Properties of action potential initiation in neocortical pyramidal cells: evidence from whole cell axon recordings

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Abstract

Cortical pyramidal cells are constantly bombarded by synaptic activity, much of which arises from other cortical neurons, both in normal conditions and during epileptic seizures. The action potentials generated by barrages of synaptic activity may exhibit a variable site of origin. Here we performed simultaneous whole cell recordings from the soma and axon or soma and apical dendrite of layer 5 pyramidal neurons during normal recurrent network activity (Up states), the intrasomatic or intradendritic injection of artificial synaptic barrages, and during epileptiform discharges in vitro. We demonstrate that under all of these conditions, the real or artificial synaptic bombardments propagate through the dendrosomatic-axonal arbor and consistently initiate action potentials in the axon initial segment that then propagate to other parts of the cell. Action potentials recorded intracellularly in vivo during Up states and in response to visual stimulation exhibit properties indicating that they are typically initiated in the axon. Intracortical axons were particularly well suited to faithfully follow the generation of action potentials by the axon initial segment. Action potential generation was more reliable in the distal axon than at the soma during epileptiform activity. These results indicate that the axon is the preferred site of action potential initiation in cortical pyramidal cells, both in vivo and in vitro, with state dependent back propagation through the somatic and dendritic compartments.
Introduction

Cortical pyramidal cells have several active zones that are independently capable of generating action potentials. These include at least the apical and basal dendrites, soma, axon hillock, axon initial segment, and more distal components of the axon, such as the nodes of Ranvier (Colbert and Johnston 1996; Colbert and Pan 2002; Mainen et al. 1995; Milojkovic et al. 2005; Stuart et al. 1997a; Stuart et al. 1997b). Which compartment is the first to initiate action potentials and how far these spikes propagate and interact with the other compartments may have a significant effect on short and long term cortical function (Golding et al. 2002; Larkum et al. 1999a; Svoboda et al. 1997; Waters and Helmchen 2004). In addition, the different compartments of cortical neurons may interact to generate complex patterns of spike generation, such as burst discharges, prolonged depolarizations, and oscillations (Helmchen et al. 1999; Kim and Connors 1993; Larkum and Zhu 2002; Larkum et al. 2001; Stuart et al. 1997b; Wang 1999).

Investigations of even low frequency single spike initiation (the simplest form of action potential generation) in cortical pyramidal cells have yielded some conflicting results, although the general consensus is that in response to somatic depolarizing current pulses or local, electrical activation of threshold synaptic inputs, fast spikes are initiated in the axon initial segment followed by back propagation into the soma and apical dendrite (Palmer and Stuart 2006; Stuart et al. 1997a; Stuart et al. 1997b). This finding is in agreement with classical studies in spinal motoneurons (Coombs et al. 1957; Fuortes et al. 1957), although some studies have suggested that the first node of Ranvier, and not the axon initial segment, is the typical site of action potential initiation in some cell types (Clark et al. 2005; Colbert and Johnston 1996; Colbert and Pan 2002).

Although these studies indicate that the axon initial segment is the preferred site of spike initiation under the relatively quiescent conditions of the in vitro slice, the site of fast spike initiation under conditions when the cortical network is active is still relatively unexplored. In vivo, cortical pyramidal neurons receive 10s of thousands of synaptic inputs, which often culminate in prolonged depolarizations and significant increases in membrane conductances of both dendritic and somatic compartments (Destexhe et al. 2001; Steriade et al. 2001). One example of complex barrages of synaptic activity is the Up state of the slow oscillation that occurs during slow wave sleep or anesthesia (Haider et al. 2006; Steriade et al. 1993; Steriade et al. 2001). The synaptic barrages arriving during the Up state display properties that are similar, but not identical, to those of the waking state (Steriade et al. 2001). The properties of fast spike initiation in cortical pyramidal cells during ongoing barrages of synaptic activity, and how these give rise to spike threshold variability (Azouz and Gray 1999), is not well understood. Simultaneous dendritic and somatic extracellular recordings in vivo indicate that action potentials are typically back-propagating from the soma into the apical dendrites, although the precise location of initiation of these spikes could not be discerned (Buzsaki and Kandel 1998). Simultaneous in vivo dendritic Ca\(^2\)^+ imaging and somatic recordings suggest that layer 2/3 pyramidal cells may generate apical dendritic spikes only following somatic spikes (Svoboda et al. 1997; Svoboda et al. 1999), while the apical dendrites of layer 5 pyramidal neurons may occasionally generate Ca\(^2\)^+ transients (presumed action potentials) in isolation of somatic spikes (Helmchen et al. 1999). Indeed, in vitro studies have suggested that action potentials may initiate first from dendritic compartments.
during strong and/or synchronous synaptic stimulation to localized or distributed portions of the dendritic tree (Gasparini et al. 2004; Golding and Spruston 1998; Larkum et al. 1999a; Larkum et al. 1999b; Losonczy and Magee 2006; Milojkovic et al. 2005; Regehr et al. 1993; Schiller et al. 1997) and these dendritic spikes may or may not propagate completely to the soma (Jarosky et al. 2005; Larkum and Zhu 2002; Larkum et al. 2001; Losonczy and Magee 2006; Milojkovic et al. 2005; Schiller et al. 1997). Dendritic and somatic interactions are even more complex during the generation of bursts of action potentials, in which rapid action potentials may be initiated in the axonal/somatic compartments, while the generation of slower spikes or other active depolarizing events in the dendrites provide the prolonged depolarization that drives the burst (Helmchen et al. 1999; Kim and Connors 1993; Larkum and Zhu 2002; Larkum et al. 2001; Stuart et al. 1997b; Wang 1999).

