Action Potential Initiation and Propagation in CA3 Pyramidal Axons

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INTRODUCTION

Action potential initiation and downstream axonal propagation have been topics of intense scrutiny over several decades and are critical to nervous system information processing (Debanne 2004). However, much of what we know about spike initiation and propagation comes from studies of larger, myelinated axons. Thin, unmyelinated axons are found throughout the hippocampus and cortex and serve the important task of interneuronal communication. Models of the proximal axon incorporating observed distributions of sodium channel staining recapitulate salient features of somatic and axonal spike waveforms, including the predicted initiation zone, characteristic spike latencies, and conduction velocity. The preferred initiation zone was unaltered by stimulus strength or repetitive spiking, but repetitive spiking increased threshold and significantly slowed initial segment recruitment time and conduction velocity. Our work defines the dynamics of initiation and propagation in hippocampal principal cell axons and may help reconcile recent controversies over initiation site in other axons.

We studied spike initiation and propagation in unmyelinated fibers of CA3 pyramidal neurons, which give rise to intensely studied synapses of the hippocampal Schaffer collateral pathway. Several critical features of spike initiation are still controversial even in myelinated fibers. For instance, in rodent cerebellar Purkinje cells, some data suggest distal initiation at the first node of Ranvier, which typically coincides with the first axon branch point (Clark et al. 2005). Other studies suggest somatic/axon hillock initiation (Khalilq and Raman 2006). Studies of myelinated subicular and layer V neocortical axons place initiation at the distal part of the initial segment (Colbert and Johnston 1996; Palmer and Stuart 2006), similar to recent estimates from unmyelinated segments of ferret cortical neurons (Shu et al. 2006a). There is also controversy regarding whether the initiation site does (Jenkins and Bennett 2001; Komada and Soriano 2002; Wollner and Catterall 1986) or does not (Colbert and Johnston 1996; Colbert and Pan 2002) correlate with a high density of sodium channels, which modeling predicts is necessary for axonal initiation (Dodge and Cooley 1973; Mainen et al. 1995; Rapp et al. 1996).

These issues are further compounded in small axons such as those of CA3 pyramidal neurons, which are unmyelinated over large portions, including the first several hundred micrometers. The initial segment lacks the structural and morphological hallmarks of myelinated axons, making a morphological correlate of initiation unclear. Here we consider the initial segment to extend from the axon hillock to the first branch point, −50–75 μm away in these cells. We have previously shown that these fibers are unmyelinated at the age of our studies, over at least the first 1 mm distal to the soma (Meeks et al. 2005). Thin, unmyelinated fibers are still typically studied with field potential measurements of compound action potentials, which obscure the specific signals of individual fibers, or with antidromic activation of fibers, which is inappropriate for studying normal initiation. Because of the difficulties in studying these fibers, there is little information on the initiation site, i.e., the site during a depolarizing stimulus that first reaches spike threshold. Furthermore, it is unclear whether action potential initiation and propagation in these fibers are themselves plastic processes, susceptible to activity-dependent changes found in other processing compartments.

To explore the dynamics of spike initiation, we used a combination of antibody staining, local drug application, and simultaneous whole cell recordings of CA3 pyramidal cell somas and extracellular recordings from their attached axons (Meeks et al. 2005). Sodium channel staining and local application of tetrodotoxin to the proximal and distal axon locations suggested a more proximal initiation zone than our previous spike latency analyses suggested (Meeks et al. 2005). Modeling and investigation of spike latency plots show a zone of initiation in the axon extending from 10 to 100 μm from the soma, presumably reflecting an initiating zone in unmyelinated fibers that classical theory suggests is necessary for faithful propagation (Rushton 1937). Analysis of spike latencies at the soma and distal axon recording locations showed little change in the location of first threshold crossing even under conditions...
that significantly alter spike threshold, such as repetitive spiking. However, initiation recruitment time and downstream conduction velocity were significantly slowed by repetitive activity. Our analyses show that appropriate latency measurement is critical for interpreting the dynamics of spike development in the axon.

**METHODS**

**Hippocampal slice preparation**

Animal use protocols were approved by the Washington University Animal Studies Committee. Eighteen–to 22-day-old Sprague-Dawley rats were anesthetized with isoflurane by inhalation and decapitated. Brains were dissected into oxygenated ice-cold slicing buffer containing (in mM) 87 NaCl, 25 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 0.5 CaCl2, 3.0 MgCl2, 25 glucose, 75 sucrose, and 0.5 kynurenic acid. Transverse slices (300 μm) were made through the hippocampus using a vibratome (World Precision Instruments, Sarasota, FL). Slices were immediately transferred to artificial cerebrospinal fluid (ACSF) containing 2 mM kynurenic acid at 34°C for 30 min and then incubated at room temperature (22–25°C) for ≥30 min prior to recording. The ACSF contained (in mM) 125 NaCl, 25 NaHCO3, 3 myo-inositol, 3 Na-pyruvate, 2.5 KCl, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, 0.4 ascorbic acid, and 10 glucose. Salts and compounds were obtained from Sigma-Aldrich (Saint Louis, MO) unless otherwise noted.

**Paired intracellular/extracellular recordings**

Paired intracellular/extracellular recordings from CA3 neurons and axons were obtained similar to previous published methods (Meeks et al. 2005). Recordings were made from slices submerged in ACSF containing postsynaptic blockers: 1 mM 2,3-dioxy-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; Tocris-Cookson, Ellisville, MO), 25 μM d(-)-2-amino-5-phosphonopentanoic acid (d-APV, Tocris-Cookson), and 100 μM picrotoxin (Sigma-Aldrich). Solution was constantly oxygenated and perfused at 2 ml/min.

Patch and extracellular recording pipettes were pulled from borosilicate glass (World Precision Instruments) with a Flaming/Brown micropipette puller (P-87 Model, Sutter Instrument, Novato, CA). Internal solution contained (in mM) 130 potassium gluconate, 4 NaCl, 0.5 CaCl2, 5 EGTA, 10 HEPES, 4 Mg-ATP, and 0.3 Na2-GTP and 10–200 μM AlexaFluor 488 or AlexaFluor 568 hydratize (Molecular Probes, Eugene, OR). Open-tip pipette resistance for patch electrodes was 4–8 MΩ and for extracellular pipettes 1.5–3.5 MΩ. Somatic recordings were obtained using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Bridge balance and pipette capacitance compensation were automatically adjusted using Multi-Clamp 700B Commander software attached to the MultiClamp 700B amplifier. Signals were acquired at 50–250 kHz, filtered using a 10 kHz (soma) or 3 kHz (axon) 8-pole Bessel filter and digitized using a DigiData 1322A 16-bit A/D converter (Axon Instruments) running on a Pentium 4-based personal computer running PClamp 9.2 acquisition software (Molecular Devices).

