BRIEF COMMUNICATION

LOCATING SPIKE-INITIATING OR SPIKE-ENTRY ZONES IN A BRANCHED MOLLUSCAN NEURON

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ABSTRACT A theoretical method for locating spike-initiating or spike-entry zones within a single neuron was experimentally verified using the cell RPI in the marine opisthobranch *Navanax inermis*. Multiple sites of spike-initiation were found to exist in RPI. Furthermore, it was demonstrated that extracellular recording alone was sufficient to map sections of cells' branching patterns and possibly locate multiple spike-initiating zones.

Specifying the number and location of spike-initiating zones within a single neuron is important to an understanding of that neuron's function (1, 2, 3, 6, 7, 8, 11). For example, the presence of spike-initiation zones in separate branches of a frog lateral-line neuron affects the sensitivity of the neuron to various types of stimuli (6). Also, multiple spike-initiating zones in a single molluscan nerve cell can result in independent activity in different branches due to selective invasion by spikes initiated in different parts of the cell (11).

We show here that is it possible to demonstrate the existence of multiple sites of spike-initiation and obtain some information concerning their location using spike conduction-time measurements obtained from peripheral axons of the neuron. However, all the spike-initiating zones may not be found; this method can be used to determine a lower bound on the number of such zones within a neuron. Spike conduction-time measurements have been used by others to locate sites of spike initiation within neuronal structures (1, 10). However, the techniques previously published require difficult electrophysiological methods and/or are limited to special types of neurons. This paper presents results obtained from measurements made both intracellularly at the soma and extracellularly from processes in peripheral roots. In general, however, intracellular recordings are not necessary to utilize this method.

Locating spike-initiation zones of synaptically or endogenously generated spikes requires two steps: First, one measures the times that stimulus-evoked spikes arrive at the various peripheral recording sites upon stimulating at each peripheral recording site in turn; these data are sufficient to determine the branching pattern and branch conduction times of the cell. Second, one observes the relative arrival-times of synaptically-generated spikes at the peripheral recording sites; these data, combined with the results from the first step, are used to

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specify where the synaptically-generated spikes arise within the branch structure of the neuron.

This technique has already been described theoretically in great detail (5); a brief review is presented here (see Appendix for numerical example).

Consider a neuron which has a monopolar soma giving rise to an axon which ramifies to produce three branches. Fig. 1 *a*, and *b* shows the two possible structural classes of such a neuron; either there is a single branch-point from which all three branches arise, or there are two branch-points. Now suppose spikes are initiated at R_3 and then at R_4 and their relative arrival times are measured at R_1 and R_2. If the structure has a single branch-point then the difference between arrival times at R_1 and R_2 will not depend on whether the spikes were

