Action Potential Reflection and Failure at Axon Branch Points Cause Stepwise Changes in EPSPs in a Neuron Essential for Learning

STEPHEN A. BACCUS,1 BRIAN D. BURRELL,2 CHRISTIE L. SAHLEY,3 AND KENNETH J. MULLER1,2
1Neuroscience Program and 2Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Florida 33136; and 3Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

INTRODUCTION

Identifying the changes responsible for learning in a given system is a complex problem. Although a number of mechanisms of synaptic plasticity have been identified (Bear and Malenka 1994), it is less clear how these mechanisms relate to learning and operate in systems with thousands or millions of neurons. Even in invertebrates, with relatively fewer cells, hundreds of neurons can participate in multiple behaviors and scores of cells can modify their activity during learning (Wu et al. 1994; Zecevic et al. 1989).

In contrast, it has been found in the leech that a single interneuron, the S cell, is required for a form of nonassociative learning (Modney et al. 1997; Sahley et al. 1994). The leech can modify its reflexes including reflex shortening (Debski and Friesen 1985; Lockery and Kristan 1991). The leech defensively shortens in response to stimuli that activate mechanosensory neurons including P cells. A prior noxious stimulus will sensitize this behavior, increasing the contraction produced by the weaker shortening stimulus (Boulis and Sahley 1988). The S cell is necessary for sensitization of the shortening reflex. Killing one S cell in the electrically coupled chain of S cells, or cutting its axon, abolishes this form of learning while leaving the basic reflex intact. The S cell is also required for complete dishabituation, another form of nonassociative learning (Sahley et al. 1994). The single S cell in each ganglion is excited by sensory neurons, is electrically coupled through nonrectifying junctions to a pair of coupling interneurons, the C cells (Muller and Scott 1981), and in turn excites motoneurons active throughout the entire length of the leech (Gardner-Medwin et al. 1973). Therefore, understanding changes in sensory transmission to the S cell is important for understanding the cellular mechanisms of learning.

The structure of neurons, including leech sensory cells, can influence synaptic transmission in a dynamic manner. One mechanism that depends on axonal branching and influences transmission is conduction block, which enables an impulse to activate some synapses within a neuron but not others, reducing transmission in a stepwise manner (Gu 1991; Macagno et al. 1987; Muller and Scott 1981). Recently it was found that reflection of action potentials, a related mechanism, allows single branch points of an axon to increase transmission (Baccus 1998). Reflection occurs when impulses are sufficiently delayed as they travel through branch points. Thus when the delay exceeds the refractory period of the conducting axon, the impulse propagates backward as well as forward from the branch point, creating a reflection. In leech mechanosensory neurons, the reflected impulse activates presynaptic terminals on one side of a branch point a second time, facilitating transmission from those synapses and not others (Fig. 1). Repetitive activity can cause these branch points to change between the states of reflection, conduction block, and the state where impulses pass through the branch point without reflection, known as full conduction (Baccus 1998), but the separate effects of these three states on synaptic transmission has not been reported.

The P to S synaptic connection, which has been cited as an unpublished observation (Shaw and Kristan 1995), is characterized here. To understand the extent that a neuron’s morphology can dynamically influence synaptic transmission, the effect of presynaptic reflection and conduction block on transmission at this synapse is examined.
METHODS