Whole cell, current-clamp recordings were achieved from somas of CA3 neurons visually identified under infrared differential interference contrast microscopy on a Nikon E600FN microscope (Nikon, Melville, NY) and with a cooled digital camera (Roper Scientific, Tucson, AZ). Cells were current-clamped to their initial resting potentials (approximately –70 mV) and held for 10–15 min to allow for dye diffusion. After positioning the extracellular pipette nearby, the axon was visualized by dimly illuminating the cell. Light suction to the extracellular pipette achieved the loose-seal, single-axon recording configuration (Raastad and Shepherd 2003). Cells that exhibited >10% change in firing frequency in response to 1-s depolarizing pulses to the soma (delivered periodically before and after establishment of the paired soma/axon recording) were considered damaged and recordings were discarded (<10% of all recordings). Axonal recordings were performed in voltage-clamp mode of the patch amplifier at a command potential of 0 mV. Axonal signals are displayed as the raw clamp current obtained by the amplifier. All presented records were obtained at room temperature. Additional recordings at 32–34°C yielded differences discussed in the Results. Local puffer applications of 500 nM tetrodotoxin were achieved by positioning a sharp patch pipette (15–20 MΩ) within 10 μm of the target region followed by a 1- to 5-s pressure application at 5–10 psi. The combined use of lower TTX concentration and higher tip resistance than in previous similar studies (Colbert and Johnston 1996; Khaliq and Raman 2006; Palmer and Stuart 2006) helped limit spread of TTX. Furthermore, the combination of positive and negative results obtained from individual neurons (Fig. 1) validated both the focal nature and the effectiveness of applications.

For excitatory postsynaptic current (EPSC) generation, a theta glass (50 μm tip diameter) filled with extracellular solution served as a bipolar stimulating electrode. The stimulus electrode was waxed in the hilus near the inner blade of the dentate granule cell layer, and stimulation (50–200 μA) in the presence of 100 μM picrotoxin and 50 μM d-APV elicited AMPA receptor-mediated EPSCs from CA3 pyramidal cells.

**Within-cell initiation site and conduction velocity estimates**

Raw somatic and axonal traces were filtered with a 5 kHz Bessel filter. First and second derivatives (dV/dr and d2V/dr2, respectively) of the somatic waveform and first derivatives of the axonal waveform were calculated using ClampFit 9.2 software. The somatic dV/dr2 waveforms contained two distinct peaks. Based on modeling results, we defined threshold for spike initiation by choosing the time at which the first of these d2V/dr2 peaks reached 5% of its maximal value. To assist in identifying this time, we fit a single-order Gaussian function to the leading edge of the d2V/dr2 first peak, which reduced noise contributions to the threshold estimate. Other latencies are as described in RESULTS and Fig. 5B. Measures of T1 and T2 latencies were derived from the second derivative of the somatic waveform (T1 and T2), compared with the first derivative of the extracellular waveform (T2). T1 represents the time from the site of first threshold crossing (denoted as 5% of the first peak in somatic second derivative, taken from Gaussian fits to the signal) to the first peak of the somatic second derivative, which we interpret as the maximum Vm acceleration at the axon hillock as explained in RESULTS. T2 represents the time from the site of first threshold crossing (taken again from the 5% inflection in the somatic second derivative) to the time of maximum Vm acceleration (peak of the 1st derivative of the extracellular signal) at the axon recording pipette. Average conduction velocity along the axon was calculated by dividing the measured three-dimensional length from the soma to the recording site by the combined T1 + T2 time. The ratio of T1/(T1 + T2) multiplied by the length of the recording site estimated the site of spike initiation. This should be considered an estimate only, however, since we know that conduction velocities in both directions are not constant (Shu et al. 2006b). For calculations of Fig. 7A, recruitment time, the average T2 value from axon recordings <100 μm distal to the soma (see RESULTS) was subtracted from T1 and T2 latencies. For Fig. 7A, a fixed 0.02-μs latency (2 sample points) was added to experimental times to prevent division by zero and associated infinite conduction velocities. Based on the results of Figs. 4, 5, and 7, we determined that approximations of the initiation site are only accurate in the context of a broader initiation zone, and do not reflect a pure “site” of initiation. Nevertheless, as indicated in Figs. 6 and 8, T1 and T2 values are not static, and changes in the relationship between these elements can indicate substantial changes in spike initiation and propagation.

Error bars and values represent ± SE for all amplitude/magnitude measurements and calculations depending on axon recording length.
FIG. 1. Focal TTX application indicates that spike initiation occurs in the proximal CA3 pyramidal cell axon initial segment. A: voltage-gated sodium channel blocker tetrodotoxin (TTX, 500 nM) was focally applied to different visualized regions of CA3 soma and axon initial segment during whole cell recordings. Short, suprathreshold current pulses delivered through the somatic recording electrode (1–2 nA × 10 ms) elicited single action potentials in all conditions. Somatic action potential threshold (arrows) was maximally depolarized when TTX was applied to proximal regions of the axon initial segment (red trace) compared with control untreated (black trace) and treated distal initial segment (blue trace). B: 2nd example of TTX application to the proximal initial segment (red trace) and the apical portion of the soma (gray trace) compared with control (untreated, black trace). Note the visible difference in the shape of the dV/dt (middle) and d2V/dt2 (bottom) waveforms across the conditions. C: summary of effects on threshold when 500 nM TTX was delivered to the indicated regions during somatic current injection to elicit a spike. Identical somatic current pulses were delivered immediately before and during focal TTX application. The difference in voltage threshold before and during TTX application is plotted for 5 individual cells. Four of the 5 cells showed a depolarization of somatic spike threshold during application to the proximal region of the axon initial segment (20–40 μm from the soma) compared with applications at the distal segment (60–80 μm from the soma).

Error bars and values for latency measurements represent ± SD to avoid variance estimates smaller than our sample interval. Tests of significance were performed using paired and unpaired Student’s t-test, where appropriate. Significance threshold was set at significance were performed using paired and unpaired Student’s t-test, where appropriate. Significance threshold was set at $P < 0.05$.

Immunohistochemistry and image analysis

Slices were fixed immediately in freshly prepared 4% paraformaldehyde for 30 min after recording. Filled cells were imaged using a Nikon C1 laser scanning confocal attachment to a Nikon Eclipse TE300 inverted microscope. Filled cells were reconstructed in three dimensions using Nikon C1 software, and three-dimensional measurements of axonal recording distance were made using MetaMorph 6 software (Molecular Devices).