These results suggest that the site of spike initiation in regular spiking cortical pyramidal cells may vary according to the nature of the initiating stimulus, with threshold stimuli preferring initiation in the axon (initial segment or first node of Ranvier) and strong, synchronized, or distal stimuli occasionally initiating spikes from the dendrites. Given the possibility of spikes being initiated in variable portions of the neuron, it is not clear where these action potentials will be initiated under the various conditions occurring in vivo, including during ongoing synaptic barrages (either spontaneous or evoked by sensory stimuli), or under pathological conditions, such as during epileptic seizures. Here, as a first step towards investigating this question, we examine the properties of spike initiation in cortical pyramidal cells during the spontaneous generation of Up states in vitro and in vivo, in response to sensory stimuli in vivo, during epileptiform discharges in vitro, and during the intradendritic and intrasomatic injection of complex patterns of current in vitro. We provide evidence that under all of these conditions, fast action potentials are preferentially initiated from the axonal initial segment and propagate reliably down the axon.
Materials and Methods

Experiments were performed on 0.3 mm thick slices of the ferret (7-10 week old) prefrontal cortex (anterior to the sylvian fissure), but also from somatosensory area, and maintained in vitro in a submerged style recording chamber at 36.5°C. Immediately after cutting, the slices were transferred to an incubation beaker filled with aerated normal ACSF containing (in mM): NaCl 126, KCl 2.5, MgSO4 2, CaCl2 2, NaHCO3 26, NaH2PO4 1.25, dextrose 25 (315 mOsm, pH 7.4) and held at 35°C until use. After at least 1 hour of incubation, slices were transferred to a submerged chamber in which both a bottom grid and a top grid were used to lift and hold the slices in position and which contained the ACSF solution as above. Cortical neurons were visualized with an upright infrared-differential interference contrast (IR-DIC) microscope (Zeiss Axioskop 2 FS plus). A light sensitive camera (OLY-150, Olympus) was used for tracing the fluorescent axonal profiles. For only those recordings where the slow oscillation or epileptic activity was examined in the submerged chamber, the ACSF solution was modified to contain 1 mM MgSO4, 1 mM CaCl2 and 3.5 mM KCl (Sanchez-Vives and McCormick 2000; Shu et al. 2003a; Shu et al. 2003b). The membrane potentials in our whole cell recordings were not corrected for Donnan liquid junction potentials, which is between 5 and 15 mV (Fricker et al. 1999).

Whole-cell recordings were achieved simultaneously from either the soma and the apical dendrite or the soma and the cut end of the main axon using a Multiclamp 700B or Axoclamp 2B amplifier (Axon Instruments, Union City, CA). Patch pipettes were formed on a Sutter Instruments (Novato, CA) P-97 microelectrode puller from borosilicate glass (1B200-4, WPI, Sarasota, FL). Pipettes for somatic recording had an impedance of 5-6 MΩ, and were filled with an intracellular solution that contained (in mM): KGluconate 140, KCl 3, MgCl2 2, Na2ATP 2, HEPES 10, pH 7.2 with KOH (288 mOsm). Calcium buffer included in the whole cell pipette was 0.2 mM EGTA. Only regular spiking neurons were analyzed in this study.

Alexa Fluor® 488 (100 µM) and biocytin (0.2%) were added to the pipette solution for tracing and labeling the recorded pyramidal cells. For simultaneous somatic and axonal (prefrontal cortex) or somatic and dendritic (typically in somatosensory cortex) whole cell recordings, approximately 10 minutes after somatic whole-cell recording was established, the course of the main axon or apical dendrite was examined under the fluorescent microscope equipped with a 40x water immersion objective and a magnifier of up to 2x. Only those pyramidal neurons in which the apical dendrite or axon was preserved and came to the upper surface of the slice were used in this portion of our study. Patch pipettes for whole cell dendritic or axonal recording were filled with a similar intracellular solution, but without fluorescent dye added; these had an impedance of 9-15 MΩ. The pipette was advanced to the apical dendrite or the cut end of the axon with a positive pressure of about 65 mbar, and guided by switching back and forth between the fluorescent and DIC images, with the total time the cell was exposed to fluorescence being kept to less than 20 seconds to minimize damage (our whole cell recordings from the soma did not reveal any evidence of changes in the electrophysiological properties of the recorded neurons during this brief exposure to fluorescence). The apical dendrite or the bleb formed at the cut end of the axon was then pressed by the pipette tip and negative pressure was applied to form a seal. As soon as a
tight seal (>10 GΩ) was achieved, pulses of brief suction were applied to break the patch, and the whole-cell configuration could be easily obtained. Thereafter, steps of positive and negative current were injected to the soma to examine the intrinsic membrane properties of the recorded neuron. During the whole period of simultaneous somatic and dendritic, or somatic and axonal recordings, access resistance was monitored frequently; recordings with access resistance higher than 25 MΩ for somatic recording, 80 MΩ for axonal or dendritic recording, were discarded. Bridge balance and capacitance neutralization were carefully adjusted before and after every experimental protocol. After a recording was completed, the slice was transferred to 4% paraformaldehyde in 0.1 M phosphate buffer for subsequent immunostaining and visualization.

All computations were performed in Spike2 (Cambridge Electronic Design, Cambridge, UK) and MATLAB (MathWorks, Bethesda, MD). All error bars were calculated as the standard error of the mean and data in the text are reported as mean +/- standard deviation.

A number of experiments were performed with a dynamic clamp technique using a DAP-5216a board (Microstar Laboratory). Noisy conductances were constructed according to an Ornstein-Uhlenbeck (colored noise) model (Destexhe et al. 2001). The standard deviation (and, concurrently, the amplitude) of the conductance was adjusted to give a 10 mV peak-to-peak membrane potential deviation, and the holding current was adjusted to keep the cell just near spike threshold.

The in vivo intracellular recordings analyzed here were from previously published studies performed in the primary visual cortex of halothane anesthetized cats (Nowak et al. 2005) or ketamine-xylazine anesthetized ferrets (Haider et al. 2006). Analysis was restricted to those neurons classified as regular spiking, according to standard criteria (McCormick et al. 1985; Nowak et al. 2003).

**Anatomical Methods**

Male ferrets, 7 weeks or 15 months old, were used for examination of the axon region of myelination, mainly in the ferret prefrontal cortex.

**Myelin staining**

*Bulk silver staining:* Because silver staining can make visible even the thinnest fibers of myelin in the cortex and it has been successfully used in various animal species. To visualize the overall pattern of myelination in the ferret brain, we used a modified version of the silver staining of myelin technique described by Gallyas (Gallyas 1979) using 40 µm thick, formalin fixed, vibratome cut brain sections mounted on gelatin subbed glass slides (Figure 5A).

*Single axon immunohistochemistry:* To visualize myelin in single axons (Figure 5C,D), layer 5 pyramidal cells in the ferret prefrontal cortex were intracellularly injected (in vitro, 300 µm thick sections as used for recordings) with 0.2% biocytin and immersion fixed in 4% paraformaldehyde. After rinsing with 0.1 M PB, sections were permeabilized in 50% cold methanol in 0.1 M PB containing 3% hydrogen peroxide and 0.5% triton X (45’, RT). Sections were then incubated in anti-myelin basic protein
Golgi staining: For Golgi staining (Figure 5B, Supplement Figure 2) we used a commercially available kit that has combined and improved the Golgi-Cox and the classical rapid Golgi methods. This kit, known as the FD Rapid GolgiStain™ (FD NeuroTechnologies, Inc., Ellicott City, MD) is convenient to use because it is highly reliable and results in excellent staining of unmyelinated axons while preserving staining of somata and dendritic spines. Also, this method works well in older specimens, an important feature since a commonly cited drawback of Golgi impregnation is that it has been sometimes observed to work well only in young animals (Millhouse 1981).

