell BD, Sahley CL, Muller KJ. Non-associative learning and serotonin induce similar bi-directional changes in excitability of a neuron critical for learning in the medicinal leech. *J Neurosci* 2001;21:1401–1412. [PubMed: 11160412]
- Burrell BD, Sahley CL, Muller KJ. Differential effects of serotonin enhance activity of an electrically coupled neural network. *J Neurophysiol* 2002;87:2889–2895. [PubMed: 12037192]
- Burrell BD, Sahley CL, Muller KJ. Progressive recovery of learning during regeneration of a single synapse in the medicinal leech. *J Comp Neurol* 2003;457:67–74. [PubMed: 12541325]
- Calabrese RL. Control of multiple impulse-initiation sites in a leech interneuron. *J Neurophysiol* 1980;44:878–896. [PubMed: 6255110]
- Calabrese RL, Kennedy D. Multiple sites of spike initiation in a single dendritic system. *Brain Res* 1974;82:316–321. [PubMed: 4374295]
- Carew TJ, Sahley CL. Learning in invertebrates: From behavior to molecules. *Ann Rev Neurosci* 1986;9:435–487. [PubMed: 2423010]
- Cleary LJ, Lee WL, Byrne JH. Cellular correlates of long-term sensitization in *Aplysia*. *J Neurosci* 1998;18:5988–5998. [PubMed: 9671684]
- Cohen TE, Kaplan SW, Kandel ER, Hawkins RD. A simplified preparation for relating cellular events to behavior: mechanisms contributing to habituation, dishabituation, and sensitization of the *Aplysia* gill-withdrawal reflex. *J Neurosci* 1997;17:2886–2899. [PubMed: 9092610]
- Constantine-Paton M. Shining light on spike timing-dependent plasticity. *Neuron* 2006;50:5–7. [PubMed: 16600848]
- Crisp KM, Muller KJ. A 3-synapse positive feedback loop regulates the excitability of an interneuron critical for sensitization in the leech. *J Neurosci* 2006;26:3524–3531. [PubMed: 16571760]
- Cruz, GE. Thesis. Miami, Florida: University of Miami; 2006. Neuronal Competition for Action Potential Initiation Sites in a Circuit Controlling Simple Learning.
- Dan Y, Poo MM. Spike timing-dependent plasticity of neural circuits. *Neuron* 2004;44:23–30. [PubMed: 15450157]
- Daoudal G, Debanne D. Long-term plasticity of intrinsic excitability: learning rules and mechanisms. *Learn Mem* 2003;10:456–465. [PubMed: 14657257]
- Destexhe A, Bal T, McCormick DA, Sejnowski TJ. Ionic mechanisms underlying synchronized oscillations and propagating waves in a model of ferret thalamic slices. *J Neurophysiol* 1996;76:2049–2070. [PubMed: 8890314]
- Edwards DH, Yeh SR, Krasne FB. Neuronal coincidence detection by voltage-sensitive electrical synapses. *Proc Natl Acad Sci U S A* 1998;95:7145–7150. [PubMed: 9618553]
- Ehrlich JS, Boulis NM, Karrer T, Sahley CL. Differential effects of serotonin depletion on sensitization and dishabituation in the leech, *Hirudo medicinalis*. *J Neurobiol* 1992;23:270–279. [PubMed: 1378084]
- Elliott EJ, Muller KJ. Sprouting and regeneration of sensory axons after destruction of ensheathing glial cells in the leech CNS. *J Neurosci* 1983;3:1994–2006. [PubMed: 6619920]
- Farley J, Richards WG, Ling LJ, Liman E, Alkon DL. Membrane changes in a single photoreceptor cause associative learning in *Hermisenda*. *Science* 1983;221:1201–1203. [PubMed: 6612335]
- Frank E, Jansen JKS, Rinvik E. A multisomatic axon in the central nervous system of the leech. *J Comp Neurol* 1975;159:1–13. [PubMed: 162801]
- Gainutdinov KL, Chekmarev LJ, Gainutdinova TH. Excitability increase in withdrawal interneurons after conditioning in snail. *Neuroreport* 1998;9:517–520. [PubMed: 9512399]
- Gardner-Medwin AR, Jansen JKS, Taxt T. The “giant” axon of the leech. *Acta Physiol Scand* 1973;87:30A–31A.
- Hartline HK, Ratliff F. Inhibitory interaction of receptor units in the eye of *Limulus*. *J gen Physiol* 1957;40:1357–1376.
- Hines ML, Carnevale NT. NEURON: a tool for neuroscientists. *Neuroscientist* 2001;7:123–135. [PubMed: 11496923]
- Hochner B, Klein M, Schacher S, Kandel ER. Action-potential duration and the modulation of transmitter release from the sensory neurons of *Aplysia* in presynaptic facilitation and behavioral sensitization. *Proc Natl Acad Sci USA* 1986;83:8410–8414. [PubMed: 16593779]