Sodium channel density was measured by counter-staining with an antibody against a conserved cytoplasmic domain of sodium channel alpha subunits (PanNav, Sigma-Aldrich, St. Louis, MO). Immediately after fixation, hippocampi were incubated for 90 min in blocking solution consisting of PBS with 6% normal goat serum, 0.3% Triton-X100, and 3 mM sodium azide (Boiko et al. 2003). Primary antibodies

FIG. 2. Voltage-gated sodium channel alpha subunit immunostaining indicates sodium channel density varies over the initial 100 μm of the axon initial segment, peaking 35–40 μm from the soma. A: maximum projection image of a filled CA3 neuron. The first ~60 μm of the axon is highlighted by the red box. B: monoclonal antibodies against a conserved region of voltage-gated sodium channel alpha subunits (PanNav) stained many axon initial segments along CA3 pyramidal cell layer—stratum oriens boundary. C: expanded view of the area boxed in A. C1: AlexaFluor 488 fill. C2: PanNav sodium channel stain. C3: merged image. Green indicates AlexaFluor 488 fill. Magenta indicates the PanNav stain. Overlapping pixels appear white (arrows). D: side-view of the axon of interest in A–C. x axis represents combined x+y dimensions along the traced axon in A–C. y axis represents the z dimension (depth) into the slice. D1: AlexaFluor 488 fill. D2: PanNav staining. D3: merged image. Arrow indicates region of dense sodium channel staining. E: quantification of PanNav sodium channel staining along the axon. The black circles represent intensity values along the length of the axon shown in A–D. The red line indicates a best-fit Gaussian curve to these data. Areas of apparent increased intensity at ~150 and ~275 μm result from axons different from the filled axon passing just above or below the axon of interest and do not reflect regions of accumulation in the axon of interest. F: top data points represent the point of peak staining intensity (solid symbols) and SDs (× error bars) from Gaussian fits to 22 immunostained axons from AlexaFluor-filled CA3 neurons. Bottom: average best-fit curve from the same 22 filled and immunostained CA3 pyramidal cells. The fit indicates an average peak sodium channel density at ~35 μm from the soma.
were diluted 1:100–1:500 in blocking solution. Slices were incubated at room temperature in primary-containing solution for 48–72 h and then washed in blocking solution for 90 min and in PBS for 15 min. Tissue was incubated with secondary antibody (AlexaFluor 467 goat anti-mouse, 1:250–1:500) for 60 min followed by a 30-min wash in blocking solution and a brief rinse in PBS. Slices were then mounted on coverslips and imaged using the Nikon C1 confocal system described in the preceding text. Tissue was not sectioned for imaging. Antibody penetration into the relevant slice depth was verified by the presence of stained axon initial segments surrounding the filled axon in the same confocal plane. Superficial somas were chosen for filling to minimize chances that the proximal axon would be lost to staining, and to help ensure good antibody access, cells were discarded from analysis if the first 100 μm of proximal axon (the portion analyzed for staining intensity) extended below 50 μm from the slice surface.

Relative sodium channel density along the axon was measured using MetaMorph software. A tracing of the axon along its visible trajectory was made in three dimensions using the Alexa 488 fill. The axon’s path was then analyzed using the “Kymograph” feature of MetaMorph, resulting in a composite two-dimensional image with the x axis representing the combined “x” and “y” dimensions of the traced axon and the y axis representing the course of the traced axon in the “z” dimension (see Fig. 2). The traced axon region was then transferred to the immunostaining channel, which was also transformed. This allowed clear isolation of the colocalized fill/sodium channel staining from other nearby stained axons. In the transformed image, the axon trajectory in the AlexaFluor 488 (fill) channel was traced and transferred to the AlexaFluor 647 (PanNav). The linescan function of MetaMorph was used to quantify the average AlexaFluor 647 intensity within 5 pixels along the calibrated length of the traced axon (see Fig. 2, D and E). Gaussian functions were used to define the peak and spread of the PanNav signal along each filled axon (Fig. 2E). A composite distribution was made for illustrative purposes by plotting the normalized average mean and SD values of the 22 Gaussian fits (Fig. 2F).

CA3 axonal initiation computer simulation

We implemented a reduced compartmental model of a CA3 pyramidal cell and its proximal 800 μm of axon using NEURON 5.9 (http://neuron.duke.edu). The morphological values used in this model are presented in Table 1. We employed a simplified dendritic morphology and limited dendritic active properties compared with dendrite-focused models (Mainen et al. 1995; Major et al. 1994; Traub et al. 1994) to reduce computational load and limit the number of model variables. The axonal tapering was chosen to match the observable taper of axons recorded from in this study as determined from confocal reconstructions.

The biophysical properties for the model compartments are indicated in Table 2. The axon had passive properties ($C_m$ and $G_m$) similar to standard values in the literature (Mainen et al. 1995; Major et al. 1994; Rapp et al. 1996; Traub et al. 1994). The $C_m$ and leak $G_m$ values of the somatic and dendritic compartments were adjusted (up and down, respectively, compared with axonal values) to match somatically recorded passive charging properties obtained experimentally from CA3 cells in this study. Part of this adjustment reflected the simplification of the entire dendritic tree and spines into a few dendritic model compartments (see Tables 1 and 2), although even realistic dendritic models have adjusted somatodendritic passive properties to account for model deviations from experimental observations (Major et al. 1994; Traub et al. 1994). The time constant (τ) obtained from the model cell soma was 111 ms compared with 133 ± 15 ms obtained from five experimentally derived somatic CA3 recordings. The model input resistance was 248 MΩ, compared with 441 ± 50 MΩ from the experimental recordings. Without adjustment of somatodendritic passive membrane properties away from more standard values used for the axon, simulation input resistance was 16 MΩ and time constant was 1.7 ms, significant deviations from experimental values. To ensure that our choice to manipulate somatodendritic membrane capacitance and resistivity values did not affect salient results from the simulations, we ran simulations with unadjusted values for dendrites and soma (Supplementary Figure 1). Although the passive properties deviated from experimentally measured responses, the shape of second derivative somatic waveforms (e.g., Fig. 3C) and of latency measurements (e.g., Fig. 5C) were nearly unaltered. These results suggest that the major results apply under a broad range of somatodendritic morphologies and membrane properties.

In our standard model the axonal length constant, measured as the steady-state voltage change in successive axonal compartments (from the hillock distal) to a current injection at the soma compartment, was ~450 μm, consistent with several studies, including (Alle and Geiger 2006; Shu et al. 2006a). The model contained a variable distribution of voltage-gated sodium channels along the soma and axon as illustrated in Fig. 3. These values were chosen to match the shape of the distributions obtained from immunohistochemical sodium channel staining (Fig. 2). The shape of the NaCh conductance distribution was scaled to a maximum value that reproduced somatic action potential voltage ($V_{max}$), first derivative ($dV_{max}/dt$), and second derivative ($d^2V_{max}/dt^2$) waveforms. The basal NaCh conductance of 0.1 S/cm² in the proximal and distal axon was approximately three times the comparable axonal value used outside the initial segment in previous studies (Mainen et al. 1995) because lower NaCh peak conductance values did not accurately reproduce spike waveforms in our CA3 model. Dendritic NaChs were added at 0.005 S/cm². Nonactivating voltage-gated potassium channels (Mainen et al. 1995) were added at densities empirically determined to preserve the shape of somatic and axonal waveforms: 0.01 S/cm² in the soma and axon, and at 0.04 S/cm² in the dendrites.