Microscopy and documentation: Bulk silver staining of myelin and Golgi preparations were observed under a Zeiss Axiophot microscope. Digital images were acquired with an AxioCam HRc camera and Axiovision software. Single stained axons double-labeled for myelin using immunohistochemistry were observed using a Zeiss LSM 510 Meta confocal microscope system direct-coupled to a Zeiss Axiovert 100M microscope. Digital photos were minimally manipulated for contrast and brightness in Adobe Photoshop®. Drawings and illustrations were made in Adobe Illustrator®.

Model Methods

Our computational model (Figure 6) was implemented using NEURON 5.8 (Hines and Carnevale 1997) and was based upon the multi-compartmental model of the full dendritic and somatic structure of a layer 5 cortical pyramidal cell (Figure 1D in Mainen and Sejnowski (Mainen and Sejnowski 1996)) coupled with a reconstruction of cortical pyramidal cell axons according to Binzegger et al. (Binzegger et al. 2005). We used this model recently to examine the passive propagation properties of intracortical axons (Shu et al. 2006). The temperature of the modeled cell was 37°C.

Dendrites and soma: The soma and dendrites contain 164 segments. The somatic surface area is 2,748 µm², while its diameter is 25 µm, and its length is 35 µm. There are 11 primary neurites, 87 branches, totaling 17,668 µm in length and 78,858 µm² in surface area.

Axon: Our model of the axon began with that of Mainen et al. (Mainen et al. 1995), and was subsequently modified to include either a reduced axonal arbor that was of similar length and branching pattern as that of our biocytin-filled neurons in vitro, or a more complete axonal arbor modeled after that of Binzegger et al. (Binzegger et al. 2005). In addition, we modified membrane capacitance and ionic conductances in order to match the spike initiation properties of our layer 5 pyramidal cells (McCormick et al. 2006).

Main axon: The soma connects to the axon hillock, which tapers from 2.4 µm to 0.92 µm and has a length of 10 µm. The hillock is followed by a 40 µm initial segment of 0.92 µm diameter. Following the initial segment, there is a 300 µm length of unmyelinated axon (0.55 µm in diameter) based on our examinations on the main axons of prefrontal
cortical neurons in 7 week old ferrets. For modeling the full axonal arbor, the next portion of the main axon is myelinated and 3000 µm in length, with internode distances of 200 µm. The diameter of the internodal axon is 0.92 µm. The diameter of axonal nodes is 0.46 µm and their length is 1 µm.

**Axon collaterals of full layer 5 pyramidal cell:** All collaterals were assumed to be unmyelinated (Shu et al. 2006). There are 8 first order collaterals (diameter 0.46 µm), 23 second order collaterals (diameter 0.37 µm), and 131 third, forth and fifth order collaterals (diameter 0.37 µm). The total length of collaterals is approximately 64 mm. Including the main axon, the total axon length is 66.7 mm.

**Axon collaterals of “slice” layer 5 pyramidal cell:** For the slice neuron simulation, the dendritic tree and soma are the same as in the simulated full neuron, but there are 6 first order collaterals, 22 second order collaterals, totaling 18.7 mm in length consisting of 35 sections composed of 3,293 compartments. With the main axon, the total axonal length for the slice neuron is 19.2 mm.

**Electrical properties:** The membrane capacitance $C_m$ for the soma, dendrites, unmyelinated axon, and axon collaterals is modeled as 0.7 µF /cm$^2$ while for myelinated axon it is 0.04 µF /cm$^2$. The axial resistance for dendrite and axon is 100 Ω-cm (Mainen and Sejnowski 1996). The membrane time constant of the soma, dendrite, hillock and initial segment is 21 msec, while for the main axon and collaterals the time constant ranges from 2 to 10.5 msec.

**Channel distributions:** The transient Na$^+$ current is present in all parts of the modeled cell and its density is high in the initial segment, hillock and node (7,500 pS/µm$^2$), but lower in the soma (750 pS/µm$^2$) and dendrites (20 pS/µm$^2$). In unmyelinated model axon and collaterals, Na$^+$ conductance is between 750-2500 pS/µm$^2$ (the thinner the collateral, the lower the value of the Na$^+$ conductance). The density of Na$^+$ conductance was low (20 pS/µm$^2$) in the myelinated portions of the axon. The reversal potential of Na$^+$ in our model is 60 mV. The fast, voltage activated K$^+$ current, $I_{Kv}$ is present in the model hillock, initial segment, and node (1000 pS/µm$^2$). The density is lower for soma (400 pS/µm$^2$), unmyelinated model axon (500 pS/µm$^2$), and axon collaterals (200 pS/µm$^2$ for 1st, 100 pS/µm$^2$ for higher order collaterals). The reversal potential of K$^+$ is -90 mV.

The slow non-inactivating potassium current (M-current; $I_{km}$), high-voltage activated Ca$^{2+}$ current, $I_{Ca}$ and one Ca$^{2+}$ dependent K$^+$ current, $I_{kCa}$ are distributed throughout the soma and dendrites (Km conductance 0.1, Ca conductance 0.3, and Kca conductance is 3 pS/µm$^2$) as per Mainen et al. (Mainen and Sejnowski 1996). The reversal potential of Ca is 140 mV. Background leak current is distributed throughout the cell. In the soma, dendrites, hillock, initial segment and myelinated axon, $g_{leak} = 0.0000333$ S/cm$^2$. In the unmyelinated axon and collaterals, $g_{leak} = 0.00006667$ S/cm$^2$. In the nodes, $g_{leak} = 0.02$ S/cm$^2$. The value of the leak conductance is modified to give time constants that are similar to what we observed in experiments. The reversal potential of the leak current is -70 mV. For the specific rate functions for the different ionic channels see(Mainen and Sejnowski 1996).

