High Threshold, Proximal Initiation, and Slow Conduction Velocity of Action Potentials in Dentate Granule Neuron Mossy Fibers

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Kress GJ, Dowling MJ, Meeks JP, Mennerick S. High threshold, proximal initiation, and slow conduction velocity of action potentials in dentate granule neuron mossy fibers. J Neurophysiol 100: 281–291, 2008. First published May 14, 2008; doi:10.1152/jn.90295.2008. Dentate granule neurons give rise to some of the smallest unmyelinated fibers in the mammalian CNS, the hippocampal mossy fibers. These neurons are also key regulators of physiological and pathophysiological information flow through the hippocampus. We took a comparative approach to studying mossy fiber action potential initiation and propagation in hippocampal slices from juvenile rats. Dentate granule neurons exhibited axonal action potential initiation significantly more proximal than CA3 pyramidal neurons. This conclusion was suggested by phase plot analysis of somatic action potentials and by local tetrodotoxin application to the axon and somatodendritic compartments. This conclusion was also verified by immunostaining for voltage-gated sodium channel alpha subunits and by direct dual soma/axonal recordings. Dentate neurons exhibited a significantly higher action potential threshold and slower axonal conduction velocity than CA3 neurons. We conclude that while the electrotonically proximal axon location of action potential initiation allows granule neurons to sensitively detect and integrate synaptic inputs, the neurons are sluggish to initiate and propagate an action potential.

INTRODUCTION

Dentate granule neurons serve as a gateway for information flow through the hippocampal formation (Coulter and Carlson 2007; Dudek and Sutula 2007; Hsu 2007; Schwartzkroin and Prince 1978). The axons of these cells, the dentate mossy fibers, form synapses onto both CA3 pyramidal neurons and interneurons. Therefore they are a key influence on the spread of normal and pathological activity through the hippocampus and beyond. In addition, mossy fibers possess a number of unusual features that make them an interesting target of experimental study, including their small diameter, their unmyelinated status, their unusually large presynaptic terminals (mossy fiber boutons), and the presence of axonal ionotropic receptors (Alle and Geiger 2007; Henze et al. 2000; Lawrence and McBain 2003; Ruiz et al. 2003; Toth et al. 2000). Therefore it is of interest to know how basic aspects of spike initiation and propagation compare with other unmyelinated and myelinated axons that have been studied recently, such as myelinated cerebellar Purkinje cell axons, myelinated layer 5 cortical cell axons, and unmyelinated CA3 pyramidal cell axons (Clark et al. 2005; Khaledi and Raman 2005; Kole et al. 2007; Meeks and Mennerick 2007; Monsivais et al. 2005; Palmer and Stuart 2006; Shu et al. 2007; Stuart and Hausser 1994; Stuart et al. 1997). Fundamental action potential properties such as the site of origination, the orthodromic and antidromic conduction velocities, and the fidelity of propagating action potentials in individual hippocampal mossy fibers are known but have recently begun to be investigated (Kress et al. 2007; Schmidt-Hieber et al. 2008).

Traditional methods for studying action potential propagation and fidelity in small fibers include population techniques such as fiber volley measurements and antidromic stimulation methods, where interactions among the many simultaneously stimulated fibers and the ectopic location of initiation may obscure the normal behavior of the fibers. To circumvent limitations of these classical techniques for studying conduction velocity and axonal spike properties, here we have used techniques to study individual dentate granule neurons and their axons. We combined fluorescent dye fills of dentate neurons with immunostaining for voltage-gated sodium channels to localize the likely site of spike initiation on the mossy fiber axon initial segment. We used electrophysiological techniques, including dual intracellular somatic and extracellular axonal recordings, to characterize spike initiation, action potential waveforms, and conduction velocity.

Our results suggest a more proximal initiation site but more depolarized action potential threshold and slower conduction velocity than in CA3 pyramidal neuron axons. Several of these features are consistent with the small, compact morphology of these cells. Together these properties are important for understanding the integration and output of dentate granule neurons, a critical relay for information entering the hippocampus. Forming a basic understanding of mossy fiber action potentials provides insight into signal processing within the classical hippocampal trisynaptic circuit and serves as a basis for comparison with other cell types.

METHODS

Slice preparation

Hippocampal slices were prepared from postnatal day 19 (P19) to P23 Sprague Dawley rats. In accordance with Washington University Animal Studies Committee, rats were anesthetized with isoflurane and decapitated. The brain was removed and glued onto a Vibratome 1000 specimen holder. Transverse (300 μm) slices were cut in ice-cold, modified artificial cerebrospinal fluid (ACSF) (in mM: 125 NaCl, 25 glucose, 25 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, equilibrated with 95%
oxygen-5% CO₂, plus 0.5 CaCl₂, 3 MgCl₂, and 2 kynurenic acid). Slices were then incubated at 32–34°C for 30 min in ACSF containing 2 mM CaCl₂ and 1 mM MgCl₂ and subsequently stored at room temperature. Except when noted, drugs were obtained from Sigma (St. Louis, MO). All Alexa Fluor reagents were from Invitrogen/Molecular Probes (Carlsbad, CA).