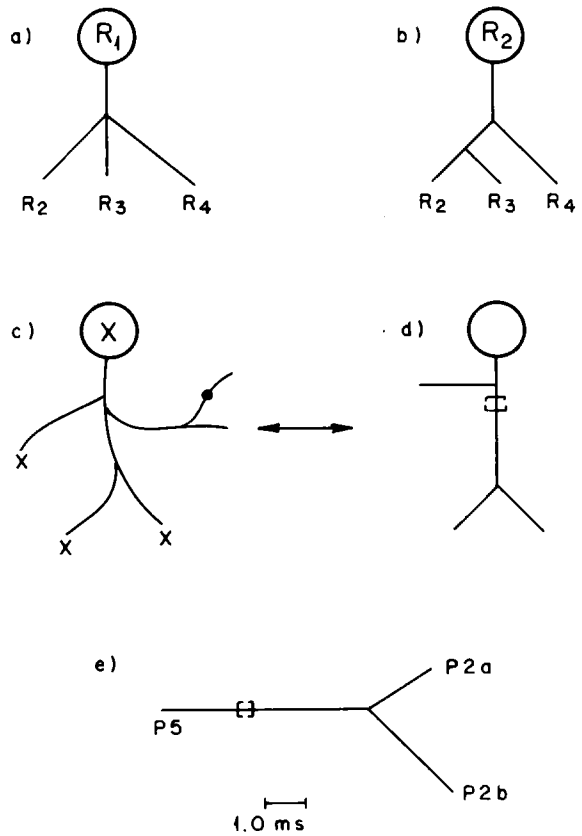

FIGURE 1 (*a*) A hypothetical neuron with a single branch-point. Spikes which arise at either R_3 or R_4 will have the same relative arrival-time between points R_1 and R_2. (*b*) A hypothetical neuron with two branch-points. In this configuration the relative arrival-time between points R_1 and R_2 will depend on whether the spike was initiated at R_3 or R_4 (*c*) A hypothetical neuron with six terminal points. However, only four (X) are available for recording and stimulating. Also, a spike-initiating zone (●) is on an unavailable branch. (*d*) The reconstruction which would result from arrival time data taken from the neuron in *c*. Note that only four branches are specified and that the spike-entry zone (marked with brackets) appears where the spike enters the reconstructed part of the branching pattern. (*e*) The scaled stick figure reconstruction from the numerical example in the appendix. Note that this is a section of the reconstruction in Fig. 4.

initiated at R_3 or R_4 However, if there are two branch-points, as in Fig. 1 *b*, then spikes arising at R_3 will arrive relatively earlier at R_2 than at R_1, as compared with spikes initiated from R_4 Therefore, a distinction between these structures can be observed by stimulating endings and measuring spike arrival times.

Theoretically, this method allows complete determination of a neuron's branching structure and branch conduction times. However, this is dependent on the following assumptions: (*a*) It is assumed that all endings of the neuron are experimentally accessable for recording and stimulating. If a branch is inaccessible, its existance will not be noticed (Fig. 1 *c* and *d*). This is particularly important if a site of spike-initiation is on an unavailable branch. Then one can only identify where the spike enters the part of the cell which has been mapped. For this reason, these areas are called spike-entry zones—they are not necessarily spike-initiation zones. Fig. 1 *c* and *d* graphically displays this situation. When two branches exist within the same nerve trunk it is possible that they will appear as a single fiber in the results. However, if the two branches have significantly different conduction-times they may be detected. When a stimulus-evoked spike is initiated from another trunk, two spike arrival-times will be recorded in the double-fiber trunk. Furthermore, stimulus of the double-fiber trunk could produce two spikes in all other recording sites. Verification could be made by observing stimulus thresholds for the two spikes. Of course, invasion failure, selective gating, and/or collisions may obscure these effects. (*b*) It is assumed that propagation of spikes proceeds freely throughout the structure. Isotropic delays will be interpreted simply as slower average conduction times; directionally-dependent delays, however, can result in erroneous reconstruction and misplacement of sites of spike-entry. Invasion failure is equivalent to making the uninvaded branch inaccessable (assumption *a*). Also, spontaneous activity may cause spike collisions, so that repeated measurements are necessary. (*c*) It is assumed that there are no loops in the branch structure (i.e., there is just one path from any point to any other point). (*d*) It is assumed that there is no unknown response latency at the site of stimulation. This assumption only affects branches which are directly connected to a recording and stimulating site (external branches). If an unknown latency exists, then the conduction-time determined for the branch connected to the stimulating site will be in error by one-half the latency time. The branch conduction-times of internal branches are completely determined by the relative arrival-times and are therefore not affected by stimulus response latencies. (*e*) It is assumed that stimulus-evoked spikes can be readily distinguished from synaptically or endogenously generated spikes. In our experiments, stimulus-evoked spikes are identified through repetition of the stimulus and waveform appearance. A spike must consistantly be observed at a specific time after stimulation. Furthermore, a stimulus-evoked spike appears to rise straight out of the baseline—synaptically-driven spikes are preceded by an EPSP.

To display the results from conduction-time measurements a stick figure is constructed (Fig. 1 *c* and *d*) which shows the branching pattern of the cell and represents the average spike conduction time within a branch by the length of the corresponding stick figure branch. The results of the relative arrival-time data from synaptically-driven spikes are graphically represented in the stick figures by brackets which indicate the maximum ranges of the calculated spike-initiation loci.