Preparations and electrophysiology

Leeches Hirudo medicinalis were obtained from a supplier (Leeches USA, Westbury, NY) or raised in the laboratory. Preparations were dissected as described (Baccus 1998; Nicholls and Baylor 1968). The bath contained leech saline composed of (in mM) 115 NaCl, 4 KCl, 1.8 CaCl₂, and 10 Tris maleate (pH 7.4 adjusted with NaOH) (Kuffler and Potter 1964). For experiments using elevated \([\text{Ca}^{2+}] - [\text{Mg}^{2+}]\) saline, the bath contained (in mM) 15 \text{Ca}^{2+} and 18 \text{Mg}^{2+}, each replacing Na⁺ mole for mole. Experiments were conducted at room temperature (20–22°C). Preparations consisted of a chain of two segmental ganglia with one ganglion attached to the skin by the dorsal peripheral nerve root. Intracellular recordings were made in the adjacent ganglion, which was either anterior or posterior to the ganglion attached to the skin, using sharp microelectrodes (20–25 MΩ) filled with 4 M potassium acetate. In some experiments the recording electrode in the P cell contained 1 M tetraethylammonium chloride (TEA⁻), which diffused into the cell and broadened the action potential by blocking potassium channels. The broader action potentials increased transmitter release in a graded fashion for the purpose of testing whether a connection was physiologically monosynaptic (see RESULTS). Medial P cells were identified by their size, shape, and position within the ganglion. The S cell was identified by its position and action potential because it is the only small cell in the central glial packet that produces a large, fast overshooting action potential in response to weak stimulation of the segmental nerve cord or stimulation of the soma. Resting membrane potentials for P cells and S cells ranged between −41 and −55 mV. P cells were peripherally stimulated using a suction electrode applied to the anterior or posterior minor receptive fields in the dorsal skin, which are contiguous with the central, major receptive field as shown in Fig. 1A. In some experiments, to prevent the S cell from firing in response to large synaptic potentials, continuous hyperpolarizing current was injected into the soma. Values of n indicate number of preparations, not measurements.

Laser axotomy

When measuring the S cell synaptic potential produced by peripherally stimulating the P cell, it was necessary to eliminate the contribution from P, touch (T), and nociceptive (N) mechanosensory neurons in the ganglion attached to the skin. This is because these sensory neurons also innervated the area of skin being stimulated and synapsed on the S cell in the adjacent ganglion where the recording was made. Therefore the peripheral axons of these neurons were cut with a laser microbeam as described (Baccus 1998; Gu et al. 1989). In brief, the cells were pressure injected with 0.17 M 6-carboxyfluorescein, neutralized to pH 7.4 with KOH, and their peripheral axons cut by irradiation for a few seconds with 488 nm light from a 20-mW argon laser attenuated two- to fourfold with neutral density filters.

Histology

In one series, S cells were injected with Lucifer yellow dye by either pressure or iontophoresis and P cells were pressure injected with either 2% horseradish peroxidase (HRP) (Muller et al. 1981) or 5% (wt/vol) biocytin (Horikawa and Armstrong 1988). Cells injected with HRP were processed as described by incubation in leech saline saturated with diaminobenzidine (DAB) (Gu 1991; Macagno et al. 1987). Biocytin injected cells were processed as described (Peinado et al. 1993) by incubation with rhodamine-conjugated streptavidin at a concentration of 0.2% wt/vol for 3 h. To count apparent synaptic contacts, ganglia were viewed at ×400 with a ×40 oil-immersion objective having a 1.3 numerical aperture. For ganglia stained using HRP, by balancing fluorescence epi-illumination and transmitted light, it was possible simultaneously to view the Lucifer yellow and HRP-stained cells. To ascertain whether cells appeared to be in direct contact, the focal plane was adjusted during viewing. Photomicrographs were not used to identify synaptic contacts. Preparations stained with rhodamine required alternate viewing through filters optimized for fluorescein, which allowed simultaneous viewing of Lucifer yellow and rhodamine and filters optimized for rhodamine. For this reason, counting synaptic contacts using HRP was preferable. In a few cases, fibers crossed each other in close proximity without an apparent presynaptic varicosity; these were not counted as a synaptic contact.

In a complementary series, 5% lysinated tetramethylrhodamine dextran (10⁴ Da, Molecular Probes) dissolved in 0.2% Fast Green FCF in 0.1 M KCl was injected into the S cell and 5% Lucifer yellow into the P cell. The dextran did not cross gap junctions and was excluded from C interneurons, permitting the identification of apparently direct contacts between the P and S cells. Preparations were fixed in paraformaldehyde, mounted in Fluormount (Gurr), and viewed with a laser scanning confocal microscope (Fluoview, Olympus) with fluorescein and rhodamine optics using a ×20 objective.