- Hodgkin AL, Huxley AF. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J Physiol (Lond)* 1952;117:500–544. [PubMed: 12991237]
- Klein M, Camardo J, Kandel ER. Serotonin modulates a specific potassium current in the sensory neurons that show presynaptic facilitation in *Aplysia*. *Proc Natl Acad Sci USA* 1982;79:5713–5717. [PubMed: 6957886]
- Meunier C, Segev I. Playing the devil's advocate: is the Hodgkin-Huxley model useful? *Trends Neurosci* 2002;25:558–563. [PubMed: 12392930]
- Modney BK, Sahley CL, Muller KJ. Regeneration of a central synapse restores non-associative learning. *J Neurosci* 1997;17:6478–6482. [PubMed: 9236255]
- Moss BL, Fuller AD, Sahley CL, Burrell BD. Serotonin modulates axo-axonal coupling between neurons critical for learning in the leech. *J Neurophysiol* 2005;94:2575–2589. [PubMed: 15987763]
- Muller KJ. Synapses between neurones in the central nervous system of the leech. *Biol Rev* 1979;54:99–134. [PubMed: 383168]
- Muller KJ, Carbonetto S. The morphological and physiological properties of a regenerating synapse in the C.N.S. of the leech. *J Comp Neurol* 1979;185:485–516. [PubMed: 438368]
- Muller KJ, Scott SA. Transmission at a “direct” electrical connexion mediated by an interneurone in the leech. *J Physiol (Lond)* 1981;311:565–583. [PubMed: 6267257]
- Nicholls JG. *Magnes Lecture Series: Volume II*. Sunderland, Massachusetts: Sinauer Associates Inc; 1987. *The Search for Connections: Study of Regeneration in the Nervous System of the Leech*.
- Nicoll RA, Malenka RC. Contrasting properties of two forms of long-term potentiation in the hippocampus. *Nature* 1995;377:115–118. [PubMed: 7675078]
- Oesch N, Euler T, Taylor WR. Direction-selective dendritic action potentials in rabbit retina. *Neuron* 2005;47:739–750. [PubMed: 16129402]
- Prescott SA. Interactions between depression and facilitation within neural networks: updating the dual-process theory of plasticity. *Learn Mem* 1998;5:446–466. [PubMed: 10489261]
- Ratliff F, Knight BW, Toyoda J, Hartline HK. Enhancement of flicker by lateral inhibition. *Science* 1967;158:392–393. [PubMed: 6061895]
- Rinzel J. Mechanisms for nonuniform propagation along excitable cables. *Ann N Y Acad Sci* 1990;591:51–61. [PubMed: 2197933]
- Roberts AC, Glanzman DL. Learning in *Aplysia*: looking at synaptic plasticity from both sides. *Trends Neurosci* 2003;26:662–670. [PubMed: 14624850]
- Sahley CL, Modney BK, Boulis NM, Muller KJ. The S cell: An interneuron essential for sensitization and full dishabituation of leech shortening. *J Neurosci* 1994;14:6715–6721. [PubMed: 7965072]
- Schmitz D, Schuchmann S, Fisahn A, Draguhn A, Buhl EH, Petrasch-Parwez E, Dermietzel R, Heinemann U, Traub RD. Axo-axonal coupling: A novel mechanism for ultrafast neuronal communication. *Neuron* 2001;31:831–840. [PubMed: 11567620]
- Shaw BK, Kristan WB Jr. The whole-body shortening reflex of the medicinal leech: Motor pattern, sensory basis, and interneuronal pathways. *J Comp Physiol [A]* 1995;177:667–681.
- Shaw BK, Kristan WB Jr. Relative roles of the S cell network and parallel interneuronal pathways in the whole-body shortening reflex of the medicinal leech. *J Neurophysiol* 1999;82:1114–1123. [PubMed: 10482731]
- Siegelbaum SA, Camardo JS, Kandel ER. Serotonin and cyclic AMP close single K⁺ channels in *Aplysia* sensory neurones. *Nature* 1982;299:413–417. [PubMed: 6289122]
- Spruston N. Axonal gap junctions send ripples through the hippocampus. *Neuron* 2001;31:669–671. [PubMed: 11567606]
- Stuart AE. Physiological and morphological properties of motoneurons in the central nervous system of the leech. *J Physiol (Lond)* 1970;209:627–646. [PubMed: 5499800]
- Wallace BG, Adal MN, Nicholls JG. Sprouting and regeneration of synaptic connexions by sensory neurones in leech ganglia maintained in culture. *Proc R Soc Lond B* 1977;199:567–585. [PubMed: 23544]
- Walters ET, Cohen LB. Functions of the LE sensory neurons in *Aplysia*. *Invert Neurosci* 1997;3:15–25. [PubMed: 9706699]