**Electrophysiology**

Slices were perfused at 2–4 ml min⁻¹ with oxygenated ACSF containing (in mM) 2 CaCl₂, 1 MgCl₂, and 2 kynurenic acid at 25 ± 1°C (mean ± SE). Granule cells of the dentate gyrus were identified using infrared differential interference contrast on an upright Nikon Eclipse E600FN microscope and a cooled digital camera (Roper Scientific, Tucson, AZ) controlled with MetaMorph software (Molecular Devices, Sunnyvale, CA). Somatic whole cell recordings and axonal extracellular recordings were made with borosilicate patch pipettes (World Precision Instruments, Sarasota, FL; Sutter Instruments, Novato, CA), having open tip resistance of 3–7 and 1.5–3 MΩ, respectively. The intracellular pipette solution contained (in mM) 115 potassium gluconate, 20 KCl, 10 HEPES, 2 EGTA, 2 MgATP, 0.3 NaGTP, 10 sodium phosphocreatine, and 100–200 mM Alexa Fluor 488. pH was adjusted with KOH to pH 7.35. In some experiments, Alexa Fluor 568 hydrazide and/or 5 mg/ml biocytin were used for axon visualization instead of Alexa Fluor 488. After a somatic recording was established, cells were allowed to fill with the intracellular solution for ~10 min. Fluorescent dye excitation with a metal halide lamp was used to track the attached axon with limited light exposure. Once located, the axon was gently pulled into the extracellular pipette with negative pressure. Recordings were obtained using a MultiClamp 700B amplifier (Molecular Devices) and pClamp 9.2 software (Molecular Devices). Somatic bridge balance and pipette capacitance were adjusted using MultiClamp 700B Commander software. Somatic access resistance was monitored continuously, and cells with unstable access resistance (>20% change) or with values >20 MΩ were excluded from analysis. When necessary, a small bias current was injected to maintain a similar baseline membrane potential (near ~84 mV in both cell types) before depolarizing current injections. Reported values of the membrane potential and action potential thresholds have been corrected for the liquid junction potential of +14 mV estimated from Igor XOP Patcher’s Calculator (Wave metrics, Oswego, OR). All dentate granule neurons studied exhibited electrophysiological and morphological properties of mature granule neurons (X. Liu et al. 2000; Y. B. Liu et al. 1996; Overstreet-Wadiche and Westbrook 2006). For Fig. 2 and Table 1, only cells with intact axons >100 µm were used for comparisons.

For local drug application, sharp patch pipettes were filled with 500 nM tetrodotoxin (TTX) dissolved in extracellular solution. The puffer pipette was positioned within 5 µm of the target region, visualized under fluorescence and IRDIC. A 50–200 ms pressure application at 5–7 psi produced a small radius (~5–10 µm) of tissue displacement during the brief drug application. The restricted nature of the application was verified as described (Meeks and Mennerick 2007).

**Electrophysiology data analysis**

Analysis was performed with custom-written programs in Igor Pro 6.0 (Wave metrics, Oswego, OR). Data were imported and filtered with Igor Filter Design Laboratory low-pass Bessel 8-pole at 5 kHz. First-, second-, and third-order derivatives (denoted dV/dt, d²V/dt², and d³V/dt³, respectively) of the somatic and axonal membrane potentials were calculated using a central differences algorithm. Phase plots were constructed from the first derivative of the somatic membrane potential (dV/dt) versus the somatic membrane potential.

Action potential threshold was calculated by two methods. The first method used the first inflection point of phase plots to assign action potential threshold. The membrane potential at which phase plot slope reached 10 mV ms⁻¹ (Naundorf et al. 2006) was denoted threshold. The second method used the second derivative of the somatic membrane potential (d²V/dt²). In this method, the somatic membrane potential corresponding to 5% of the first peak amplitude in the d²V/dt² somatic waveform denoted the action potential threshold (Meeks and Mennerick 2007). We also examined the slope of phase plots at action potential threshold (Naundorf et al. 2006; Shu et al. 2007). Slopes of the phase plot at the three data points bracketing spike threshold at 10 mV ms⁻¹ were calculated using linear regression. For calculation of threshold and slope from phase plots, the baseline dV/dt value resulting from the passive membrane response to current injection was subtracted.

**Imaging and immunocytochemistry**

After recordings, a fluorescence photograph was taken of the filled cell to document axon recording position. Slices were then fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 30–35 min at room temperature (RT, 25°C) immediately following recording. Using a Nikon C1 laser scanning confocal system, three-dimensional images were acquired of the fluorescent, dye-filled cell to measure the precise distance of the axonal recording site to the axon hillock/soma junction with MetaMorph software.

For studies of voltage-gated sodium channel localization, slices were fixed with 4% PFA in PBS for 30–35 min, permeabilized, and blocked with 6% goat serum, 5% bovine serum albumin, 0.3% tritonX-100, 3 mM sodium azide for 2 h at RT. Primary antibody incubation included the preceding solution plus 4 µg/ml of monoclonal anti-sodium channel antibody, clone K58/35 (PanNav antibody, Sigma-Aldrich, St. Louis, MO), for 32–34 h at RT. Secondary antibody incubation was with a goat anti-mouse Alexa Fluor 555 or goat anti-mouse Alexa Fluor 633 and streptavidin Alexa Fluor 488 conjugate for 2 h at RT. Slices were then incubated with 10 µM Hoechst 33342 for 20 min at RT, washed with PBS, and mounted with ProLong Gold Antifade Reagent (Invitrogen/Molecular Probes) onto microscope slides. For studies of ankaryn G immunoreactivity, the