Sodium and potassium conductances were similar to those of Mainen et al. (1995) and implemented using the channel builder function of Neuron 5.9. Sodium and potassium conductance kinetics were specified by $m^h$ and $m^I$ formalisms, respectively. The $m$ and $h$ voltage dependence of these channels were specified by the ($\alpha$, $\beta$) strategy of Channel Builder. The $\alpha$ and $\beta$ values were constructed

### Table 1. Morphological values of key compartments in the CA3 initiation model

<table>
<thead>
<tr>
<th>Component</th>
<th>Length μm</th>
<th>Diameter μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soma (1)</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Apical dendrite (1)</td>
<td>150</td>
<td>3.0</td>
</tr>
<tr>
<td>Basal dendrites (2)</td>
<td>150</td>
<td>2.5</td>
</tr>
<tr>
<td>Distal dendrites (3)</td>
<td>300</td>
<td>1.5</td>
</tr>
<tr>
<td>Axon initial segment (20)</td>
<td>5</td>
<td>1 (distal)-3.0 (proximal)</td>
</tr>
<tr>
<td>Proximal axon (20)</td>
<td>10</td>
<td>0.75</td>
</tr>
<tr>
<td>Distal axon (50)</td>
<td>10</td>
<td>0.5</td>
</tr>
</tbody>
</table>

1 The online version of this article contains supplemental data.
using the equation: 
\[ (\alpha, \beta) = A'x/(1 - e^{-x}(r)) \]  
where \( x = k^*(v - d) \).
The constants supplied for these conductances are given in Table 3.

Model output was assessed at each compartment from the soma down the axon. Model output was converted for analysis in the ClampFit 9.2 program used to analyze experimental recordings. Point current- and voltage-clamp processes were implemented to initiate simulations at the model soma. Raw axonal signals are shown as negative-going to reflect the polarity observed in voltage-clamp mode. Corresponding simulation outputs are displayed as positive because of their derivation from intracellular \( V_m \) waveforms.

**RESULTS**

**Local tetrodotoxin application to identify initiation site**

We recently estimated a fairly distal initiation site in CA3 pyramidal neurons of hippocampus from bilinear fits to axonal latency versus recording distance plots. As an empirical test of these estimates, we used local applications of the sodium channel blocker tetrodotoxin. Previous experiments have shown that somatic first and second derivative waveforms (Colbert and Johnston 1996; Coombs et al. 1957; Khaliq and Raman 2006; Palmer and Stuart 2006), and second derivative (Fig. 1, middle) of somatic spikes. Analyses of other cell types have shown that somatic first and second derivative waveforms contain multiple components, particularly evident as double peaks in the second derivative (Fig. 1, A and B). The early component in other cells has been associated with the axonal spike and the later component with the somatic spike (Colbert and Johnston 1996; Coombs et al. 1957; Khaliq and Raman 2006; Stuart and Hausser 1994). The time lag between the two components represents the impedance mismatch between the two compartments and the associated time for somatic channel recruitment. Consistent with this interpretation, tetrodotoxin significantly reduced the first (axonal) component of the second derivative waveform when applied to the axon 20–40 \( \mu \)m away from the soma (Fig. 1, A and B, middle). The later component of the second derivative waveform (Fig. 1, A and B, bottom) was greatly reduced when tetrodotoxin was applied to distal axon (Fig. 1A). Tetrodotoxin applied to the soma selectively reduced the second component of the second derivative waveform (Fig. 1B).

**TABLE 3.** Values used to describe \( Na^+ \) and \( K^+ \) conductance voltage-gated kinetics

<table>
<thead>
<tr>
<th>Channel, variable</th>
<th>Subvariable</th>
<th>Constant</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCh m</td>
<td>( \alpha )</td>
<td>A</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>k</td>
<td>0.111</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>-40</td>
<td></td>
</tr>
<tr>
<td>NaCh h</td>
<td>( \beta )</td>
<td>A</td>
<td>1.668</td>
</tr>
<tr>
<td></td>
<td>k</td>
<td>-0.111</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>-40</td>
<td></td>
</tr>
<tr>
<td>KCh m</td>
<td>( \alpha )</td>
<td>A</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>k</td>
<td>0.111</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \beta )</td>
<td>A</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>k</td>
<td>-0.111</td>
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**FIG. 3.** CA3 computer simulations help interpret somatically measured spike latencies. A: 1st 100 \( \mu \)m of the CA3 initiation model used in the computer simulations. The initial segment was modeled as a tapering axon comprised of 20 5-\( \mu \)-long segments, each with independent sodium current densities (indicated by color). The passive properties of the soma and dendrites were chosen to match subthreshold responses during actual CA3 recordings and are consistent with other simulations of pyramidal neurons (Mainen et al. 1995; Major et al. 1994; Traub et al. 1994). B: peak dV/dr (indicated by arrowheads) in individual compartments occurred at different time points. The 1st compartment to reach peak dV/dr was the compartment 40–45 \( \mu \)m from the soma, near the modeled peak sodium channel density. C: simulated somatic dV/dr (soma) reproduced the hallmark double-peak profile recorded experimentally. X axis scale bar represents 0.2 ms, y axis scale bar represents 1,000 mV/ms².
Initiation site corresponds to a sodium channel-dense region of axon

Computational and experimental evidence have previously indicated that the proximal region of the axon is the spike initiation site in several neuronal types (Fohlmeister and Miller 1997; Mainen et al. 1995; Stuart et al. 1997). Modeling studies suggested that for initiation to occur in a region of the axon in response to direct somatic depolarization, a combination of increases in sodium channel sensitivity and/or density are required (Dodge and Cooley 1973; Mainen et al. 1995; Rapp et al. 1996). Although some immunohistochemical (Boiko et al. 2003; Jenkins and Bennett 2001) and physiological evidence (Colbert and Pan 2002) supports these hypotheses, much about the site of greatest tetrodotoxin sensitivity observed in Fig. 1. Interestingly, the most proximal segments of axon had weak immunoreactivity for sodium channels.

Spike latency considerations

Several recent estimates of the location of spike initiation have relied on comparing spike arrival time from somatic recordings with arrival time in the axon (Clark et al. 2005; Meeks et al. 2005; Palmer and Stuart 2006; Shu et al. 2006a). Latency evaluations are generally made by comparing times at which spikes reach their peak amplitude (Shu et al. 2006a) or by comparing times at which spikes reach their maximum rate of rise (Clark et al. 2005; Meeks et al. 2005). We constructed a reduced compartmental CA3 pyramidal neuron model to help us interpret spike latencies in various subcellular compartments (Fig. 3). We spatially distributed sodium conductances similarly to empirically observed antibody staining, with a peak current density 46-fold higher than the basal density modeled in the axon (Fig. 3A). The model recapitulated salient features of the measured spike waveforms. The time of maximum intracellularly recorded membrane potential rate of rise (dV/dt) was predicted to occur nearly simultaneously in the soma and ~160 μm from the soma on the axon (Fig. 3B). This was nearly identical to the experimentally observed latencies (cf. Fig. 4E). Furthermore, the second derivative (d²V/dt²) of simulated somatic waveforms shared overall properties with actual recordings, including the characteristic double peak (Figs. 1 and 3C).