- White JA, Ziv I, Cleary LJ, Baxter DA, Byrne JH. The role of interneurons in controlling the tail-withdrawal reflex in *Aplysia*: a network model. *J Neurophysiol* 1993;70:1777–1786. [PubMed: 8294952]
- Zecevic D. Multiple spike-initiation zones in single neurons revealed by voltage-sensitive dyes. *Nature* 1996;381:322–325. [PubMed: 8692270]
- Zhang W, Linden DJ. The other side of the engram: experience-driven changes in neuronal intrinsic excitability. *Nat Rev Neurosci* 2003;4:885–900. [PubMed: 14595400]
- Zhang W, Shin JH, Linden DJ. Persistent changes in the intrinsic excitability of rat deep cerebellar nuclear neurones induced by EPSP or IPSP bursts. *J Physiol (Lond)* 2004;561:703–719. [PubMed: 15498810]

Comprehensive list of abbreviations

ANOVA	analysis of variance
g	ganglion
H-H	Hodgkin-Huxley
HN	heart interneuron
ISI	interstimulus interval
N cell	nociceptive cell
P cell	pressure sensory cell
s	segment
T cell	touch sensory cell
SEM	standard error of the mean
5-HT	5-hydroxytryptamine

Figure 1.

S-cell action potential initiations at multiple sites in the behaving preparation. (A) Diagram of the semi-intact preparation used to study behavioral and electrophysiological responses simultaneously. Ganglia in segments 2, 3, 9 and 10 are labeled g2, g3, g9 and g10, respectively. A tension transducer attached to the head monitored shortening. Paired silver wires in the skin in segment 5 or 6 (s5 or s6) evoked shortening (test stimulus); sensitizing shocks were applied to s11 or s12. S-cell action potentials were recorded from anterior (cuff electrodes, between g2 and g3) and posterior (suction electrode between g9 and g10) locations. The sites of action potential initiations were located and plotted on an arrival time difference histogram, as shown below the preparation. Each peak corresponds to initiations in an S-cell in one segment, as indicated by a dashed line. A second x-axis shows the estimated distances between ganglia after converting time differences to locations (see Methods). (B) Sample traces of S cell action potentials recordings in posterior (top trace) and anterior (bottom trace) extracellular electrodes in the behaving preparation, with single initiations matched by dashed lines, used for histograms as in A and in Fig. 3. (C) Typical S-cell (large action potentials in top 2 traces) and behavioral (bottom trace) responses to test stimuli before (left panel) and after (right panel)