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**TABLE 1. Action potential (AP) parameters for dentate granule (DG) and CA3 neurons**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Relative AP Height, mV</th>
<th>AP Duration @ 50% Height, ms</th>
<th>AP Threshold, mV</th>
<th>Max dV/dt, mV ms⁻¹</th>
<th>Inflection Rate, ms⁻¹</th>
<th>Avg Axon Length, µm</th>
<th>Stationary Inflections (2nd derivative)</th>
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<tbody>
<tr>
<td>DG</td>
<td>110.6 ± 2.9*</td>
<td>1.60 ± 0.14</td>
<td>−52.85 ± 1.51*</td>
<td>2.39 ± 0.14*</td>
<td>247.4 ± 29.3</td>
<td>8.24 ± 0.63*</td>
<td>281.1 ± 17.7</td>
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<tr>
<td>CA3</td>
<td>127.5 ± 1.1</td>
<td>3.74 ± 1.99</td>
<td>−63.31 ± 1.29</td>
<td>3.10 ± 0.16</td>
<td>310.6 ± 17.5</td>
<td>21.18 ± 1.49</td>
<td>262.6 ± 51.8</td>
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Cells included in the analysis all had axons intact for at least the first 100 µm distal to the soma to help ensure that severed axons did not influence the results. Relative action potential height was measured from a baseline membrane potential of ~84 mV (liquid junction potential corrected), achieved with small bias current injection when necessary. Maximum dV/dt and inflection rate were derived from phase plots. Stationary inflection points refer to whether the first component of the second derivative achieves a slope of zero (at 1 in Fig. 5A and B3), reflecting well-resolved axon and soma components of the action potential. These values reflect the number of cells exhibiting a slope of zero in each group. See also Fig. 5 and associated discussion. *P < 0.05, Student’s t-test. **P < 0.05, Fisher’s exact test.
preceding immunohistochemistry protocol was utilized with minor modifications. Monoclonal anti-ankyrin G (4G3F8) (Santa Cruz Biotechnology, Santa Cruz, CA) antibody (0.8 μg/ml) incubation was overnight at RT. For measurement of proximal axon diameters based on ankyrin G immunoreactivity, a naïve rater picked 10 well-isolated axons from confocal projections of each subregion for analysis. The diameter of ankyrin-positive staining was quantified 5 μm from the onset of proximal axon staining.

To measure the location and relative density of voltage-gated sodium channels and ankyrin G immunoreactivity, analysis was performed with Metamorph software using the kymograph add-in tool. A three-dimensional mask of the Alexa Fluor 488 filled axon trajectory was created and placed onto either the voltage-gated sodium channel or ankyrin G immunoreactivity kymograph and a line-scan of maximal intensity was plotted for the first 100 μm of mossy fiber. In our case, the kymograph function of Metamorph was used to create a two-dimensional image with the x axis representing the combined “x” and “y” dimensions of the traced axon within the confocal stack and the y axis representing the course of the traced axon in the “z” dimension from a confocal x-y-z stack. For each mossy fiber analyzed, the average proximal dendritic (15–40 μm from the cell body) voltage-gated sodium channel staining intensity was measured in the preceding fashion, then subtracted from the axonal staining intensity. Axonal fluorescence intensity was then normalized to the maximum intensity found within the first 100 μm of mossy fiber. Data were quantified and displayed as the average normalized voltage-gated sodium channel fluorescence intensity along the first 100 μm of mossy fiber, such that immunoreactivity above the zero line is the normalized axonal voltage-gated sodium channel staining intensity above what is found on the proximal dendrite. Axonal ankyrin G immunoreactivity intensity was normalized to the maximum staining along the first 100 μm of mossy fiber. An equation describing an exponential decay was fit to the falling phase of the average fluorescent intensity profiles ≤100 μm of mossy fiber. The resultant decay constant was termed the distance decay constant.

### Analysis and statistics

Graphs were constructed with Sigma Plot 10.0 (Systat, San Jose, CA). Variability is indicated in the text and figures as standard error of the mean. Unless otherwise indicated, statistical comparisons were made using two-tailed Student’s t-test.

### RESULTS

Figure 1 confirms and highlights differences in cellular morphology, particularly axonal diameter, between dentate granule and CA3 pyramidal neurons, another cell type our group has recently studied (Meeks and Mennerick 2007). Figure 1A shows an Alexa Fluor 488 fill from a whole cell recording of a typical dentate granule neuron, demonstrating the dendritic tree within the molecular layer and a small diameter axon (Fig. 1A gray arrowhead) originating from the soma and extending into the hilus. Figure 1B shows a fluorescent, dye-filled CA3 pyramidal neuron at the same magnification. Particularly note the large diameter proximal axon of the CA3 neuron (Fig. 1B, gray arrowhead) when compared with the mossy fiber. The generality of the small-diameter dentate granule proximal axon was confirmed with ankyrin G immunoreactivity to identify the axon initial segment of the two cell types (Kordeli et al. 1995; Lai and Jan 2006) (Fig. 1, C and D).

We found that the diameter of proximal axons, measured in 10 stained axons from each subregion, was significantly larger in CA3 cells (2.5 ± 0.09 μm) than in dentate granule neurons (1.4 ± 0.08 μm; P < 0.05).

We examined properties of somatically recorded action potentials in dentate granule neurons and CA3 neurons with axons of greater length than 100 μm (Fig. 2, Table 1). Raw somatic action potential waveforms showed several differences in height and width (Table 1). Phase plots, graphs of the

**FIG. 1.** Morphological comparison of dentate granule and CA3 pyramidal neurons, 2 cell types giving rise to unmyelinated intrahippocampal fibers. A: confocal 3-dimensional reconstruction of a mature dentate granule neuron filled with Alexa Fluor 488, scale bar: 50 μm. Gray arrowhead points to the small diameter dentate granule axon, the mossy fiber. B: confocal 3-dimensional reconstruction of a mature CA3 pyramidal neuron filled with Alexa Fluor 488, scale bar 50 μm. Gray arrowhead indicates the larger-diameter CA3 pyramidal axon. Fluorescent debris in the field labeled during the approach of the whole cell pipette has been digitally removed from the panel. C and D: immunohistochemistry for ankyrin G from a dentate granule and CA3 subfield, respectively, scale bar 25 μm.
membrane potential slope (dV/dt) versus membrane potential, highlighted several strong differences between granule neurons and CA3 pyramidal neurons (Fig. 2, C and D). First, action potential threshold differed significantly (Fig. 2, C–G). Using the membrane potential at which dV/dt reaches a criterion of 10 mV ms⁻¹ to estimate action potential threshold (Naundorf et al. 2006), granule neuron threshold was 52.85 ± 1.51 mV (n = 11) under our recording conditions (Fig. 2, C, E, and G), while threshold in CA3 pyramidal neurons was 63.31 ± 1.29 mV (n = 9; P < 0.0001; Fig. 2, D, F, and G). These results are summarized in Table 1.