When we compared latencies on the basis of peak amplitudes of spikes, we obtained a v-shaped relationship with respect to distance (Fig. 4D), as other simulations have predicted (Shu et al. 2006a). However, we identified several problems with using simple Vm peaks for latency analysis. First, on the time scales considered here (tens of microseconds), the spike peak is broad, leading to potential experimental error in identifying the timing of the true peak (Fig. 4B). Second, it becomes clear when examining peak latencies (and also 1st temporal derivatives, see following text) that the somatic Vm peak occurs after a temporal lag representing the impedance mismatch between hillock and soma (Fig. 4A). This is apparent in the slower rise of the somatic spike compared with the axonal spike (Fig. 4B) and in the multiple components of the first derivative somatic waveform (Fig. 4C). Although certainly passive somatic properties contribute to proximal spike development, including this lag in temporal analysis may obscure the contribution of axon-specific channels in spike development. A final practical disadvantage of using spike peak amplitudes for latency measures is that obtaining experimental data corresponding directly with the simulations would require multiple intracelluar axonal recordings, which are impractical in small axons, except in the special case of cut axon blebs (Shu et al. 2006a,b) and possibly with noninvasive optical recordings (Palmer and Stuart 2006).

For these reasons, we also investigated the effects of using the time of maximum slope (dV/dt peak) to estimate spike arrival in the various compartments. Our previous estimate of a distal action potential initiation site was derived from such measurements, obtained from extracellular axonal and whole cell somatic recordings (Meeks et al. 2005). Because the raw extracellular axonal signal corresponds directly with the first derivative of intracellular Vm when the extracellular signal is capacitive (Meeks et al. 2005), experimental data are readily obtained. The method is advantageous because the maximum slope of the intracellular somatic signal is readily identifiable with little ambiguity about the temporal location (Fig. 4C).

We compared simulation output with actual records from 53 paired soma/axon recordings. These included 33 paired recordings analyzed previously (Meeks et al. 2005) plus 20 additional recordings obtained to improve plot resolution. Unlike results using raw Vm peak amplitude to measure latency (Fig. 4D), we found that simulation-derived first derivatives predicted a zone of axon that reached the maximum change in Vm nearly synchronously (Fig. 4E). Further, the simulation matched experimentally measured latencies quite well. The zone of minimum latency, identified by the red-boxed region in Fig. 4, E and F, extended from the axon compartment 15 μm from the soma to the segments ~100 μm distal. Interestingly, this zone of minimum latency extends beyond the region of dense sodium channel staining and beyond the point we previously identified as the initiation site in CA3 axons (~75 μm distal). The results suggest that the region of densest sodium channels (centered 35 μm from the soma) supplies enough current to rapidly charge adjacent segments of the axon.

The relatively flat region of little latency change between axon recording site and axon-soma spike latency observed in real data and in simulations can be interpreted as a zone of initiation. The membrane potential exceeded threshold first at a site within the densest region of sodium-channel staining (~40 μm, Figs. 3A and 4F). However, a zone of the axon, extending from compartments 20–60 μm reached peak dV/dt within one sample point in our experimental measurements (10 μs). Further, a zone of axon extending 40 μm beyond the area of densest sodium channel density reached peak dV/dt within 40
of linear relationship with an estimated spike conduction velocity recording distance relationship assumed a positively sloping most negative time. Note the pronounced "jump" from near 0 to strongly negative values between the 5- to 10- and 10- to 15- attributed to the substantial latency caused by the somatic impedance mismatch and capacitive load seen in differences. The strong slowing effect imparted by this mismatch can be seen in the substantial negative latency \( V_t \) at each segment is shown in the \( V_d/dt \). Note the broadness of the soma waveform \( V_t \) at discontinuous times due to the somatic mismatch. Waveforms are scaled and temporally aligned based on their peak to highlight temporal features of the simulation matched the \( V_m \) segment waveform compared with waveforms of more distal axonal segments. \( D \): peak latency plot derived from model intracellular \( V_m \). The CA3 initiation model indicated a V-shaped curve with a nadir at 40–45 \( \mu \)m from the soma. \( E \): simulated axon-soma \( dV/dt \) latency measurements at each segment of the model axon (blue line) match experimental paired soma/axon records. The solid symbols reflect data from Meeks et al. (2005). The open symbols are additional, new data points. The initial 160 \( \mu \)m of the simulation output (highlighted by the red box) is shown in an expanded view \( F \). \( F \): expanded view of the soma-axon latency over the initial 160 \( \mu \)m of the simulated axon. Many simulated axon segments around the 40- to 45-\( \mu \)m segment (the first to peak) reach peak \( dV/dt \) within the 0.01-ms sample interval used in standard intracellular/extracellular soma/axon recordings. The shallow profile of the developing spike indicates that the region 20–60 \( \mu \)m from the soma reaches peak \( dV/dt \) nearly simultaneously. An additional 40 \( \mu \)m of the axon reaches peak \( dV/dt \) within 40 \( \mu \)s of the most negative time. Note the pronounced "jump" from near 0 to strongly negative values between the 5- to 10- and 10- to 15-\( \mu \)m segment. This jump can be attributed to the substantial latency caused by the somatic impedance mismatch and capacitive load seen in \( A-C \).

\( \mu \)s of the most sensitive area. Beyond this region, the latency-recording distance relationship assumed a positively sloping linear relationship with an estimated spike conduction velocity of \( \sim 300 \mu \text{m/ms} \). These features of the simulation matched the measurements from real cells remarkably well (Fig. 4E).

However, this method also suffers from inclusion of a temporal lag between hillock invasion and somatic recruitment (Fig. 4A, bottom, C, and F). In simulations, as in actual records (e.g., Fig. 1) of proximal and somatic compartments, there are two components observed in the first derivative (Colbert and Johnston 1996; Coombs et al. 1957). It is clear that the first component, although not dominant in the somatic record, corresponds to the spike in the axon, whereas the second (later) component, the amplitude of which dominates the somatic and first two proximal axonal compartments records (Fig. 4A, bottom), represents the somatic spike that emerges after charging of the much larger somatic capacitance. In latency plots, the switch between axonal versus somatic dominance is evident as a discontinuity in the proximal limb of the latency plot (Fig. 4, E and F). To summarize, the first-derivative method of latency measurement has advantages over the raw \( V_m \) peak method. The first derivative can be derived noninvasively from extracellular axonal records, the waveforms contain a readily identifiable peak, and the method makes progress toward separation of axonal from soma-derived signals, but precise identification of the axonal derived component is still difficult.
Spike emergence and propagation estimated from second derivative waveforms

Spike development in the initial segment depends on complex interactions of active and passive processes in the region that may be obscured by analysis of somatic $V_m$ and $dV/dt$. To circumvent the above limitations of somatic $V_m$ and $dV/dt$ latencies, we turned to estimates based on the second derivative waveform of the somatic spike. Although this signal gives only indirect information about the axonal spike, it appears to yield reliable information about spike development in the axon initial segment as well as information about the spike as somatic channels are eventually recruited. As depicted in Figs. 1 and 3, the somatic second derivative most easily allows separation of the two components (axonal spike vs. somatic spike) of the somatic signal. A waveform corresponding to the second derivative of the axonal signal is readily obtained from the extracellular recordings. Because the extracellular axon signal corresponds to the first derivative of membrane voltage (Meeks et al. 2005) (Fig. 4E), the first derivative of this signal can be compared with the somatic second derivative waveform (Fig. 5, A and B).