sensitizing shocks. Test stimuli produced additional initiations and a concomitant increase in the tension (shortening) after sensitization. Different animal than for **B**.

Figure 2. S-cell firing increased in parallel with enhanced shortening in sensitized preparations. After 3 test trials separated by 2 min (averaged at b (baseline) on the abscissa), a sensitizing stimulus was delivered (or not, for controls) at point indicated by arrow, and then 20 test trials done, also separated by 2 min, with the response to each normalized to baseline. Those trials were grouped in blocks of 4 and averaged. The dotted lines mark the baseline level set to either 100% or 1.0 for comparison of responses by sensitized (filled circles) and nonsensitized (open circles) preparations. **A.** Mean percentage initial shortening. Leeches in the sensitization group (n=18) had significantly stronger responses to the repeated presentations of the test stimulus compared to those in the nonsensitization group (n=7, $p<0.01$). **B.** Number of spikes fired by the S-cell relative to baseline level. Overall S-cell activity response was significantly higher in the sensitization group ($p<0.01$). In contrast, S-cell firing declined progressively in the nonsensitization group. In the sensitized group, S-cell firing was above baseline in the first trial block following the sensitizing shocks. Error bars are Standard Error of the Mean (S.E.M.). Data in **A** and **B** were from the same experiments and groups of animals.

Figure 3.

Location and timing of action potential (AP) initiations in the S cell network with behavioral sensitization. The location (**A**, **B**) and timing (**C**, **D**) of spike initiations in response to 3 test stimuli were determined for the stimulated segment (*c*, central) and neighboring sequential anterior and posterior segments (*a1*, *a2*, *p1* and *p2*) before and after the sensitizing shocks in semi-intact preparations. The central S-cell initiated the most impulses and, in this example, two additional sites appeared after sensitization, but without significantly more spike initiations in other S-cells. **C** and **D** show post-stimulus time (PST) histograms of the mean S-cell firing rate (AP/sec) and total network activity (see Results) for 3 test stimuli before (**C**) and after (**D**) sensitization. Bin width for **A** and **B** = 0.5 ms, and for **C** and **D** = 20 ms.

Figure 4.

Action potential initiations in the S-cell chain spread little to other ganglia with sensitization. **A.** The number of spikes initiated in all 3 trials during baseline and in the first 3 trials following sensitization were counted for each S-cell (initiation site) for all animals in the sensitization group and averaged. Spike initiations immediately following the sensitizing stimulus (black bars) increased significantly (*) compared to baseline (white bars) only in the central segment, based on the nonparametric Wilcoxon Matched Pairs Test ($p < 0.05$, $n = 18$). **B.** The total number of sites that initiated impulses in each trial was counted and averaged in blocks of 3 trials each, which were then averaged for all animals. The average number of initiation sites did not change significantly between animals in the experimental (sensitized) and control (not sensitized) groups across trial blocks or between groups and within trial blocks (Repeated measures ANOVA, where $n = 18$ for sensitized and $n = 7$ for nonsensitized preparations). Error bars, S.E.M.

Figure 5.