A second difference between these cell types was in phase plot shapes during the action potential upstroke (Fig. 2, C–F). In particular, the slope of the phase plot differed between the two cell types near action potential threshold. The slope of the phase plot at a y-axis value of 10 mV ms⁻¹ (Fig. 2, E and F) was steeper in CA3 neurons (21.18 ± 1.49 ms⁻¹, n = 9) than in granule neurons (8.24 ± 0.63 ms⁻¹, n = 11, P < 0.00001; Fig. 2, E, F, and H).

Previous studies found average phase plot slopes at spike threshold from a variety of neocortical neuron classes that were similar to CA3 pyramidal neuron slopes (20 ms⁻¹ at 10–15 mV ms⁻¹) (Naundorf et al. 2006; Shu et al. 2007). This makes dentate granule neurons outliers among cell classes evaluated in this manner. The sudden onset of the phase plot at threshold has been interpreted to suggest cooperativity of sodium channel gating (Naundorf et al. 2006). However, more recently the steep phase plot slope has been shown to result from the distal, axonal initiation site of the action potential, combined with backpropagation into the soma (Bean 2007; McCormick et al. 2006).

FIG. 2. Phase plot analysis suggests action potential initiation site to be more proximal to the soma in dentate granule neurons when compared with CA3 pyramidal neurons. A: dentate granule somatic membrane potential record of an action potential. The action potential was elicited by sustained current injection of 50 pA. B: CA3 somatic membrane potential record of an action potential in response to sustained current injection (325 pA). In both A and B, a bias current was used to maintain a similar baseline membrane potential before depolarizing current injection (near −84 mV). In both cases, an action potential developed within 150–200 ms from the start of the current injection. C and D: phase plots, the 1st derivative of the somatic membrane voltage (dV/dt) vs. membrane voltage (V_m), for a dentate granule (C) and CA3 pyramidal neuron (D). ψ, the threshold for the action potential. After the action potential threshold, 2 inflection points are indicated with (*, ○). See also Fig. 5, A3 and B3, arrow and plus sign. E and F: threshold estimated from phase plots (C and D), and differences in initial phase plot slope from the 2 cell types. Examination of action potential threshold at 10 mV ms⁻¹ (ψ), data points used to calculate the slope and average threshold from the phase plots. Baseline dV/dt due to the passive response to current injection has been subtracted from the plots. G: summary of action potentials thresholds at 10 mV ms⁻¹ in both dentate granule (DG) and CA3 neurons (n = 11 and 9, respectively). *, P < 0.0001. H: summary of phase plot slopes (inflection rate) at 10 mV ms⁻¹ in DG and CA3 neurons. *, P < 0.00001. Dentate granule neurons exhibit a shallower phase plot slope at threshold than CA3 pyramidal neurons.
A third difference between phase plots of granule neurons and CA3 pyramidal neurons was the prominence of several inflections in the rising phase of the phase plot (Fig. 2, C and D, * and ○). As discussed in detail in the following text (see Fig. 5, Table 1), the inflections arise as a result of the remote initiation of the action potential in the axon, followed by full soma invasion (Colbert and Johnston 1996). Thus the shallow slope of the phase plot at threshold and the less prominent rising phase inflections in dentate granule neurons may suggest that the action potential originates electrotonically closer to the soma than in other cell types and is consistent with an initiation site more proximal to the recording site than in CA3 neurons.

The shallow phase plots of granule neurons could suggest that action potential initiation is somatodendritic rather than axonal. One criterion for the location of action potential initiation is sensitivity of action potential threshold to pharmaceutically reduced sodium channel density (Colbert and Johnston 1996; Khaliq and Raman 2006; Meeks and Mennerick 2007; Palmer and Stuart 2006). We found that local tetrodotoxin application to the proximal axon of granule neurons (15–30 μm) produced a shift in spike threshold, assessed by phase plot analysis (Fig. 3, A and C). In the same cells, application to either the distal axon (70–90 μm) or to the proximal dendrite just apical to the soma did not produce significant threshold shifts (Fig. 3). It might be expected that

**FIG. 3.** Pharmacological evidence for robust action potential initiation in the proximal mossy fiber. A: effect of local TTX application (gray traces) on phase plots of action potentials elicited by just-suprathreshold current injection (minimal stimulation, 80 pA). Baseline traces (absence of TTX) are black. A shift in threshold is indicated by a shift of first inflection of the phase plot along the x-axis. Distal axon local application was 70–90 μm from the soma; proximal axon local application was 10–30 μm from the soma; proximal dendrite local application was at the primary dendrite adjacent to the soma. B: strong (1.0 nA) stimulation was used to elicit the action potential. Again, the proximal axon was the site at which local TTX application elicited a significant change in threshold. C and D: summary of the change in threshold taken from phase plots at a slope of 10 mV ms⁻¹ from 4 to 5 dentate granule neurons in each condition. *P < 0.05.
very strong current injections could influence the locus of action potential initiation to a more proximal or distal location. However, we found that even with very strong stimulation (~1 nA), proximal axon tetrodotoxin application produced a significant shift in threshold while application to the other locations did not (Fig. 3, B and D). Therefore our pharmacological approach confirms recent direct axonal recordings that have suggested a robust axonal action potential initiation site in dentate granule neurons (Schmidt-Hieber et al. 2008).