Simulations also suggested that the first detectable inflection of the somatic second derivative (5% of the initial peak) corresponded temporally with threshold crossing in the region of highest sodium channel density (Fig. 5C). The first peak of the somatic second-derivative waveform was a good approximation of the time of hillock invasion (maximum hillock $d^2V/dt^2$). We denoted the latency between the 5% inflection point and the peak of the first component as $T_1$ (Fig. 5B). The second peak of the somatic second derivative waveform corresponded to the recruitment of somatic channels, which we omitted from formal latency analysis.

The sum of $T_1$ and $T_2$ gives the total latency from axon hillock to axon recording site, including the time needed for full spike development in the axon initiation zone. The sum of $T_1$ and $T_2$ can then be compared with the three-dimensional distance, reconstructed in confocal $z$ stacks, between the soma and axonal recording site to estimate the axonal conduction velocity from an individual paired soma/axon recording. Also a strong change in the ratio between $T_1$ and the average conduction velocity may suggest a change in the location of dynamics of spike initiation (see Fig. 6).

As expected, $T_1$ did not vary significantly with axonal recording distance (Fig. 5D), but $T_2$ showed a marked dependence on recording distance past recording distances of $\sim 100 \mu m$ (Fig. 5E). $T_2$ (the time between estimated 1st-threshold crossing and maximum $V_m$ acceleration at the recording site) did not fall to zero in the initiation zone (Fig. 5E) because of the time associated with initial spike development at the initiation zone, which we call recruitment time, is built into the $T_1$ and $T_2$ measures (Fig. 5C). Use of the second derivative measures shows even more clearly than single derivatives (Fig. 5E) that the region of axon from 10 to 100 $\mu m$ from the soma exhibits a nearly fixed spike latency ($T_2$). Again, the enhanced resolution gained from second derivative-based latencies results from the minimized contribution of somatic channels to these latency estimates.

If our interpretation of $T_1$ is correct, one would expect that $T_1$ could be altered by ectopic initiation distally in the axon or by a significant change in the dynamics of initiation that would alter the emergence of the spike (i.e., the backpropagation of the spike) toward the hillock region. To test whether more distal initiation can be detected as a change in $T_1$, we antidromically stimulated six axons. We found that $T_1$ was significantly lengthened in all six cells (Fig. 6A), consistent with the idea that altering the initiation location alters $T_1$. Simulations of antidromic initiation at the same axonal distance produced a similar result (Fig. 6B). We point out that $T_1$ in the case of
neglects the time needed for full spike development in the initial segment toward latency measurement time (minimum T2 time, from axon records or following stimulation at the axon segment 230–240 μm from the soma (gray trace). The model reported a similar increase in T1 latency. This difference indicates that the T1 value changes when the site of stimulation is reversed.

antidromic stimulation will not yield an accurate estimate of the initiation site several hundred microns from the soma because passive axonal decay will be too strong for the time of first initiation to be detected at the soma. These interpretations are in line with previous analyses of other cell types, which found that antidromic spikes in myelinated motorneuron axons produce a component of the somatic waveform that is not normally present with orthodromic stimulation and that likely represents the regenerated spike at the first node of Ranvier (Coombs et al. 1957). In addition to the T1 latency increase, antidromic stimulation produced an increased delay between hillock invasion and somatic threshold crossing (the separation between the peaks of the 2nd derivative waveform in Fig. 6, A and B). This is expected because the soma during antidromic stimulation is resting further from somatic spike threshold than during somatic depolarization.

Because the latency measurements in Fig. 5 include the time required for development of the spike at the site of first threshold crossing, we estimated directly the time lag associated with channel recruitment over the zone of initiation (recruitment time in Fig. 5C). For this analysis, we examined our records of axonal recordings from within the zone of initiation (<100 μm proximal to the soma) to measure recruitment time directly. We examined the first derivative of axon signals (dV/dt) and measured the time from inflection (5% of peak) to the peak of the waveform, analogous to our T1 time measurement from somatic d²V/dt². By subtracting this recruitment time (minimum T2 time, from axon records <100 μm) from T1 and T2 and expressing the result as apparent conduction velocity (Fig. 7A), one can see that both simulations and experimental data suggest nearly instantaneous “conduction” near the zone of initiation. Because distance (numerator in the calculation of conduction velocity) increases linearly with axonal recording length, but the denominator (latency) is near zero over the initiation zone, the peak conduction velocity appears shifted distal from the site of first threshold crossing (~35 μm) in this analysis. This approach indicates that if one neglects the time needed for full spike development in the initiation zone, electrotonic spread over this zone is nearly instantaneous (Fig. 7A). With distance, however, passive coupling from the region of high channel density is weaker, and the combination of active and passive propagation combine to yield a stable conduction velocity of 300–400 μm/ms (Fig. 7A), consistent with other estimates from small, unmyelinated fibers (Soleng et al. 2003b).

By contrast, when the recruitment lag of channels in the initiation zone is included in the estimates of conduction velocity, overall conduction velocity appears less variable over the axon length (Fig. 7B). The differences in apparent conduction velocity over the zone of initiation, depending on whether recruitment time is included, highlight the dominance of spike development in the initial segment toward latency measurements over this region.

Changes in spike development and propagation under different stimulus conditions

CA3 neurons typically fire action potentials in short trains and bursts. During brief, low-frequency trains, we previously reported that axonal action potential waveforms remained reliable despite strong changes in somatic dV/dt (Meeks et al. 2005). To determine the temporal characteristics of spike initiation and propagation in the context of variable inputs, we compared somatic and axonal responses to variable patterns of somatic stimulation. To test the robustness of initiation and propagation during variable input amplitudes, we compared second derivative spike latencies in response to short, just-suprathreshold somatic current injections (750–1250 pA delivered for 10 ms) to those produced in response to strong suprathreshold current injections (4,000–4,250 pA delivered...
for 10 ms; Fig. 8, A and B). To test the robustness of initiation and propagation during short frequency trains, we also compared the first spike with the 10th spike in a 15- to 30-Hz spike train elicited with sustained current injection (200–600 pA delivered for 0.5–1 s; Fig. 8, C and D).