Reducing action potential initiations in one S-cell increased initiations in adjacent ganglia. **A.** Preparation was a chain of 5 ganglia with skin attached on one side; S-cell spikes were recorded at cut ends of the nerve cord. Initiation sites were identified and labelled as in Figs. 1 and 3. Test shocks on the skin were given as usual on the central segment; its S-cell received <1 nA, 500-ms duration hyperpolarizing pulses (see Methods). The arrowhead indicates the timing of the test stimulus applied to the skin ~0.6 ms after the onset of the hyperpolarizing pulse. **B.** Impulses that initiated in *a1* and *p1* (immediate neighbors of *c*) during the first 150 ms following the test stimulus were added together ($n = 10$ experiments). Reducing impulse initiations in the central S-cell by hyperpolarizing it (Hyperpolarized) led to a significant increase in impulse initiations in its adjacent segments (*, two-tailed t-test for dependent samples, $p < 0.05$). Error bars are S.E.M.

Figure 6.

Evidence for a refractory period from the distribution of interspike intervals (ISIs). The intervals between action potential initiations in ganglia neighboring the central ganglion and the arrival beforehand in those ganglia of impulses initiated in the central ganglion are shown. *Inset:* Trains of S-cell spikes in response to a test stimulus, as recorded from anterior or posterior electrodes, are shown diagrammatically. Impulses initiating in the central S-cell (c) that immediately preceded impulses initiating in adjacent S-cells on either side (a or p) were identified in records of 5 preparations under control conditions, a subgroup of preparations used in Fig. 5. A total of 21 trials were pooled and interspike intervals (ISI) were measured up to 300 ms. The distribution of 31 ISIs obtained was determined using time bins of 5 ms width. These data suggested a refractory period of ~10 ms for the S-cell.

Figure 7.

Modifications of the Hodgkin-Huxley (H-H) model for use in the S-cell model. The standard model was modified so as to emulate the action potential (AP) of the S-cell (red traces and vertical scales in A) particularly its rising phase and afterhyperpolarization. All of the graphs compare the effect of the modifications (gray) with the standard model (black). The *inset* in each graph is a magnified view of the respective waveforms, beginning at 12 ms, to show the changes at the end of the impulse as the membrane potential (V_m) returns to resting levels. The parameters of the model of the squid axon AP were altered primarily to affect the events occurring at the end of the impulse. The final AP model was within the range of values for the actual S-cell AP, including the duration of the AP and its afterhyperpolarization (see Results).

A. Comparison of AP waveforms. The notable differences shown in the inset were the elimination of the oscillation and a slower return of V_m to resting level. **B.** Underlying kinetics of the “h” variable of the H-H model, describing the inactivation of the fast sodium channel as the AP progressed. The effect of the modified H-H model was to make the recovery from inactivation slower but more stable. **C.** The underlying kinetics of g_k , describing the change in conductance of the potassium channel responsible for repolarization of V_m . In the modified model, a higher level of g_k at the end of the AP was maintained, making the membrane less excitable for a period of time.

Figure 8.

Refractory period following S-cell action potentials: experiment and model. **A.** The S-cell membrane's refractory period was determined by impaling an S-cell soma in a chain of 4 ganglia. The cut end of the connectives on one end was stimulated using a suction electrode to evoke a single S-cell spike, which propagated to the soma under study. A second impulse was evoked in the soma by passing a depolarizing current through the microelectrode (0.5 ms pulse, just above threshold). **B.** The first impulse recorded in the soma was the propagated impulse, followed by the intracellularly evoked impulse. As the interval between the 2 impulses shortened, more current was required to be injected into the soma to reach threshold (lower pair of traces). Inter-impulse intervals were measured from the peak of one action potential to

the next. **C.** Excitability curve for the S-cell. The amount of current required to reach threshold at each interval was normalized to initial levels obtained at long inter-impulse intervals of 18-25 ms. The S-cell membrane was relatively refractory to a second impulse at intervals of <12 ms (experimental data points, filled circles). The experiment was simulated using a compartmental model of the S-cell (open circles).

Figure 9.