**Results**

In cortical slices the main axon of pyramidal cells travels to the surface of the slice where it forms a patchable(Shu et al. 2006) spherical 3-6 µm bleb owing to being
cut during the slice procedure (Figure 1D). Here we perform whole cell recordings from the axon, apical dendrite, and soma to examine the properties of action potential generation in layer 5 cortical regular spiking pyramidal cells in the prefrontal cortex in response to current injections and during spontaneous synaptic activity of the Up state (Shu et al. 2006; Shu et al. 2003b) as well as during epileptiform discharges. We then compare our results with action potentials recorded in vivo during Up states and in response to visual stimulation.

Whole cell recordings were obtained simultaneously from either the cell body and apical dendrite (n=7) or cell body and axon (n=16) of cortical pyramidal cells during spontaneous Up and Down states in ferret prefrontal or somatosensory cortical slices maintained in vitro (Figure 1). These Up states are characterized by 5-15 mV amplitude, 0.25 to 1 second duration barrages of synaptic activity containing approximately an equal mixture of excitatory and inhibitory potentials (Figure 1B, E) (Haider et al. 2006; Shu et al. 2003b). This synaptic activity arises from the discharge of neighboring neurons and results in the activation of action potentials in the soma at the rate of 5-31 Hz (10-90th percentiles), with an average discharge rate of 11.2 +/- 3.2 Hz (Mean +/- SD; n=7) (Supplement Figure 1A).

Simultaneous recordings from pyramidal somata and apical dendrites (50-580 µm from the soma) during the generation of spontaneous Up states revealed that action potentials were generated in both the cell body and apical dendrite. Examination of the timing of these action potentials revealed that all action potentials were generated in the soma prior to the apical dendrite (n=637 spikes in 7 neurons; Fig. 1C), even at all frequencies of action potential generation from 5-75 Hz. The dendritic action potentials decreased in amplitude and broadened in duration with increases in distance from the cell body (not shown). Consistent with previous findings, these recordings revealed that action potentials propagate from the soma up the apical dendrite at a speed of 0.66 +/- 0.18 m/sec (n=6) (Larkum et al. 2001; Stuart et al. 1997a).

Simultaneous recordings from the axon and soma of pyramidal cells during the spontaneous generation of Up states revealed a spike timing relationship that varied with the distance of the axonal recording site from the soma (n=16). Recordings in which action potentials arrived at the axonal recording site prior to the soma were obtained at axonal distances of between 40 and 157 µm (Figure 1D-G), while axonal recordings that exhibited spikes occurring after those in the soma were recorded at distances of 111-311 µm (see below; Figure 1G). Plotting the time difference between the axonal and somatic spike times versus location of the axonal recording site, revealed a relationship that fell to zero difference at a distance of approximately 80-110 µm (Figure 1G). If the action potentials were to propagate orthodromically and antidromically from their site of origin with equal speed, then spikes that arrive at the same time at the axonal and somatic recording sites are likely to have initiated at a point halfway between the two. Therefore, these results suggest that during spontaneous Up states, action potentials are initiated in the axon somewhere between 40 and 55 µm from the soma. Our computational model suggests, however, that action potentials propagate orthodromically away from their site of origin at an average speed of 0.83 m/sec, while they propagate antidromically towards the cell body at an average speed of 0.7 m/sec (see Figure 6 below). These simulations
suggest that our present method of estimation of the site of spike initiation should be compensated by approximately 10 µm towards the soma.

**Action Potentials Initiated by Artificial Up States**

In order to control the precise location of the arrival of the currents initiating action potentials, we used the dynamic clamp technique to inject artificial barrages of synaptic activity in the apical dendrite or soma while performing dual dendritic-somatic or somatic-axonal recordings (n=10; Figure 2). As with naturally occurring barrages of synaptic activity, these artificial barrages resulted in action potentials that occurred first in the soma followed by propagation into the apical dendrite (Figure 2A), at all frequencies of action potential generation from 2-34 Hz (Supplementary Figure 1B), for both somatic and apical dendritic injections. The injection of artificial Up states into the soma resulted in action potentials whose timing difference between the soma and axon (Figure 2C, D) exhibited a zero difference at approximately 110 µm (Figure 1G), indicating that these spikes are initiated at approximately 55 µm from the cell body, in the axon initial segment. Similar results were obtained with action potentials generated in response to current pulses and during epileptiform activity (see Figure 1G and below).

**Responses to current pulse injection**

By initiating spikes with the intracellular injection of short duration current pulses at either the somatic or axonal recording sites, and examining the timing relationship of these action potentials versus distance between the recording locations, we were also able to calculate the apparent action potential initiation site using simple algebra (Figure 3). Our calculation revealed that in response to somatic current pulses action potentials initiate approximately 46 +/- 5.6 µm (n=16 cells) down the axon from the edge of the soma. This location, however, should be compensated for possible differences in antidromic versus orthodromic conduction velocities in our experiments (see below).

We investigated the properties of action potential generation further through the initiation of spikes with the intracellular injection of long duration (100-500 msec) current pulses of 0.1 to 5 nA amplitude, delivered through either the soma or axon whole cell recording electrodes (Figure 4; n=22 cells). We found that there was a gradation of electrophysiological properties between three active zones: the soma, axon initial segment, and distal axon. The injection of prolonged current pulses into the soma or proximal axon initial segment (22-97 µm from the soma) resulted in the repetitive generation of action potentials in both the somatic and axonal recording sites. Increasing the amplitude of somatic injected current resulted in an increase in action potential frequency and a broadening of action potential duration in both the soma and the axon (Figure 4A-D,F). Action potential amplitude in both the soma and axon initial segment decreased with large current injections (Figure 4B, D), while action potential amplitude in the distal axon was less susceptible to strong depolarization of the soma (Figure 4C,D). With very large somatic depolarizations (/>= 5 nA), action potential generation occasionally failed in either the axonal or somatic recording site (Figure 4D). In addition, action potentials were observed to fractionate into components, which were especially
pronounced when $dV/dt$ of the action potential was examined (Figure 4F). There appeared to be at least three components to these action potentials. The smallest component, as recorded from the soma or distal axon, preceded spike generation in both the distal axonal and somatic recording sites (Figure 4F). Because of this timing, and the presence of this smaller spike even when action potentials in the soma and distal axonal recording sites failed (Figure 4F, asterisk), we presume that this component represents the generation of an action potential in the axon initial segment. This IS spike was followed, in the soma, by the initiation of a somatodendritic (SD) spike. Similarly, in the distal axon (DA), local action potential generation was also preceded by the IS spike (Figure 4F).