Voltage-gated sodium channel clustering has been used as an indicator of the action potential initiation site in many neuron types (Kole et al. 2008; Lai and Jan 2006; Meeks and Mennerick 2007). To test explicitly whether initiation is likely to be more proximal in dentate granule neurons than in other neuron types, we examined immunohistochemistry profiles to measure site(s) of highest voltage-gated sodium channel clustering. We filled individual dentate granule neurons with Alexa Fluor 488 hydrazide dye, fixed the brain slices, and stained with an antibody directed against all voltage-gated sodium channel alpha subunit isoforms (PanNav antibody). By combining neuron fills with antibody staining, we localized voltage-gated sodium channel staining on an identified axon. Figure 4A, I and 2, shows representative confocal images of the fills (A1) and immunohistochemistry (A2). These images are confocal projections, from which individual fiber staining is difficult to discern. However, the example in Fig. 4A is

![Image of neuronal structure](http://example.com/neuronimage.png)

**Fig. 4.** Voltage-gated sodium channel and initial segment visualization with combined cell fills and immunohistochemistry. A1: representative Alexa Fluor 488 filled dentate granule neuron. Scale bar is 30 μm. A2: corresponding voltage-gated sodium channel alpha subunit (PanNav) immunoreactivity. The arrows in A1 and A2 denote the soma/hillock junction from which measurements of staining were made. B1–B3: axonal stretched side views (see METHODS) of the 1st 100 μm from the representative mossy fiber from A. This rotated view highlights the axonal localization of PanNav staining within the filled axon. In these images the x axis represents combined x and y planes from the confocal stack. The y axis represents the z plane, the depth within the confocal stack. B1: side view of Alexa Fluor 488 filled mossy fiber (green) Scale bar: y = 5 μm. B2: corresponding side view of PanNav immunoreactivity (magenta) B3: overlay of B1 and B2 shows overlapping region (white) of Alexa Fluor 488 fill and PanNav staining. C: quantification of fluorescence pixel intensity for PanNav along the 1st 100 μm of the representative mossy fiber from B1, shown by circles over the binned area. D: summary of average voltage-gated sodium channel localization from 10 granule neurons, squares plus error bars represent SE. The red line is an exponential decay function, fit from the peak through the falling phase of the mean PanNav staining. Values above the dotted line at 0 pixel intensity denote voltage-gated sodium channel immunoreactivity above that found on the proximal dentate granule neuron dendrite. E1–E3: side views of ankyrin G immunoreactivity. E1: side view of another representative mossy fiber Alexa Fluor 488 fill (green). E2: corresponding side view of ankyrin G immunoreactivity (magenta). E3: overlay of side views E1 and E2 shows localization of ankyrin G immunoreactivity on the mossy fiber (white). F: quantification of fluorescence pixel intensity of the representative mossy fiber from E1, represented by diamonds over the binned regions. G: summary of average ankyrin G staining compiled from 7 mossy fibers. Red line illustrates an exponential decay fit from the peak through the falling phase of the mean ankyrin G staining along the initial 100 μm of mossy fiber.
magnified and rotated in Fig. 4B, as described in METHODS, to highlight the single filled axon. Figure 4B3 shows an overlay of the fluorescent fill and the voltage-gated sodium channel labeling. The mossy fiber voltage-gated sodium channel staining from the example cell shown in Fig. 4, A and B, is quantified in C. A region of high-voltage-gated sodium channel immunoreactivity is observed from the hillock \( (x = 0 \ \mu m; \ \text{Fig. 4, A and B, arrow} \) to 30 \( \mu m \) on the mossy fiber. Figure 4D shows the average staining profile versus distance from 10 dentate granule neurons. A high density of voltage-gated sodium channels extended from the soma/hillock junction \( (x = 0 \ \mu m) \). The average peak staining occurred at 18 \( \mu m \) distal to the soma. A falling exponential was fit from the peak. This fit indicated a distance decay constant of 13.2 \( \mu m \) for the decrease in staining (Fig. 4D). Interestingly, in comparable staining for voltage-gated sodium channels in CA3 axons, immunoreactivity above baseline extended \( \leq 80 \ \mu m \) from the CA3 pyramidal soma (Meeks and Mennerick 2007). Also the location of average peak voltage-gated sodium channel staining differed between dentate granule and CA3 axons (Meeks and Mennerick 2007). On mossy fibers, the average peak voltage-gated sodium channels staining occurred at 18 \( \mu m \) while on CA3 axons it was located 40 \( \mu m \) from the soma (Meeks and Mennerick 2007).

Finally, while a clear gap in staining was noticed in the hillock region of CA3 pyramidal neurons (Meeks and Mennerick 2007), this same gap was not as prominent in staining from dentate granule neurons. Taken together these observations are consistent with a more proximal action potential initiation site in dentate granule mossy fibers than in CA3 pyramidal axons.