RESULTS

Synaptic transmission from P cell to S cell

Activation of the P cell produced a synaptic potential in the S cell of 1.1 ± 0.1 (SE) mV (n = 17) (Fig. 2). The synaptic potential had an excitatory early component and later more variable components that were excitatory, inhibitory, or both. To determine whether the synaptic potential was physiologically monosynaptic, or direct, the excitatory postsynaptic potential (EPSP) was measured with the ganglion bathed in physiological saline containing 1.8 mM Ca²⁺ and the bath was switched to saline containing 15...
mM Ca\(^{2+}\) and 18 mM Mg\(^{2+}\) (Nicholls and Purves 1970). This solution reduced postsynaptic excitability without reducing transmission at chemical synapses thereby eliminating or reducing polysynaptic transmission involving spiking interneurons. The early component of the synaptic potential had a constant latency and persisted in high [Ca\(^{2+}\)]–[Mg\(^{2+}\)] saline, indicating it was direct (Fig. 2, A and B). The later, variable components were eliminated in high [Ca\(^{2+}\)]–[Mg\(^{2+}\)] saline, indicating they were polysynaptic. P cells also produced a synaptic potential of ~0.5 mV in S cells in adjacent ganglia (n = 2, data not shown). In contrast to the touch mechanosensory cell (T cell) to S cell connection, there was no electrical coupling detectable between the P and the S cells because strong hyperpolarizing current (2 nA) injected into either cell did not pass to the other (n = 4, data not shown).

As an additional test to confirm that the early component was effectively monosynaptic, 1 M TEA\(^{+}\) was included in the presynaptic microelectrode and allowed to diffuse into the cell. TEA\(^{+}\) prolongs the action potential in a graded fashion by blocking presynaptic K\(^{+}\) channels in a concentration dependent manner, thereby increasing transmitter release. Monosynaptic potentials increase gradually as the concentration of TEA\(^{+}\) increases, whereas polysynaptic potentials do not increase or increase in a stepwise manner as an interneuron fires multiple times. During repeated trials, the presynaptic action potential recorded at the soma broadened (Fig. 2, C–E). As TEA\(^{+}\) diffused to presynaptic terminals, the early component of the synaptic potential increased steadily, confirming that it was monosynaptic (n = 6).

These criteria to verify that a connection is monosynaptic cannot rule out the presence of an intervening electrically coupled interneuron (Deschênes and Bennett 1974; Muller and Scott 1981). In fact, many contacts between the P and S cells are mediated by two coupling interneurons (C cells) that are strongly electrically coupled to the S cell (see Fig. 6) as described previously for T cells. Such a connection is functionally monosynaptic, is not distinguished physiologically from a true monosynaptic connection, and is termed here a direct connection (Muller and Scott 1981) included with the monosynaptic connection.

The pharmacology of the P to S synapse was determined by applying CNQX, which blocks glutamate receptors of the AMPA/Kainate type. CNQX blocks input from the P cell to the swim interneuron TR1 (Thorogood and Brodfuehrer 1995) and blocks the chemical monosynaptic connection between the P cell and the Anterior Pagoda (AP) cell (Wessel et al. 1999). The S cell synaptic potential produced by the P cell was eliminated by 25 μM CNQX (Fig. 3). CNQX blocked both the monosynaptic component isolated using high [Ca\(^{2+}\)]–[Mg\(^{2+}\)] saline (Fig. 3, A–C; n = 4) and the polysynaptic component recorded in physiological saline (Fig. 3, D–F; n = 4).

### Branch point conduction states

The conduction state of P cell central branch points was determined by stimulating the minor receptive field and recording the impulses that arrived at the P cell soma. Reflection was distinguished from full conduction by stimulating the periphery twice at levels above threshold to generate a pair of impulses in the periphery (Baccus 1998). When the branch point was fully conducting, the second impulse reached the soma (Fig. 4A). Otherwise, if the first impulse reflected, the reflection...
traveled back toward the periphery, colliding with the second impulse and preventing it from reaching the soma (Fig. 4B). The absence of the second impulse thus indicated that the branch point was reflecting. The second impulse could always be recovered by depolarizing the soma to produce full conduction (Fig. 4D) or by hyperpolarizing the soma to produce conduction block (data not shown). This confirmed that the absence of the second impulse was not because the peripheral stimulus was below threshold (Baccus 1998). Additionally, action potentials that reflected had a longer initial rising phase, or “foot”, indicating a delay in traveling through the branch point (Baccus 1998) (Fig. 4B, arrow). Reflection, full conduction, and conduction block (Fig. 4C) occurred at rest in different preparations as previously described and all states occurred in all cells examined (n = 12) (Baccus 1998). The conduction states were changed by injecting the soma with steady depolarizing or hyperpolarizing current, or in some cases depolarizing current pulses, to change membrane potential by <10 mV. Pulses were used to produce full conduction in some cells that exhibited reflection at rest (Fig. 4D), including those cells that with steady depolarization fired repetitively in response to a skin stimulus (data not shown).