The intracellular injection of depolarizing current pulse into the distal axon (110-309 µm from the soma) resulted in the initiation of single spikes only, which back-propagated into the soma (Figure 4E; n=16). Even intense, prolonged depolarization of the distal axon failed to initiate trains of action potentials (Figure 4E), a property previously reported for axons and which appears to result from a high density of low threshold $K^+$ channels in the axon (Dodson et al. 2003; Waxman 1995). As mentioned above, prolonged depolarization of the proximal axon (22-97 µm from the soma; n=6) resulted in repetitive spike discharge, similar to that seen with somatic depolarization (not shown). Thus, the soma, proximal axon, and distal axon all exhibited distinct electrophysiological features.

Properties of myelination of axons in ferret prefrontal cortex

Previous reports have compared the site of spike initiation with the pattern of myelination to demonstrate that spikes are initiated either in the axon initial segment or the first node of Ranvier (Clark et al. 2005; Palmer and Stuart 2006). Here we also compared the pattern of myelination in layer 5 pyramidal neurons in the ferret prefrontal cortex with our calculation of the spike initiation point. The initial portions of the principal axon of cortical pyramidal cells contains four components that we will consider here: 1) the axon hillock, which is characterized by the initial tapered component of the axon at its origin (typically the cell body but on occasion a dendrite); 2) the axon initial segment; 3) the myelinated segment of the axon; and 4) the first node after myelination. To determine these components in layer 5 cortical pyramidal cells of the ferret prefrontal cortex, we examined the myelination of these axons through both Golgi-stained material (Figure 5B) as well as immunofluorescence staining against myelin on neurons that were also filled with biocytin and Alexa-488 (Figure 5C,D; Supplement Figure 2).

First, we examined the overall pattern of myelination of the 7-week old ferret prefrontal cortex through silver-stain visualization of myelin (Figure 5A; Methods). This method revealed dark staining only within the white matter, with relatively little staining in the medial or lateral banks of the prefrontal cortex (Figure 5A; n=6 ferrets). In contrast, silver-stain based visualization of myelination in the primary visual cortex from the same animals revealed typical bands of myelin in both the white matter as well as within layers 4 and 5 (not shown).

To examine the issue of myelination of layer 5 pyramidal cell axons in the ferret prefrontal cortex further, we examined Golgi-stained material (see Methods). Golgi staining reveals axons only if they are unmyelinated (Fairen et al. 1977). Therefore the
point at which axons emanating from the cell body lose Golgi staining should indicate the approximate beginning of myelination. Examination of Golgi stained sections revealed that in the 7 week old ferret prefrontal cortex the principal axon extends for a long distance (average of 212 +/- 118 µm; n=38) from the soma before staining is lost. Examination of Golgi-stained material from the prefrontal cortex of older ferrets (15 months) revealed that these also appear to be unmyelinated for a long distance (138 +/- 103 µm; n=46), although this distance is less than in 7 week old animals. The long length of axon that is visualizable with Golgi staining in the ferret prefrontal cortex is in contrast to that previously reported for many neocortical pyramidal cells (Farinas and DeFelipe 1991). Indeed, when we examined Golgi-stained layer 5 pyramidal cells in the ferret primary visual cortex, the axons became unlabeled at a much shorter distance (36 +/- 19 µm in 7 week old; n=30; 54 +/- 46 µm in 15 month old; n=30) (Supplementary Figure 2).

To examine the location of myelination more directly, we performed immunofluorescent staining of myelin basic protein (MBP) on prefrontal cortical layer 5 neurons that had been previously filled with biocytin and a fluorescent dye (Alexa 488; 7 week old ferrets). Of 15 neurons examined, the axons of 12 were cut at the surface of the slice at an average distance of 289 (+/- 144 µm) before myelination became evident. The axons of the three remaining cells became myelinated at an average distance of 453 (+/- 87) µm, corresponding roughly with the location of their entry into the white matter (Figure 5C,D). These results indicate that in layer 5 pyramidal neurons of the ferret prefrontal cortex, action potentials are initiated in the initial, unmyelinated portion of the axon and that this location does not correspond to the beginning of myelination as it does in some preparations (Palmer and Stuart 2006).

Location of First Axon Collateral

The first axon collateral is often associated with the first node of Ranvier (Fraher and Kaar 1984; Sloper and Powell 1979), which has been suggested to be the location of action potential generation in some cell types (Clark et al. 2005; Colbert and Johnston 1996; Coombs et al. 1957). Here we examined the distance from the cell body to the first axon collateral in Golgi stained sections of ferret prefrontal cortex. Of 38 cells examined in 7 week old ferrets, 26 exhibited at least one axon collateral, and the average distance to the first collateral from the soma was 104.3 +/- 32.1 µm. This distance is similar to that obtained by examining the distance to the first axon collateral in biocytin/Alexa 488 filled pyramidal cells (112.5 +/- 25.7; n=14). In adult (15 month old) ferrets, the distance to the first axon collateral in Golgi-stained sections was similar (105 +/- 26 µm; n=10). Thus, the calculated location of action potential initiation is significantly proximal to the location of the first axon collaterals in our layer 5 pyramidal neurons.

Computational Model of Action Potential Initiation and Propagation

One possible modifying factor in calculating the location of spike initiation, is the assumption that orthodromic and antidromic spikes propagate at the same velocity from their site of origin. We examined this using computational models of either a restricted, “slice-like” axonal arbor, or a complete arbor (Mainen et al. 1995; Mainen and Sejnowski 1996; Shu et al. 2006). In both the partial and complete axonal arbor models, spikes...
initiated at 38 μm from the root of the axon in response to somatic current injection (Figure 6). Increasing the axonal density of gNa⁺, starting with levels equal to that of the soma, moved the action potential initiation point along the axon away from the soma. This progression down the axon exhibited a plateau of approximately 40 μm (not shown). In our model of axonal action potential generation, the spikes were found to propagate orthodromically down the axon and antidromically towards the cell body at different rates (Figure 6C). In simulations in which the main axon terminated at lengths similar to those in our actual recordings, we calculated that this difference in orthodromic and antidromic propagation velocity will cause the calculated point of initiation in real recordings to be on average about 10 μm farther down the axon than the actual initiation point. Including this correction factor results in an estimated initiation point of between 30 and 45 μm from the soma in our recordings from real neurons.