The strongest suprathreshold stimuli caused no significant shift in T1 or T2 compared with weak, just-suprathreshold stimuli (Fig. 8, A, B, E, and F). When these values were combined to produce a simple estimate of the initiation site, we found no significant change between the two conditions (4.9 ± 3.8 μm shift toward the soma with strong suprathreshold stimuli, n = 20, P = 0.23; Fig. 8G). Both initiation estimates (50.2 and 45.3 μm, respectively) are within the range of the dense sodium channel staining and well within the expected initiation zone, although we note that the physiology-based estimates are prone to some error because of assumptions of constant propagation velocity along the axon. The result suggests that the proximal initial segment is the first to reach threshold even under conditions of strong somatic stimulation. Additionally, there was no significant change in the estimated conduction velocity along these axons in response to strong, suprathreshold stimulation (Fig. 8H). The only latency value that changed between the weak and strong stimulation conditions was the latency between the two somatic d²V/dr² peaks, which decreased from 190 ± 40 to 130 ± 30 μs (n = 20, P < 0.001). This decrease in temporal lag caused by the soma impedance mismatch is expected, as strongest stimuli more rapidly charge the somatic capacitance and bring somatic channels nearer their own threshold (Colbert and Johnston 1996).

To determine whether the spike initiation site was robust with variable intensity synaptic input, AMPA receptor-mediated excitatory postsynaptic potentials (EPSPs) were manipulated by altering the amplitude of an extracellular stimulus applied to presynaptic inputs to CA3 neurons. We found no change in T1/T2 ratio (n = 3 cells) over a 3- to 10-fold increase in stimulus intensity, beginning with stimuli eliciting single EPSPs that were just suprathreshold (data not shown). These results suggest that weak and strong synaptic inputs to CA3 dendrites also elicit spike initiation in the axon with little spatial variability.

Repetitive spiking can alter the excitability of fibers studied with antidromic or extracellular stimulation techniques (Meeks and Mennerick 2004; Soleng et al. 2003a; Swadlow et al. 1980). It also alters spike threshold, measured at the soma (Henzé and Buzsáki 2001; Meeks et al. 2005). We examined spike development during 15- to 30-Hz spike trains initiated during 0.5–1 s sustained somatic current injections to determine whether spike initiation and/or conduction velocity were affected during train activity. The average somatically measured threshold increased by 9.9 ± 2.8 mV between spikes 1 and 10 under these conditions. Repetitive spiking causes strong spike waveform depression in somatic records but raw axonal waveforms remain largely unaffected (Khaliq and Raman 2005; Meeks et al. 2005; Monsivais et al. 2005; Shu et al. 2006a; Williams and Stuart 1999). When we examined d²V/dr² latencies, we found significant changes between the 1st and 10th spikes. Both T1 and T2 were shifted toward longer latencies (although the T2 shift did not reach statistical significance; Fig. 8, C–F). We found that the T1/T2 ratio was significantly decreased by 3.8 mV between spikes 1 and 10 during 0.5–1 s sustained somatic current injections to determine the 1st and 10th spikes in a train.
similarly unaffected by repetitive spiking, remaining well within the initiation zone (Fig. 6G).

Despite the robustness of the site of action potential initiation in the face of repetitive spiking, the increase in combined T1 + T2 latency indicated a strong slowing of overall conduction velocity along the axon at the 10th spike compared with the 1st spike (103 ± 35 μm/ms slower at AP10 than AP1, P < 0.001, n = 20, Fig. 8H). The slowing of conduction velocity was observed in all 20 cells studied with axon recording distances reaching >400 μm from the soma. To determine whether the apparent slowing was accounted for by a change in recruitment time in the initiation zone, we examined latency data from the initiation zone (<100 μm from the soma) in more detail. In the seven dual recordings from this region, we found that rise time from the 5% inflection point to the peak of the axon first derivative was slowed by spike 10, indicating an increased recruitment time (Fig. 9, A and B). As a secondary verification of this increase in recruitment time, we measured the minimal T2 values from these recordings nearest the initiation zone, finding that the minimal T2 value increased by a similar amount. Both estimates indicated a 40- to 60-μs increase in recruitment time (Fig. 9B).

This increase in recruitment time, however, did not simply result in a static conduction delay downstream in the axon. We found that the change in T2 over the course of 10 action potentials was strongly correlated with recording distance (Fig. 9C), suggesting that a cumulative conduction velocity change contributed to spike latency changes along with the fixed-latency change in recruitment time. This distinction indicates that conduction velocity is altered over a substantial length of the proximal axon.

We also observed a pronounced delay of somatic invasion apparent in the second derivative waveform during repetitive spiking. The time between the two peaks in somatic d²V/dt² increased from 200 ± 40 μs at the 1st spike to 340 ± 80 μs at the 10th spike (P < 0.001, n = 20). Three cells challenged with repetitive synaptic stimulation showed similar trends to those seen with sustained somatic current injection (data not shown).

As a comparison with a condition that is expected to speed conduction velocity (Soleng et al. 2004), we tested temporal changes in T1 and T2 with increased temperature to further explore properties of spike emergence in the initial segment and soma and downstream propagation in CA3 axons. We found that raising the slice temperature from 24–26 to 32–34°C caused a significant speeding of average conduction velocity along the axon (from 361.0 ± 29.1 to 446.7 ± 36.0 μm/ms, n = 8, P < 0.01). Again, the change in T2 latency was significantly correlated with distance, although the relationship was opposite that of repetitive firing (Fig. 9C, ○). Raising slice temperature did not significantly affect the ratio between T1 and T2, and therefore the estimated initiation zone was apparently unaffected by temperature. (n = 8, P = 0.36, paired comparison with 24–26°C). Although this explanation is the most parsimonious, we cannot formally exclude the possibility that compensatory changes in both conduction velocity and initiation site occurred with temperature manipulation.

DISCUSSION

We have used detailed analysis of somatic and concurrently recorded axonal spike waveforms, along with several empirical tests, to test the location, dynamics, and propagation of spikes at their natural origin of initiation site in small, unmyelinated axons of the hippocampus. Convergent evidence suggests that the axon ~35–40 μm from the soma is the first to cross spike threshold and contains a high density of voltage-gated sodium channels. Nevertheless, in contrast to recent studies implying a “site” or point of initiation, our empirical measurements and simulations suggest a zone of axon surrounding the densest sodium channels that depolarizes nearly simultaneously. Our results, along with other previous work, suggest caution in interpreting latency changes from somatic Vₘ and dV/dt records because latencies include signals from both axonal and somatic channels, which are not recruited synchronously. Our results also suggest caution in applying a bilinear model to latency estimates based on first or second derivatives (Clark et al. 2005; Meeks et al. 2005). Finally, our results show that threshold changes of ~10 mV during repetitive stimulation do not alter the zone in which spikes originate but significantly slow axonal channel recruitment and propagation over the axon. Together, our results yield a detailed picture of the dynamics of development and propagation of axonal spikes of a heavily studied cell type within the hippocampus.