To determine if the region of high-voltage-gated sodium channel density was restricted to the axon initial segment, we utilized the combination of cell fills and immunohistochemistry for ankyrin G, a protein primarily localized on the axon initial segment, to probe this question (Lai and Jan 2006). Figure 4E shows reconstructions of a represented filled mossy fiber (green, Fig. 4E, I and 3) and its ankyrin G staining (magenta, Fig. 4E, 2 and 3). The quantification of the single example and of average ankyrin G staining found on mossy fibers is illustrated in Fig. 4, F and G, respectively. Strong ankyrin G immunoreactivity extended from the dentate granule axon hillock to \( \sim 60 \ \mu m \) on mossy fibers. From the average peak staining at 15 \( \mu m \), staining fell with a distance decay constant of 29.4 \( \mu m \), obtained from an exponential decay fit. Thus the high density of voltage-gated sodium channel staining overlaps and is restricted within the axon initial segment as defined by ankyrin G immunolocalization. These results suggest the proximal voltage-gated sodium channel staining is unlikely to represent restricted subcompartmentalization of sodium channels within a longer axon initial segment (Van Wart et al. 2007). Instead the axon initial segment itself is shorter than in pyramidal neurons.

To estimate the action potential initiation site physiologically, we performed conventional somatic whole cell recordings combined with simultaneous loose-seal extracellular recordings from the cell’s axon (Khaliq and Raman 2005; Meeks et al. 2005; Monsivais et al. 2005; Raastad and Shepherd 2003). Action potentials were elicited with somatic current injection (\( < 200 \ pA \)) in current-clamp mode. In many neurons, the somatic waveform is composed of multiple components, which can be resolved by examining the first (Colbert and Johnston 1996; Coombs et al. 1957) or second (Meeks and Mennerick 2007) temporal derivative of the membrane voltage (Fig. 5, A3 and B3, \( \downarrow \) and \( + \); also evident in the phase plots of Fig. 2, * and \( \searrow \)). Classical work shows that the first component (Fig. 5, A3 and B3, \( \downarrow \)) arises from the axonal spike registered in the somatic recording, while the lag between the components (Fig. 5, A3 and B3, \( \downarrow \) to \( + \)) represents the slower recruitment of somatic channels. This sluggishness arises from the time to charge the large capacitance of the soma from the local current arising from the axon initial segment. The second peak (Fig. 5, A3 and B3, \(+\)) represents a maximal recruitment of somatic sodium channels (Colbert and Johnston 1996; Coombs et al. 1957).

In CA3 neurons, the somatic second derivative contains well-separated peaks as described in the preceding text (Fig. 5B3, \( \downarrow \) and \( + \)) (Meeks and Mennerick 2007). By contrast, granule neuron second derivative waveforms typically did not contain well-resolved peaks (Figs. 5A3, \( \downarrow \) and \(+\), and 2), although two inflection points were typically discernible. Inspection of second derivative waveforms from the two cell types suggested a smaller time lag between the two components (Fig. 5A3, \( \downarrow \) to \( + \)) in granule neurons. This shorter latency typically resulted in a merging of the two components of the second derivative waveform. That is, there was less bumpiness in the second derivative waveform of granule cells.

We compared “bumpiness” in each cell type by assessing the number of dentate neurons and CA3 neurons exhibiting a stationary inflection point (slope = 0) in the initial (axon) component of the second derivative somatic membrane potential waveform, (Fig. 5, A4 and B4, between \( \ldots \cdot \cdot \cdot \cdot \cdot \cdot \)) in dentate neurons, 36% of cells met this criterion, whereas in CA3 neurons, 89% of cells exhibited a stationary inflection point (Table 1, \( P < 0.05\); Fisher’s exact test). This quantitative assessment matches the qualitative observation from the phase plot analysis of Fig. 2: dentate neurons exhibit less “bumpiness” in their phase plot and second derivative waveforms, consistent with a more proximal initiation site.

From dual recordings of somatic and axonal action potentials, we obtained two latencies. From somatic records, we used the somatic membrane potential second derivative to estimate the latency from spike initiation to invasion of the axon hillock. We have previously shown through modeling that the time at which the second derivative waveform reaches 5% of the first peak amplitude represents a good estimate of the time of first threshold crossing in the axon (Meeks and Mennerick 2007). Further validation of this conclusion comes from analysis of threshold in dentate granule neurons calculated using the method in Fig. 2 (phase plots) versus the second derivative 5% method. Granule neuron threshold values calculated by the two methods were similar (\( -52.9 \pm 1.5 \ mV \) versus \( -54.4 \pm 1.5 \ mV \), \( n = 11 \)). Therefore we used the second derivative 5% time point to denote the time of action potential initiation for this analysis. The latency between this value and the time of first peak in the somatic membrane potential second derivative (Fig. 5, A3 and B3, \( \downarrow \)), was taken as the time from initiation to development/backpropagation of the spike to the axon hillock (Time 1, Fig. 5, A7 and C), before full invasion into the soma.

The axon signal was used to identify the time of arrival of the spike at the site of the local extracellular recording. Axon recordings were performed in voltage-clamp mode with the pipette tip potential clamped to the bath potential. The recorded
axonal currents correspond closely to the first time derivative of intracellular somatic membrane potential (Meeks et al. 2005). Therefore we compared the first derivative of the axon signal to the somatic second derivative waveform to calculate the time between the threshold and action potential backpropagation into the axon hillock (time 1; Fig. 5, A7 and C). We denoted the time between initiation (somatic 2nd derivative 5% value) and peak of the axonal first derivative waveform as the forward propagation time (time 2; Fig. 5, A7 and D).

To summarize, time 1 (Fig. 5, A7 and C) estimates the latency between first threshold crossing in the axon to arrival/development of the spike in the axon hillock. Note that time 1 includes time for the spike to develop from the time of initiation to the time of maximum voltage acceleration as well
as any true “backpropagation” time. Time 2 (Fig. 5, A7 and D) represents the time from initiation to the time of maximum intracellular voltage acceleration at the site of axon recording. Again, this value is expected to include the time necessary for the spike to develop from threshold to maximum acceleration as well as forward propagation time.