Effect of reflection and conduction block on synaptic transmission

The effect of presynaptic reflection and conduction block on transmission to the S cell was measured by stimulating the anterior or posterior P cell minor fields and injecting current into the P cell soma to change the branch point conduction state. This level of current injection (<0.5 nA) does not directly affect transmission from the P cell to the AP cell (Baccus 1998). Indeed, there was no change in transmission with increased current injection except when the conduction state of the P cell branch point changed. Figure 5A shows the synaptic potential recorded in the S cell when the P cell anterior branch point was in the state of full conduction, activating all presynaptic terminals, but only once. Reflection increased transmission by firing a subset of synapses rapidly a second time, causing facilitation (Fig. 5B). Conduction block decreased transmission by activating only a subset of synapses (Fig. 5C). Other sensory cells that innervated the stimulated region of skin had been axotomized with a laser (see METHODS). However, to confirm that the synaptic potential in the S cell was produced by the P cell and not by another presynaptic cell excited by the skin stimulus, an impulse was generated in the P cell soma just before the peripheral stimulus. This outgoing impulse produced an early synaptic potential and collided with the incoming peripheral impulse, eliminating the synaptic potential at the time it would otherwise have been seen, indicated by an arrow in Fig. 5D.

Table 1 shows the effect of anterior and posterior reflection and conduction block in the P cell on the magnitude of the S cell synaptic potential. On average, anterior reflection increased the synaptic potential 1.8 times, whereas anterior conduction block reduced the synaptic potential to 0.55 times the value during full conduction (n = 4). Posterior reflection increased transmission 2.31 times and posterior block decreased transmission to 0.51 times that during full conduction (n = 5). The conduction state of two different branch points can thus change synaptic transmission between different levels.

It has been previously reported that in ~12% of medial P cells, the anterior and posterior thin axons join directly together before forming a single central branch point with the thick axon (Gu 1991). Impulses can thus pass directly from anterior...
to posterior thin axon and conduction block at the single branch point between thin and thick axons does not reduce transmission. Two such P cells were encountered (Table 1, asterisks) and were excluded from analysis. Reflection did however increase transmission from these cells as would be expected.

During conduction block, by measuring the size of the synaptic potential relative to its size when all terminals are activated, the functional distribution of the synapse on both sides of the central branch points can be measured (Gu 1991; Gu et al. 1991; Macagno et al. 1987). These values indicate that on average the P to S synapse was divided functionally so that one-half of the synaptic potential was produced anterior of the P cell branch points and one-half was produced posterior of the branch points ($49 \pm 7\%$ anterior, $n = 10$).

**P cell to S cell synaptic contacts are spatially distributed**

To examine directly the spatial distribution of the synapse, apparent synaptic contacts of the P cell with the S cell were seen by injecting separate intracellular tracers into the presynaptic and postsynaptic cells. Previous studies using HRP as a marker have confirmed that contacts of the P cell identified in the light microscope are indeed synapses when examined in the electron microscope (Macagno et al. 1987; Muller and McManah 1976).

