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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.

Key words: interneuron, integration, sensitization, reflex, sensory cells, leech.

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., 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, 1995, 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, 1995, 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

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Abbreviations: ANOVA, analysis of variance; g (+ number), ganglion; H-H, Hodgkin-Huxley; HN, heart interneuron; ISI, interstimulus interval; LTP, long-term potentiation; P cell, pressure sensory cell; s (+number), segment; S.E.M., standard error of the mean; T cell, touch sensory cell.

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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.

EXPERIMENTAL PROCEDURES

Animals and electrophysiological recordings

Leeches (*Hirudo medicinalis*, ~3 g) were obtained from a supplier (LeechesUSA, Westbury, NY, USA), 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. Animals were dissected on ice for anesthesia, and the U.S. Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals for invertebrates was followed. Every effort was made to minimize the number of animals used and their suffering. Cuff electrodes recorded S-cell action potentials extracellularly through the blood sinus within the body using two thin, Teflon-coated silver wires (75 μm uncoated diameter; AM Systems, Carlsborg, WA, USA) bared at the ends, which were ~300 μm apart, coiled halfway around a minuten pin, and embedded in Sylgard 184 rubber (Dow Corning, Midland, MI, USA) 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 (Suppl. Fig. 1) was identified by its ~20 μm 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 s5 and sensitizing shocks were given at a distance from it on s11 or s12 using published parameters and electrodes (Modney et al., 1997; Burrell et al., 2001; Cruz, 2006).

For each preparation, 1 h 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 five to six action potentials, as reported previously. The stimulus was a single, capacity-coupled 3 ms pulse of 4–7 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 min, 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 10 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 min after the baseline shocks, followed 2 min 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 four 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 (bin width=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 Suppl. Fig. 2). The S-cell impulses were the largest recorded, and their timing at their peaks were measured and matched (spike sorting) with Axoscope (Axoscope 9; Molecular Devices Corporation, Sunnyvale, CA, USA). 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 five to seven 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.