The preceding considerations regarding latencies predict that time 1 would not vary with axon recording distance and that time 2 should vary with axon recording location. Indeed, time 1 did not vary with axon recording location (Fig. 5C), indicating that axon recordings near the soma did not disrupt normal development of the action potential. Time 2 did vary with recording location (Fig. 5D). In the time 2 versus distance plot (Fig. 5D), the location at which the time 2 latency reaches a minimum represents an estimate of action potential initiation site location. In CA3 pyramidal neurons, a minimum latency is observed over a zone of axonal recording distances from near the soma to ~100 μm distal to the soma (Meeks and Mennerick 2007). This zone represents membrane regions adjacent to the point of first threshold crossing, all reaching a maximum membrane potential acceleration nearly synchronously. By contrast, an extended zone of minimum latencies was not apparent in dentate granule neurons. Instead granule neuron latencies reached a minimum <30 μm distal to the soma (Fig. 5D). The markedly different mossy fiber action potential latency versus distance relationship compared with CA3 pyramidal neuron axons is consistent with a proximal axon initiation site in dentate granule neurons. Based on confidence limits of the fit to time 2 versus distance, we conclude that the site of first threshold crossing is certainly <40 μm from the dentate granule soma, corresponding closely to the pattern of voltage-gated sodium channel localization (Fig. 4D). The same conclusion is borne out by a simple axon to soma max dV/dt latency plot [Supplemental Fig. S1; compare with Fig. 4E from Meeks and Mennerick (2007)].

To determine mossy fiber conduction velocity, we calculated the axonal recording distance (from 3-dimensional confocal images of the fluorescent dye filled axon) divided by the sum of latencies (time 1 plus time 2). As evident from Fig. 5E, the conduction velocity varies depending on the axonal recording site location. As the action potential develops from the site of initiation toward the soma, the conduction velocity (time from initiation to maximum acceleration at the point of recording) is much slower than the orthodromic velocity. Between 100 and 400 μm, the average mossy fiber action potential conduction velocity was 0.27 ± 0.02 m s⁻¹. Over this same range, CA3 axonal conduction velocity conduction velocity is 0.38 ± 0.02 m s⁻¹ (Meeks and Mennerick 2007). Thus mossy fibers conduct action potentials ~30% slower than CA3 neurons.

**Discussion**

Our results suggest, by several measures, dentate granule neurons have a more proximal axonal action potential initiation site than other cell types previously examined. We highlighted this contrast by comparing dentate granule neurons with CA3 pyramidal neurons, another hippocampal unmyelinated fiber type that we have studied under similar experimental conditions. The more proximal initiation in granule neurons might at face value suggest easier spike initiation because the initiation site may be electrophysiologically closer to the sites of excitatory synaptic input, and an overall more compact morphology would tend to maximize the influence of individual synaptic inputs. However, the proximal initiation site appears to be counterbalanced by a rather depolarized action potential threshold, ~10 mV more positive than in CA3 pyramidal neurons. Also lending to an overall view of weak axon excitability is the slow conduction velocity measured in mossy fibers.

A proximal axon action potential initiation site was suggested by four independent methods of estimation. First, phase plot inflections were significantly shallower at threshold and less bumpy during the rising phase in dentate recordings than in CA3 neuron recordings. Second, local TTX application influenced threshold when applied to the proximal axon but not when applied to the apical dendrite or to the distal axon. Third, voltage-gated sodium channel immunoreactivity peaked more proximally in mossy fibers than in CA3 axons. Fourth, direct dual somatic/axonal recordings suggested that the initiation site must occur 40 μm or closer to the dentate granule neuron soma, significantly closer than in CA3 neurons. Recently a proximal granule neuron initiation site was also suggested by direct intracellular recordings from balled-up mossy fiber blebs <100 μm from the soma, arising at the cut end of severed axons (Schmidt-Hieber et al. 2008).

**Fig. 5.** Latency analysis from simultaneous axon and soma recordings. A1–A7: data from a representative dentate granule neuron. A1: the somatic membrane potential (V_m) during an action potential. Scale bar is 25 mV. A2: 1st derivative of the somatic membrane potential (dV/dt). Scale bar is 100 mV ms⁻¹. A3: 2nd derivative of the somatic membrane potential (d²V/dt²). Scale bar is 100 mV ms⁻². A4: 3rd derivative of the membrane potential (d³V/dt³) is used as an aid to determine the first peak inflection on the d²V/dt² trace. Scale bar is 1 x 10⁶ mV ms⁻³. A5: axonally recorded extracellular signal at 280 μm from the soma. Scale bar is 10 pA. A6: 1st derivative of the axonal signal (dV/dt). Scale bar is 70 pA ms⁻¹. A7: 2 latencies are indicated. Time 1 represents the time from the action potential threshold until the membrane potential reaches the 1st (nonstationary) inflection point of d²V/dt² (at the ↓). The nonstationary inflection in the 2nd derivative coincides with a 3rd derivative value that does not pass through 0 in A4 (↓). Time 2 represents the time from the action potential threshold until the peak in the 1st derivative in the axonal action potential signal (A6). B1–B4: data from a representative CA3 pyramidal neuron. B1: the CA3 somatic membrane potential (V_m) during an action potential. Scale bar is 25 mV. B2: 1st derivative of the somatic membrane potential (dV/dt). Scale bar is 100 mV ms⁻¹. B3: 2nd derivative of the somatic membrane potential (d²V/dt²) shows a waveform with 2 inflection points (indicated with ↓ and +) occurring after the action potential threshold (---). The nonstationary inflection in the 2nd derivative coincides with a 3rd derivative value that does not pass through 0 in A4 (↓). Time 2 represents the time from the action potential threshold until the peak in the 1st derivative in the axonal action potential signal (A6). B4: 3rd derivative of the membrane potential (d³V/dt³) is used as an aid to determine the 2nd derivative stationary inflection point (3rd derivative = 0). Scale bar is 1 x 10⁶ mV ms⁻³. C: a summary of time 1 from 31 paired recordings to show that the time between 1st threshold crossing and the development/invasion of the action potential into the axon hillock is not dependent on the axonal recording location. Mean ± SEM for time 1 is 0.172 ± 0.004 ms; n = 31. **D:** a summary of time 2 from 31 paired recordings shows that the time between the 1st and 2nd threshold crossing and the detection of the action potential in the axon ≤400 μm from the soma varied linearly with distance. Regression line (---) is plotted from 70 to 450 μm of axon recording distance (R² = 0.83). ---, the 95% confidence bands of all possible straight lines through the data points. E: summary of the average conduction velocity over a distance of 400 μm from the soma on mossy fibers. Note the average conduction velocity is calculated from the velocity from the action potential initiation site to the axon recording site and the velocity from the action potential initiation site to the soma.
The proximal axon initiation site may be seen as integral to an overall compact cellular morphology of dentate granule neurons. The combination of limited dendritic arbors, small soma, and proximal axon action potential initiation site may conspire to have synaptic inputs and spike initiation occur in an electrotonically compact compartment, thus maximizing the contributions of synaptic depolarization. These conclusions are consistent with a recent comparison of dendritic processing in dentate granule neurons versus pyramidal neurons (Schmidt-Hieber et al. 2007). In particular, the proximal initiation site may help to compensate for the very thin diameter of the mossy fiber, which will result in a short length constant and rapid loss in fidelity of synaptic membrane potential changes (although see Alle and Geiger 2006).