When Lucifer yellow was injected in the S cell soma, the

![FIG. 4. P cell central branch point conduction states. A: P cell was stimulated twice at times indicated by fast biphasic artifacts. Full conduction was demonstrated when 2 closely spaced impulses reached the soma. B: when the 1st of 2 impulses reflected, if the 2 stimuli were separated by less than twice the conduction time from skin to soma as here, the second impulse collided with the reflected impulse and failed to reach the soma. Arrow indicates prominent initial rising phase, or foot, of impulse that reflected. Peripheral stimuli were well above threshold. C: conduction block at the central branch point was visible as a failed impulse at the soma. D: full conduction could also be produced by applying a depolarizing pulse to the soma during peripheral impulse’s arrival. This depolarizing pulse applied on its own was below threshold (lower trace below 1st action potential).](http://jn.physiology.org/)

![FIG. 5. Reflection and conduction block produce multilevel synaptic transmission. Anterior minor field was stimulated. Transmission from other sensory cells to the S cell was eliminated by cutting their peripheral axons with a laser. Conduction states of reflection and full conduction were distinguished by applying 2 peripheral stimuli (see Fig. 4) between traces displayed here. Top traces: left, averaged synaptic potentials; right, superimposed synaptic potentials. A: P to S synaptic potential when anterior branch point was fully conducting; 6 traces averaged and superimposed. Conduction state was changed from reflection to conduction block by injecting a pulse of depolarizing current that was subthreshold in absence of skin stimulation (bottom trace below action potential). B: reflection increased transmission to the S cell; 10 traces averaged and superimposed. C: conduction block decreased transmission; 7 traces averaged and superimposed. D: collision of peripherally generated P cell impulse with an impulse previously initiated in the P cell soma eliminated transmission to the S cell at the time the synaptic potential would have otherwise appeared (arrow); average of 2 traces. Transmission during a collision was tested both before and after an experiment.](http://jn.physiology.org/)
small molecular tracer filled the S cell and the two lateral C cells (Fig. 6A). HRP was injected into the P cell to label all processes of the cell, including presynaptic terminals. Apparent synaptic contacts could be seen in the light microscope (Fig. 6, B and C). The P cell made $56 \pm 6$ ($n = 4$) contacts within a ganglion; $52 \pm 0.5$ (SD) % of the contacts were anterior of the P cell branch points, which agreed with the physiological results that the synapse was approximately equally distributed on both sides of the branch points. Because the synapse was spatially distributed (see Fig. 1A), reflection and conduction block at both branch points influenced transmission to the S cell.

Because Lucifer yellow easily passes between the S cell and C cells, processes of these cells could not clearly be distinguished in all regions of the ganglion. The S cell was alternatively filled with lysinated tetramethylrhodamine dextran, which does not fill electrically coupled C cells. P cell was filled with Lucifer yellow. Arrows identify examples of apparent synaptic contact between the P and S cells, without the C cell intervening. B: view of region with arrow in A at higher magnification. Inset: enlargement of contact indicated by right arrow.

**DISCUSSION**

These results demonstrate that branch points can have multiple dynamic effects on transmission from a single neuron, extending the known influence that neuronal morphology has over signaling and plasticity. P cell branch points control transmission produced by impulses arising in a single branch while not affecting transmission by impulses arising in other branches. Reflection and conduction block only affect impulses arising in the minor receptive fields, as impulses from the major field or the soma cross the central branch points in the direction of safe propagation from the thick to the thin axons.

**TABLE 1.** S cell synaptic potentials during full conduction, conduction block, and reflection of impulses in the P cell

<table>
<thead>
<tr>
<th></th>
<th>Full Conduction, mV</th>
<th>Conduction Block, mV</th>
<th>Ratio of Block to Full Conduction</th>
<th>Reflection, mV</th>
<th>Ratio of Reflection to Full Conduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>0.61</td>
<td>0.27</td>
<td>0.44</td>
<td>0.93</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>0.86*</td>
<td>0.85</td>
<td>0.99</td>
<td>1.76</td>
<td>2.05</td>
</tr>
<tr>
<td></td>
<td>1.33</td>
<td>1.09</td>
<td>0.82</td>
<td>2.26</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>0.82</td>
<td>0.31</td>
<td>0.38</td>
<td>1.06</td>
<td>1.29</td>
</tr>
<tr>
<td></td>
<td>1.46</td>
<td>0.80</td>
<td>0.55</td>
<td>3.64</td>
<td>2.49</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>0.55</td>
<td>1.75</td>
<td></td>
</tr>
<tr>
<td>Posterior</td>
<td>0.75</td>
<td>0.37</td>
<td>0.49</td>
<td>0.98</td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td>1.86</td>
<td>1.44</td>
<td>0.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.89</td>
<td>0.22</td>
<td>0.25</td>
<td>3.29</td>
<td>3.70</td>
</tr>
<tr>
<td></td>
<td>0.72</td>
<td>0.62</td>
<td>0.86</td>
<td>2.36</td>
<td>3.28</td>
</tr>
<tr>
<td></td>
<td>1.60</td>
<td>0.62</td>
<td>0.39</td>
<td>2.02</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td>0.57</td>
<td>0.32</td>
<td>0.56</td>
<td>1.13</td>
<td>1.98</td>
</tr>
<tr>
<td></td>
<td>0.99*</td>
<td>1.07</td>
<td>1.08</td>
<td>2.28</td>
<td>2.30</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>0.55</td>
<td>2.31</td>
<td></td>
</tr>
</tbody>
</table>