S-cell membrane refractory period can account for suppression of action potential initiations in adjacent segments. **A.** Diagram of the S-cell compartmental model (black), with the P-cell including its synapses (yellow). The model reproduced the S-cell impulse's conduction velocity (~ 1.2 m/s experimentally) and conduction time between ganglia (~ 5 msec experimentally; upper green arrow). Conduction of an impulse centrally between ganglia in the P-cell was determined experimentally to be ~ 14 msec (lower green arrow). Thus, initiation of a second impulse in an adjacent S-cell likely failed when input from the P-cell arrived ~ 9 msec after the preceding spike because the S-cell membrane was still relatively refractory. **B.** Simulation

of the experiment in Fig. 8, confirming that a second impulse was likely to fail if evoked within less than ~ 10 ms after the first impulse, which was initiated from an adjacent S-cell. Synaptic input was delivered at intervals graded by 0.5 ms in this simulation. **C.** The effect of serotonin (5-HT) was modeled by adding a potassium leak channel (k_{leak}), at a distance of 300 μm from the soma, whose gating could be modulated (see Results). Baseline (black): The channel had high conductance ($g_{\text{max}} = 15 \text{ mS/cm}^2$) and the S-cell membrane refractory profile was similar to that in **B.** The + 5-HT mechanism (orange): The channel had low conductance ($g_{\text{max}} = 1 \text{ mS/cm}^2$) and the S-cell membrane became more excitable. More impulses were initiated in the + 5-HT mechanism and the refractory period was shifted to shorter intervals. Orange and black arrows indicate timing of inputs that first initiate impulses for the 5-HT and baseline mechanisms, respectively. Note the difference in the scale of abscissas between **B** and **C**.

Table 1

S cell model parameters and constants

Table 1a. S-cell Model Parameters

Geometry	Soma	Axon
Diameter (μm)	20	8
Length (μm)	20	6000
No. of compartments	1	3000

BiophysicsMembrane capacitance (C_m), $1\mu\text{F}/\text{cm}^2$ Axial resistance (R_a), $100\Omega\text{-cm}$

Maximum conductances

Sodium ($\bar{g}_{Na}$), $120\text{ mS}/\text{cm}^2$ Potassium, ($\bar{g}_K$), $36\text{ mS}/\text{cm}^2$ Leak ($\bar{g}_{leak}$), $0.3\text{ mS}/\text{cm}^2$

Equilibrium potentials

Sodium (E_{Na}), 50 mV Potassium (E_K), -70 mV Leak (E_{leak}), -54.3 mV Resting membrane potential (V_{rest}), -63 mV Temperature, 12°C

Table 1b. Membrane Mechanisms

[1] Hodgkin-Huxley Model Parameters (Modified vs. Standard)*

Equation Constants	α_m	β_m	α_h	β_h	α_n	β_n
A (/ms)	0.1 (0.1)	4 (4)	0.07 (0.07)	1 (1)	0.01 (0.01)	0.125 (0.125)
B (mV)	45 (40)	55 (65)	62 (65)	31 (35)	53 (55)	63 (65)
C (mV)	10 (10)	18 (18)	39 (20)	10 (10)	12 (10)	79 (80)

[2] Additional mechanism: K_{leak} (neuromodulator-sensitive K^+ leak conductance)

	Baseline (no 5-HT)	+ 5-HT mechanism
Maximum conductance ($\bar{g}_{max}$, mS/cm^2)	15	1
Equilibrium potential (E_{leak} , mV)	-80	-80
Distance from soma (μm)	300	300

* Values for the standard model are in parentheses.

Rate equations:

$$\alpha_m(V) = -A(V+B)/\exp[-(V+B)/C] - 1$$

$$\beta_m(V) = A \exp[-(V+B)/C]$$

$$\alpha_h(V) = A \exp[-(V+B)/C]$$

$$\beta_h(V) = A / \exp[-(V+B)/C] + 1$$

$$\alpha_n(V) = -A(V+B)/\exp[-(V+B)/C] - 1$$

$$\beta_n(V) = A \exp[-(V+B)/C]$$

$$\text{Equation: } I_{leak} = \bar{g}_{max}(V - E_{leak})$$