Using current pulses to initiate action potentials at the cut end of the axon can also result in a miscalculation of the point of initiation of action potentials. This is because the spikes recorded at, for example, 300 μm from the soma propagate at different velocities according to whether they are initiated locally and propagate antidromically, or initiated at the axon initial segment and propagate orthodromically (Figure 6C). Antidromic spikes propagate more quickly at their site of initiation (300 μm from the soma in this model), owing to the depolarization of the axon by the current pulse during antidromic activation. In contrast, initiation of spikes with somatic current pulse initiation did not result in such a large effect at the site of spike initiation (axon initial segment). The differences in average propagation velocity suggest that our algebraic calculations from the data obtained in real neurons (Figure 3) may estimate the location of spike initiation to be distal to the actual site of spike initiation by approximately 8 μm, resulting in a corrected location of approximately 38 μm from the soma.

**Spike ThresholdVaries with Distance from the Soma**

To examine the possibility that the axon initial segment has a lower threshold for action potential initiation, we plotted the membrane potential at which spikes were initiated with intra-axonal current pulses versus distance along the axon from the soma (Figure 7). This plot revealed a relationship in which the threshold increased with distance down the axon, after a distance of approximately 100 μm (Figure 7; n=20 axonal recordings). Plotting the best linear fit for all points distal to 50 μm and the best linear fit for points at 75 μm or less revealed in intersection at approximately 60 μm. Although there is considerable variation in this plot of spike threshold (each point represents a different cell), the data suggests that the axonal region up to approximately 100 μm from the soma has a lower spike threshold than the more distal axon.

**Spikes Occurring in vivo Have Initiation Properties Suggestive of an Axonal Origin**

Recently we have demonstrated that somatic recordings of action potentials that originate in the axon reveal an unusually rapid rate of rise at the foot of the spike(McCormick et al. 2006), giving rise to the so-called action potential “kink”(Naundorf et al. 2006). Here we examined whether or not this was also true for action potentials that were initiated in the apical dendrite of layer 5 pyramidal cells.
(Figure 8). Through the intracellular injection of large (0.8-2 nA; 100-500 msec) current pulses into the proximal to middle (150-420 µm) portions of the apical dendrite, we were able to cause the dendritic initiation of fast action potentials that then propagated to the soma. Examination of these somatic spikes revealed a relatively slow, or smooth, transition at the initiation point of the spike (Figure 8A; n=105 spikes in 4 cells). In contrast, examination of somatic action potentials generated in response to somatic current injection and that were known, from dual somatic/axonal whole cell recordings, to have originated in the axon exhibited a prominent kink at spike initiation (Figure 8B; n=2671 spikes in 13 cells). Plotting the slope of the phase plot at spike initiation for action potentials known to be initiated in the axon or the apical dendrite revealed very distinct distributions (Figure 8E). Next, we compared the distributions of the slopes at spike initiation of action potentials occurring in vivo during either Up states in the prefrontal cortex of the ferret (Haider et al. 2006) or during visual stimulation in the cat primary visual cortex (Figure 8E)(Nowak et al. 2005). Interestingly, both spikes initiated during Up states in the ferret prefrontal cortex (n=2364 spikes in 11 cells) and spikes initiated by visual stimuli in the cat primary visual cortex exhibited a prominent kink at spike onset (Figure 8C; n=4298 spikes in 10 cells). This result was quantified by measuring the slope of the phase plot of spike dV/dt versus membrane potential (McCormick et al. 2006; Naundorf et al. 2006) (Figure 8D). The distributions of the slope of the phase plot at spike initiation for in vivo spikes during Up states as well as visually evoked spikes were very similar to that for in vitro somatic recordings of axonally initiated spikes in layer 5 pyramidal cells (Figure 8E). In contrast, the distribution of in vivo spike phase plot slopes were completely different from those for spikes known to have originated in the apical dendrite in vitro (Figure 8E). These results indicate that spikes occurring in cortical neurons in vivo, either during spontaneous Up states in anesthetized ferrets or during visual stimulation in anesthetized cats are primarily initiated in the axon.

Properties of Axonal Spike Initiation during Epileptiform Activity

Previous studies have suggested that dendritic spike initiation may occur when the dendrites are strongly and/or synchronously depolarized (Golding and Spruston 1998; Kloosterman et al. 2001; Stuart et al. 1997b; Turner et al. 1991). In addition, the pattern and properties of spike generation in axons may be very important in the generation of epileptiform activity (Meeks et al. 2005; Noebels and Prince 1978). Here we examined the point of initiation of fast action potentials during the generation of epileptiform activity in prefrontal cortical slices. Following the bath application of 50 µM picrotoxin, and in the presence of 1 mM Mg$^{2+}$ and 1 mM Ca$^{2+}$ (Sanchez-Vives and McCormick 2000; Shu et al. 2003a; Shu et al. 2003b), cortical slices generated prolonged (0.4 - 8 second) epileptiform bursts consisting of an initial large depolarization and inactivation of somatic spikes, followed by repetitive bursts of action potentials (Figure 9). This epileptiform activity is known to be generated through recurrent excitatory networks within the cortical slice and are associated with very strong barrages of excitatory and inhibitory (although GABA_A receptors are blocked here) synaptic activity (reviewed in (McCormick and Contreras 2001)).
Simultaneous somatic and apical dendritic (50-90 µm from the soma) recordings during the generation of epileptiform activity revealed that each fast action potential occurred in the soma prior to the apical dendrite (Figure 9A). We failed to observe any spikes that exhibited an onset in the proximal apical dendrite prior to the soma, indicating that even under this situation of strong dendritic depolarization, fast spikes still are back-propagating between the soma and proximal apical dendrite. To examine if these spikes were initiated in the axon initial segment, we performed whole cell recordings from axons that were relatively short in length (50-77 µm; n=6), with the hypothesis that action potentials initiated in the axon would occur first at these short axonal distances in comparison to the soma. Indeed, these dual somatic/axonal recordings demonstrated that during the generation of epileptiform activity, all action potentials, whether they were in the initial, or subsequent, bursts, exhibited an earlier onset in the proximal axon than in the soma (Figure 9B). The timing difference between the axon and the soma was consistent with axonal initiation at approximately 45 µm from the soma of these epileptiform spikes (Figure 1G).

Simultaneous whole cell recording from the soma and proximal to distal parts of the axon (60-300 µm) during epileptiform activity demonstrated that these portions of the axon followed action potential generation in the soma on a nearly one to one fashion with failures of spike initiation and conduction being rare (Figures 9, 10; n=6). The large depolarization (paroxysmal depolarization shift; PDS) associated with epileptiform bursts was also present in the axon, although the amplitude of this decreased with distance from the soma (Figures 9, 10). The large depolarizations, particularly the first PDS, associated with epileptiform activity could strongly reduce the amplitude of spikes generated in the soma, and to some degree, in the axon initial segment (Figure 10). However, the number of action potentials generated in the proximal and distal axon during the initial PDS was often greater than in the soma (Figures 9C,D, 10B).