The experiments were performed on an identified cell in the right pedal ganglion of the marine opisthobranch *Navanax inermis*. This cell, denoted RP1 (4), is found anteriorly on the

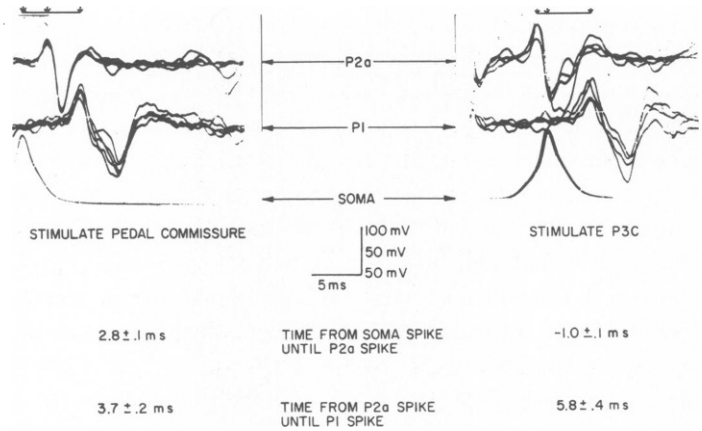

FIGURE 2 Examples of conduction-time measurements from oscilloscope traces. While recording from three sites (p2a, pl, soma) stimulation is first applied to the pedal commissure (left half of the figure). Relative conduction times are recorded–2.8 ms from the soma spike arrival until the p2a spike arrival; 3.7 ms from the p2a spike arrival until the pl spike arrival. When p3c is stimulated, the relative arrival times change. Now the soma spike arrives after the p2a spike.

ventral surface of the ganglion. RP1 has a large soma (70–200 μm), is easily identified, and has several processes in peripheral nerves which are available for stimulating and recording. It was possible to reliably produce stimulus-evoked spikes from the peripheral roots and obtain data allowing the reconstruction of the axonal branching pattern as a stick figure. See Fig. 2 for examples of conduction-time measurements from oscilloscope traces. By activating excitatory synapses on RP1 through stimulation of other cells, synaptically-evoked impulses were obtained. The relative arrival-time data from these spikes were used to locate the spike-entry zones in the stick figure model of the cell.

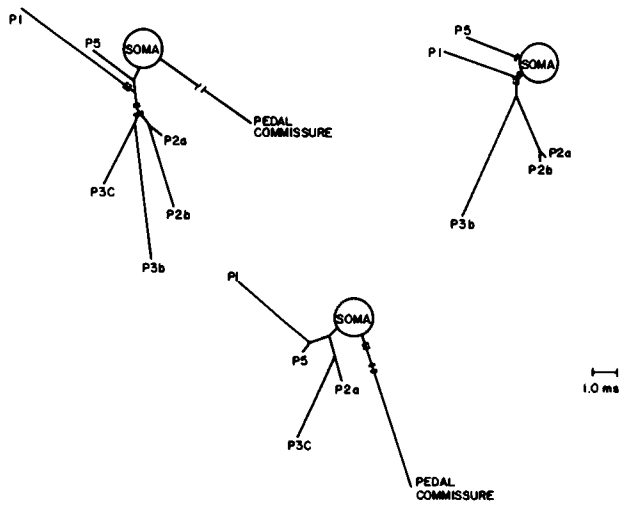

FIGURE 3 Stick figures from three experiments (RP1 from three different animals) in which two spike-entry zones were located. The brackets in the stick figures delimit the spike-entry zones found.

Fig. 3 shows stick figure models of three different RP1 cells in which more than one spike-initiation zone was found. The three figures look similar overall but contain some obvious individual differences. If a process was not available for stimulating and recording it cannot show up in the stick figure reconstruction as was explained earlier and as depicted in Fig. 1 *c* and *d*. This explains why the three trees do not all have the same processes. Spike-entry zones do not show up in the same places; this may be because not all the spike-entry zones were detected in any one experiment, e.g., some zones were not activated. Also, the locations of the spike-initiation zones may vary from animal to animal. Another difference in the figures is the placement of the soma with respect to the branches. This occurs because stimulus-evoked spikes could not be reliably generated from the soma of RP1. Therefore, in the stick figure reconstructions it is undetermined whether the soma attaches directly to the remainder of the tree or connects via an interposed branch.