CA3 neurons are known for their repetitive spiking and susceptibility to seizures (Miles and Wong 1986). Our present investigations spanned the lower range of firing capabilities of
these cells, but our previous studies of waveform changes in axons explored extreme conditions of epileptiform conditions (Meeks et al. 2005). Unlike axonal spike amplitude changes, which develop under epileptiform conditions and during long spike trains (Meeks et al. 2005), here we detected rapid latency changes within short, 10- to 30-Hz trains. Such latency/conduction velocity changes accumulated over the length of CA3 axons. Over longer stimulus trains these accumulated changes will lead to significant changes in the timing between presynaptic initiation and postsynaptic target activation; temporal information encoded at the initiation site will not be accurately preserved at the synapse. These changes may influence the degree of frequency-dependent plasticity at the synapse and therefore the strength of the resulting postsynaptic signal.

Our latency analysis of somatic and attached single-axon signals allowed us to separate events in the axon and soma into several stages: channel recruitment in the initial segment, back-development/propagation in the axon hillock and soma, and forward propagation in the axon. These phases could be separately analyzed during manipulations (repetitive spiking and temperature manipulation) that alter conduction velocity. We found evidence for a change in recruitment time at the initial segment during repetitive spiking, which will result in a fixed latency change propagated down the axon, as well as a propagation change that accumulates over the range of axon lengths we explored. The change in spike threshold and initial-segment recruitment time could in part result directly from passive spread of the sustained somatic depolarization as opposed to spiking per se. However, repetitive axonal spikes likely contributes to the cumulative propagation deficits because latency changes increased with increasing electronic distance from the soma (Fig. 9C).

Over long distances this cumulative effect may significantly delay spike arrival at downstream presynaptic terminals. In CA3 axons of rodent, transverse distances of 2.5 mm are likely (Soleng et al. 2003b). Longitudinal paths of these axons are even longer (Ishizuka et al. 1990), potentially resulting in latency changes of several milliseconds over the course of the short trains we explored. A linear estimation of the increase in latency along the axon indicates that the 10th action potential in our spike trains added an additional 0.5–0.6 μs of lag per micrometer of axon traveled. If this slowing effect persists over the estimates 2–3 mm of CA3 axon, this latency could amount to as much as 1.8 ms. Even if the slowing effect persisted for only one axonal length constant, the 10th spike would accumulate 0.3 ms of lag on account of this activity-dependent slowing. In some species and at some developmental stages, these fibers may be myelinated, which of course will minimize latency delays.

We found that a reduced compartmental model CA3 neuron recapitulated salient features of our experimental results. The model incorporated simplified dendritic geometry and only modified Hodgkin-Huxley type active conductances. The main results were robust over a range of passive properties. This suggests that the features we explored are salient and are likely to apply to many cell types with similar axonal morphology and channel distributions.

Other recent estimates of initiation site in other cell types have relied on latency analysis of electrophysiological and optical spike waveforms (Clark et al. 2005; Meeks et al. 2005; Palmer and Stuart 2006; Shu et al. 2006a). Our analysis suggests several considerations are warranted with these approaches. In our case, a bilinear fit to peak first-derivative latencies was inappropriate (Clark et al. 2005; Meeks et al. 2005) (Fig. 4E). It would seem that in myelinated fibers, such as Purkinje cell fibers, this latency analysis is further complicated by saltatory conduction, which would result in discontinuities in the plot not accommodated by linear fits. A minimum latency derived from peak $V_m$ yields an estimate of initiation near the site of densest sodium channels, but it is experimentally impractical to make the necessary intracellular axonal recordings, except in the case of cut axon blebs (Shu et al. 2006a,b), which have unknown effects on channel function. We found that use of second temporal derivatives to strip away the contribution of somatically derived channels yields a latency measurement free of the lag between somatic and axon spike development. This method shows evidence for a zone of initiation, characterized by the first $\sim 100$ μm of axon that reaches maximum $V_m$ acceleration nearly synchronously (Fig. 5, C and E). Recent estimates of initiation site in cerebellar Purkinje cells have varied from quite distal (Clark et al. 2005) to quite proximal (Khalilq and Raman 2006). Although a “zone” explanation is a tempting partial resolution to the discrepancy, it is possible that the zone may be a less prominent attribute of myelinated fibers.

Interestingly, previous evidence in myelinated axons has suggested sodium channel density that is uniformly high from the axon hillock to the distal initial segment (Jenkins and Bennett 2001; Komada and Soriano 2002; Wollner and Caterall 1986). In these cases, spike initiation in the distal initial segment is caused by the large capacitive load of the somato-dendritic compartment, which helps prevent more proximal initiation (Mainen et al. 1995). In the case of CA3 neurons, the proximal axon is largely devoid of dense sodium channel immunoreactivity. This may be an additional factor in distal initiation. A gap in sodium channel staining between the soma and initial segment staining, similar to that reported here, has been recently suggested in retinal ganglion cells (Boiko et al. 2003). Because we filled individual cells, we were able to conclusively demonstrate this gap in proximal axonal staining in CA3 neurons. Simulations of ganglion cells suggest that this distally high density is needed to reproduce ganglion cell spiking properties (Fohlsmeister and Miller 1997).

In addition to sodium channel density, potassium channel density and location will sculpt the dynamics of spike initiation. Indeed, KCNQ2 subunits, contributors to m-type potassium currents, are localized to a similar axonal domain as sodium channels (Chung et al. 2006; Pan et al. 2006). Also, Kv1 channels are clustered in the initial segment of axons of at least several cell types (Dodson et al. 2002; Popratiloff et al. 2003; Van Wart et al. 2007). Because of the generally slow kinetics of potassium channels relative to sodium channels, we might expect that such strategically placed potassium conductances may influence the signature firing pattern of a cell but not the location of the first threshold crossing. Our analysis suggested little alteration in the location of spike initiation during trains of spikes, conditions expected to recruit potassium conductances.

In summary, we present evidence that spike initiation occurs in the initial segment 20–60 μm from the soma in unmyelinated CA3 pyramidal cell axons. The initiation site remains constant in the face of varied stimulus strength and with
repetitive activity, but timing of axonal signaling is significantly altered by short bursts of repetitive activity associated with shifts in spike threshold. Such changes are likely to have an important downstream influence on timing and efficacy of transmission.

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REFERENCES


Chung HJ, Jan YN, Jan LY. Polarized axonal surface expression of neuronal KCNQ channels is mediated by multiple signals in the KCNQ2 and KCNQ3 C-terminal domains. Proc Natl Acad Sci USA 103: 8870–8875, 2006.


Miles R, Wong RK. Excitatory synaptic interactions between CA3 neurons in the guinea-pig hippocampus. J Physiol 373: 397–418, 1986.