Previously, simultaneous somatic whole cell and axonal extracellular recordings suggested that cortical axons do not faithfully follow or generate epileptiform bursts as well as pyramidal cell bodies (Meeks et al. 2005), which is in direct contrast to our results here. To test the possibility that this discrepancy may result from differences in the recording techniques, we recorded extracellularly (as per (Meeks et al. 2005)) from the same axons that we had previously performed whole cell recordings and compared the results. We found that axonal action potentials recorded extracellularly could suffer from a significant signal to noise problem. Many of the spikes that were previously observed with axonal whole cell recording disappeared into the background noise of the recording when recorded extracellularly (cf. Figure 9C,D).

**Discussion**

Cortical neurons in vivo undergo a constant bombardment of synaptic activity arriving throughout the soma, dendrites, and axon initial segment. Detailed in vitro investigations indicate that each of these compartments of the neuron are capable of generating action potentials and simultaneous whole cell recordings from the soma and apical dendrites or soma and even distal axons (Shu et al. 2006) indicate that this ongoing synaptic activity can propagate long distances (> 0.5 mm) along these processes. Which
compartment then will initiate the fast action potentials that propagate down the axon in response to this synaptic bombardment?

Prior in vitro studies suggest that fast action potential initiation will most often occur in the axon in pyramidal cells, followed by back propagation into the somatic and dendritic compartments (Stuart et al. 1997a; Stuart et al. 1997b). However, exactly which compartment of the axon will initiate the action potential has been an open question, with studies favoring both the axon initial segment (Palmer and Stuart 2006; Stuart et al. 1997a) as well as the first node of Ranvier (Clark et al. 2005; Colbert and Johnston 1996; Colbert and Pan 2002). A recent imaging study using voltage sensitive dye indicates that action potentials initiated in response to current pulses and single EPSPs evoked with local electrical stimulation occur first approximately 35 µm down the main axon from the soma (Palmer and Stuart 2006). To put this in perspective, it is worth reviewing the structure of cortical axons. Axons from pyramidal neurons typically arise from the cell body, although they can also emanate from a basal dendrite (Palay et al. 1968). The transition between the axon and the soma/dendrite from which it originates is associated with a conical, approximately 5 µm long, transition segment, the axon hillock. The axon initial segment follows the axon hillock. It is typically a 17-40 µm long segment of axon that on the electron microscopic level contains a layer of dense granular material underneath the membrane, microtubules in the cytoplasm, and scattered clusters of ribosomes (Farinas and DeFelipe 1991; Inda et al. 2006; Palay et al. 1968). Immunocytochemical localization of Na⁺ channels, K⁺ channels, and cytoskeleton and adhesion molecules spectrin BIV and Caspr2 reveal a high density in the axon initial segment (as well as at nodes of Ranvier) (Colbert and Pan 2002; Inda et al. 2006). Often, but not always, the end of the axon initial segment (as defined by the loss of the submembranous structures typical of the initial segment on the EM level) is associated with the beginning of myelination (Farinas and DeFelipe 1991; Palay et al. 1968). In the layer 5 neurons studied here, the main axons were unmyelinated for approximately 200 µm or more (see Figure 5, Supplementary Figure 2). Our results indicate that action potential typically initiate in a region that is approximately 35-50 µm from the soma, long before the axons are myelinated in these cells. Although Palmer and Stuart (2006) have suggested that the action potential initiation site corresponds to the beginning of myelination, these authors also observed a proximal initiation site at early postnatal ages when axons are unmyelinated. Together, these and our results suggest that the region of myelination by itself does not determine the site of spike initiation.

Immunocytochemical studies suggest that the axon initial segment may contain a high density of Na⁺ channels, K⁺ channels, causing it to have a low threshold for action potential initiation (Inda et al. 2006; Komada and Soriano 2002). Our high resolution recordings of action potentials in the axon initial segment reveal a very high rate of rise for these spikes, indicating a relatively high local density of Na⁺ channels (McCormick et al. 2006). In contrast, direct recordings of Na⁺ and K⁺ channels from the initial segment suggest an alternative interpretation. The Na⁺ channels at the axon initial segment may have a more negative activation curve than those in the soma (Colbert and Pan 2002). In addition to the presence of a high density of Na⁺ channels, and/or the presence of Na⁺ with low threshold, the electrical characteristics of the axon initial segment are conducive to the initiation of action potentials. The large capacitive and conductance load of the soma and dendrites decreases with increased distance down the axon, thereby decreasing the
amount of current required to initiate an action potential. As synaptic activity arriving in the dendrites and soma of the neuron propagates down the axon (Alle and Geiger 2006; Shu et al. 2006), the axon initial segment forms the location that is most favorable to action potential initiation. This region of the cell is strongly affected by the summated membrane potential arriving from the various dendritic branches and soma (Shu et al. 2006) and its electrical properties (e.g. channel densities and properties, electrical isolation, etc.) facilitate the production of action potentials.

Where do action potentials initiate in vivo? This is a difficult question to answer since simultaneous recordings with high temporal resolution from different portions of pyramidal cells, that also include the axon, in vivo are not yet possible. Prior extracellular recording studies indicate that action potentials typically are back propagating from the soma into the apical dendrites (Buzsaki and Kandel 1998), indicating that the spikes initiated somewhere within the soma, basal dendrites, or axon compartments. One property of action potentials offers an interesting possibility to decipher the region of origin of action potentials in vivo. Recently it was noted that spikes occurring in cortical neurons exhibit a prominent and characteristic rapid rate of rise at their initiation, giving rise to a so-called “kink” (Naundorf et al. 2006) (Figure 8). Detailed recordings and simulations of cortical pyramidal cells reveal that this kink in the action potential arises from spike initiation in the axon (McCormick et al. 2006), owing in part to the high density of Na+ channels apparently present in the axon initial segment (Inda et al. 2006; Komada and Soriano 2002). Our recordings reveal that spikes initiated in the apical dendrite do not share this property of axonally-initiated action potentials (see Figure 8). When we examined the properties of spike occurring in vivo, either during spontaneous Up states in anesthetized ferrets, or during visual stimulation in anesthetized cats, we found a distribution of rate of rise at the foot of the spike that was very similar to spikes initiated in the axon in layer 5 pyramidal neurons in vitro. The strong overlap in these distributions suggests that spikes that we recorded in vivo were preferentially initiated in the axon. The small difference in spike slope distributions (see Figure 8) between in vivo and in vitro suggests that a small percentage of spikes in vivo may have originated in the dendrites. However it is also possible that these differences arise from comparing the spikes of different cell types (e.g. layer 5 pyramids in vitro versus multiple cell types in vivo) or differences in important physiological parameters between in vivo and in vitro (e.g. modulators, ion concentrations, anesthetics, etc.).