Somewhat paradoxically to these considerations, we found that granule neurons have a significantly depolarized action potential threshold relative to CA3 pyramidal neurons. Past estimates of spike threshold in mature granule neurons using patch recording methods have varied from approximately −57 mV (Howard et al. 1998), to −49 mV (Staley et al. 1992) and −43 mV (Y. B. Liu et al. 1996; St. John et al. 1997). One problem with comparing estimates of threshold is the variety of methods used to estimate threshold. Previous estimates include “by-eye” estimates or methods based on first-, second-, or third-order derivatives of the membrane potential (Henze and Buzsaki 2001; Meeks and Mennerick 2007; Naundorf et al. 2006; Shu et al. 2007). We primarily focused on the phase plot method as a relatively straightforward, objective metric. Another problem is variation in the experimental conditions under which comparisons are made. Therefore we focused on comparing dentate granule neurons and CA3 neurons under essentially similar experimental conditions.

There are several likely contributors to the difference in action potential threshold between cell types. First, it seems unlikely that differential inaccuracies in threshold measurements arising from electrotonic differences between cell types account for the threshold difference. One might imagine that the more proximal location of granule cell initiation could lead to a more accurate estimate of threshold from somatic recordings than in CA3 neurons, particularly with strong current injections where the membrane potential is rapidly changing. However, initiation site in both fiber types falls well within estimated length constants for small CNS fibers (300–400 μm) (Alle and Geiger 2006; Shu et al. 2006). Also action potential threshold differences persisted even with weak current injections where cable filtering considerations should be minimized. It seems more likely that true differences in excitability of these cells account for the differences in threshold. These could include differences in sodium channel density or modulation (Kole et al. 2008), differences in potassium channel expression, differential participation of tonic GABA currents (Coulter and Carlson 2007), or a variety of other factors.

The depolarized spike threshold in the face of a proximal initiation site would seem to indicate that although excitatory synaptic inputs are not strongly attenuated by cable filtering, strong excitatory input is necessary to exceed spike threshold. The depolarized threshold could play a significant role in the often-noted “gateway” function of dentate granule neurons, a rather seizure-resistant cell type (Dudek and Sutula 2007; Schwartzkroin and Prince 1978). One is left with a picture of a dentate granule neuron that “listens” intently to incoming information but is not easily “persuaded” to pass the information by action potentials to target neurons.

As expected from the small diameter of dentate mossy fibers, we found evidence for slow conduction velocity of 0.27 ± 0.02 m s⁻¹. It is possible that the large mossy fiber boutons themselves contribute to the slowing of action potential propagation along mossy fibers as a result of impedance mismatch between the small axon and large bouton. On the other hand, a high density of active conductances in the boutons may participate in maintaining propagation fidelity in these fibers (Engel and Jonas 2005). With the aid of previous anatomical studies of granule neuron axons and presynaptic terminals (Acsady et al. 1998; Amaral et al. 2007), we estimated the latency of information arriving at the first mossy fiber bouton (∼200 μm from the dentate granule soma) versus the last mossy fiber bouton (∼3,300 μm). The delay for the same information traveling in the same mossy fiber axon will be ∼11 ms assuming constant distal conduction velocity. It is possible that this staggering of information transfer is another facet of granule neurons’ filtering function, making the development of pathological synchrony in target cells less likely. The implications for normal information transfer are less clear.

Our results suggest a more proximal initiation site, elevated threshold, and slower conduction velocity of dentate granule action potentials than observed in CA3 pyramidal axons. These properties are likely to significantly affect the strength and timing of information relay within the hippocampus. Obtaining fundamental characteristics of mossy fiber action potentials provides insight into signal processing within the classical hippocampal trisynaptic circuit and serves as a basis for comparison with other brain regions containing small-diameter, unmyelinated axons. This study also sets the stage for the exploration of developmental and modulatory cues influencing these unique features of dentate granule neuron information processing.

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