To determine whether the stick figure reconstructions truly reflected the cells' geometries, $CoCl_2$ was injected intracellularly (9) after the electrophysiological measurements were completed. The anatomy of the cell was then recorded in camera lucida drawings from the whole-mounted ganglion. As shown in Fig. 4, the stick figure is an accurate representation of

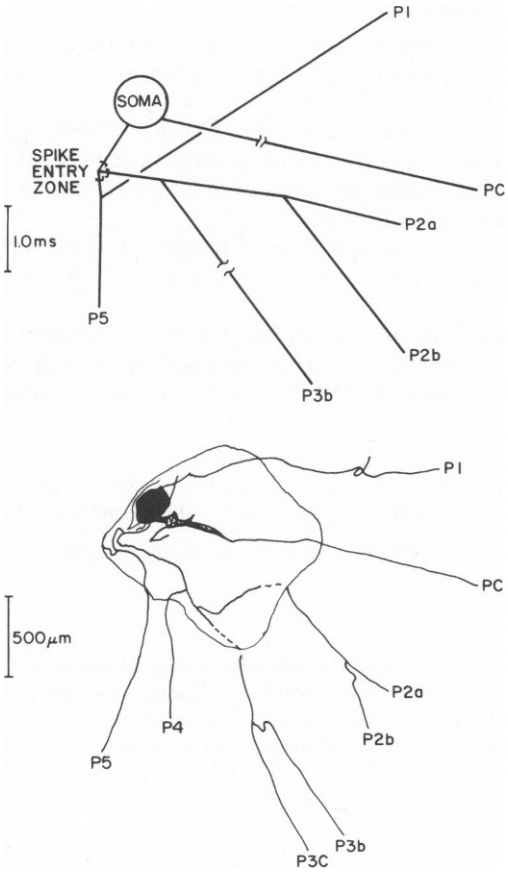

FIGURE 4 Comparison of a stick figure reconstruction with a camera lucida drawing of the cell after cobalt staining. The stick figure is an accurate representation of the actual cell.

the actual cell; the only anatomical point in question is where the process in peripheral root "Pl" connects to the rest of the cell. It disappears into a fold of the soma and its connection-point cannot be located in the whole-mount preparation.

Some of the difficulties encountered in performing these experiments are likely to confront other experimenters. If several peripheral processes are available for recording and stimulating then the large number of measurements required make the experiment somewhat time-consuming and tedious. Also, stable and reliable axon reflexes are not always present; this limits the completeness of the reconstruction and hence limits the precision with which spike-entry sites can be located.

Nonetheless, these results establish that this procedure can be used to determine the branching pattern of a cell and to locate spike-entry zones within that pattern.

APPENDIX

A numerical example will help to clarify the procedure used. Here is the construction of a section of the stick figure in Fig. 4 from the data: Stimulate p2a: (arrival time at p5)-(arrival time at p2b) = 6.6 ± 0.1 ms −4.6 ± 0.2 ms = 2.0 ± 0.22 ms; Stimulate p2b: (arrival time at p5)-(arrival time at p2a) = 8.0 ± 0.2 ms − 4.7 ± 0.2 ms = 3.3 ± 0.28 ms; Stimulate p5: (arrival time at p2a)-(arrival time at p2b) = 6.7 ± 0.1 ms −7.9 ± 0.1 ms = −1.2 ± 0.14 ms.

A tree of three branches must contain a single node. Since all three branches are external, one of the stimulus → arrival-time measurements must be used in addition to the relative arrival-time measurements (see assumption *d*).

Let the conduction-time from the node to the end of p5 equal x. Then the conduction time from the node to the end of p2a equals x-3.3 ms and the conduction time from the node to the end of p2b equals X-2.0 ms. Using 6.7 ms as the conduction time from p5 to p2a, one finds that x = 5 ms.

The resulting reconstruction is shown in Fig. 1 *e*

Now consider spikes generated synaptically from stimulation of p4 and let the arrival-time at p2a be the arbitrary origin: arrival-time at p2a = 0 ± 0.2 ms, arrival-time p2b = 1.3 ± 0.2 ms, arrival-time at p5 = −2.7 ± 0.1 ms.

The arrival-time at p2b is 1.3 ms after the arrival-time at p2a. Considering the reconstruction done above, this indicates that the spike-entry zone is located along p5. The relative arrival-time at p5 specifies the location along that branch. Refer to Fig. 1 *e* for the placement of the spike-entry zone within the tree.

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