* Excluded from analysis (see text).
Although this delay can be produced by an abrupt increase in membrane conductances is not necessary to account for the presence of reflection and conduction block at rest, and that morphology is sufficient to produce reflection (Baccus 1998). However, models based on experimental results further indicate that when reflection is produced after repetitive firing, spatially nonuniform Ca$^{2+}$-dependent K$^+$ conductances and Na$^+/K^+$ ATPase currents develop as a result of differential accumulation of Ca$^{2+}$ and Na$^+$ in different diameter axons and that these spatial properties tend to maintain the cell in a reflecting state (Baccus 1998). In neocortical pyramidal cells, impulses arising in the region of the soma can lead to slower Ca$^{2+}$ action potentials in apical dendrites that reexcite the soma and axon. These Ca$^{2+}$ action potentials and the resultant reflections can be gated by synaptic inputs (Larkum et al. 1999).

In this study, single brief stimuli were used to measure transmission during full conduction, reflection, and conduction block. Single brief stimuli have also been used during behavioral experiments to measure sensitization and dishabituation (Modney et al. 1997; Sahley et al. 1994). During trains of impulses however, the effects of reflection on transmission will be more complex. If the interval between peripheral impulses decreases to less than twice the conduction time, reflected impulses will begin to collide with peripheral impulses, thus reducing central transmission. Additionally, at increased firing frequencies, synaptic transmission from P cells facilitates (Muller and Nicholls 1974) so that at higher frequencies, each fully conducting impulse will produce greater transmission. This may reduce the difference in transmission produced by reflection as compared with full conduction. Therefore the greatest enhancement by reflection will be produced when reflection occurs at low firing frequencies.

Although the effects of conduction state examined here involve transmission in the same ganglion that contains the soma, P cells, like other mechanosensory neurons, form synaptic connections in several ganglia (Fig. 1A). Because reflection and conduction block change the way impulses propagate throughout the presynaptic cell, these mechanisms will affect transmission to multiple S cells.

Depletion of 5-HT throughout the leech eliminates sensitization and disrupts dishabituation, which are behavioral effects very similar to killing the S cell, although the S cell is not itself serotonergic (Ehrlich et al. 1992). Noxious stimuli of the type that produce sensitization also activate cells that release 5-HT (Sahley 1988). Because one effect of 5-HT is to relieve conduction block in mechanosensory neurons, 5-HT may increase sensory transmission to the S cell during nonassociative learning.

Changes in transmission are thought to underlie various types of learning. Neuronal branching pattern can dynamically change transmission from subsets of synapses in multiple ways under cellular control; these properties are sufficient to influence synaptic and behavioral plasticity.

We thank Drs. J. Barrett, R. Bookman, A. Chen, D. Johnston, W. Nonner, and R. Rotundo for helpful discussions, and Dr. A. Caiço for help in use of Dr. S. Roper’s confocal microscope.

This work was supported by National Institute of Neurological Disorders and Stroke Grant ROI-NS-34927 to K. J. Muller and C. L. Sahley. S. A. Baccus was supported by a Howard Hughes Medical Institute Predoctoral Fellowship (Baccus 1998).
REFERENCES