Prior studies have suggested that strong depolarization of dendritic compartments may give rise to local spike initiation that then propagates to the soma/axon initial segment (Gasparrini et al. 2004; Golding and Spruston 1998; Larkum et al. 1999a; Larkum and Zhu 2002; Larkum et al. 2001, 1999b; Losonczy and Magee 2006; Milojkovic et al. 2005; Regehr et al. 1993; Schiller et al. 1997). Given this, you might expect that epileptiform discharges, which are mediated by very strong excitatory synaptic barrages arriving in pyramidal cell dendrites, would be associated with dendritic action potential initiation. Perhaps surprisingly, we found that during epileptiform activity following disinhibition in vitro, fast action potentials were always initiated in the axon initial segment, followed by back-propagation into the soma and apical dendrite. Presumably, this axonal site of initiation results from the arrival of large barrages of EPSPs in the basal dendrites, which are electronically closer to the axon initial segment than the distal apical dendrite. Indeed, cortical pyramidal cells receive approximately 75-95% of their
excitatory synaptic (spinous) inputs in the basal and nearby oblique apical dendrites in the neocortex, within approximately 200 µm from the soma (Larkman 1991), indicating that the distal apical dendrite may form a relatively minor, but unique, source of excitatory input to these cells. Voltage sensitive dye imaging of action potentials generated in pyramidal cells in response to somatic current injection or local applications of glutamate to the basal dendrites indicate that these spikes typically back-propagate from the soma into the basal dendrites (Kampa and Stuart 2006; Milojkovic et al. 2005). In a minority of cells, fast pre-potentials (spikelets) originating in the basal dendrites were observed to precede spike initiation in the soma/axon (Milojkovic et al. 2005). Although we did not observe these spikelets in our in vitro or in vivo recordings, spikelets (termed fast prepotentials) have been observed in a minority of other intracellular recordings in vivo (Crochet et al. 2004). However, there are multiple possible sources for these spikelets other than action potential generation in the basal dendrites, including action potential generation in other neurons that are coupled to the recorded cell either through gap junctions, or artificially through a “double impalement” with the intracellular electrode, or distal axonal spike initiation. Thus, spikelets are relatively rare events and their occurrence is not restricted to spike initiation in the basal dendrites.

Do all fast action potentials initiate in the axon initial segment? We do not deny the existence of action potentials that are initiated in isolated dendritic locations, nor the possible occurrence of action potentials that propagate forward to the soma and axon initial segment and result in the initiation of spike discharge (for example, the generation of intrinsic bursts is likely to result in part from the prolonged depolarization of the soma by active conductances in the dendrite). Rather, our data emphasize the strong and dominant influence of the axon initial segment in the initiation of fast action potentials in cortical layer 5 pyramidal cells during periods of relatively moderate frequency, synaptically driven activity as well as during epileptiform bursts. Prior in vitro studies have repeatedly demonstrated the ability of particular patterns or locations of dendritic inputs to initiate action potentials in local dendritic branches that may or may not initiate spikes in the axonal/somatic compartments (Gasparini et al. 2004; Golding and Spruston 1998; Kampa and Stuart 2006; Larkum et al. 1999a; Larkum and Zhu 2002; Larkum et al. 2001, 1999b; Losonczy and Magee 2006; Milojkovic et al. 2005; Polsky et al. 2004; Regehr et al. 1993; Schiller et al. 1997; Wei et al. 2001). Our present studies do not rule out the occurrence of dendritic action potentials, especially in the distal apical tuft or distal basal dendrites, that failed to propagate to the portions of the neuron from which we recorded (main shaft of the apical dendrite, soma, axon).

Even if all or nearly all fast spikes are initiated in the axon initial segment, the generation of regenerative potentials in the dendrites (e.g. spikes) can significantly modulate the overall pattern of discharge. For example, intrinsic burst generation in cortical neurons can result form the interaction of fast spike initiation in the axon modulated by the occurrence of slower Na$^+$ and/or Ca$^{2+}$ mediated spikes in the dendrites that are initiated either by synaptic input or through interactions with the back propagating spikes from the axon/soma (Helmchen et al. 1999; Kim and Connors 1993; Larkum and Zhu 2002; Larkum et al. 2001; Stuart et al. 1997b; Wang 1999) and the activation of dendritic spikes can significantly alter the timing of spike initiation in the axon/somatic region (Ariav et al. 2003).
The precise pattern of spike generation within neurons undergoing synaptic barrages in various behavioral circumstances in vivo will be a complex, with interactions between all of the different compartments of each cortical neuron. Our present results indicate that under a variety of naturalistic situations, the axon initial segment is the preferred site of fast spike initiation in cortical pyramidal cells. Further investigations are required before the full complexity of spike initiation and propagation in cortical neurons will be understood.

Acknowledgements

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References


Figure Legends

Figure 1. Action potentials are initiated in the axon initial segment and propagate to the soma and apical dendrite during spontaneous network activity. A. Combined fluorescence and DIC image illustrating the simultaneous whole cell patch clamp recordings from the soma and apical dendrite of a layer 5 pyramidal cell in the submerged slice in vitro. B. Example of a spontaneous Up state in the apical dendrite and soma. C. Expansion of action potentials recorded in the apical dendrite and soma during Up states. Note that all spikes occur in the soma prior to the apical dendrite (apical dendritic recording site was 290 µm from the soma). D. Photograph from a biocytin filled neuron that was recorded simultaneously from the cell body and axon bleb. E. Example simultaneous recordings from the axon and soma during the spontaneous generation of an Up state (axonal recording 118 µm from the soma). F. Overlay of action potentials recorded from the axon and soma reveal that they occur at this axonal site only slightly before they occur in the soma, indicating that the site of action potential generation is approximately half way between the two recording sites. G. Plot of timing interval between axonal and somatic spikes versus axonal recording distance for spikes generated during natural (blue dot) and artificial (light blue triangle) Up states, in response to somatic current injection (star), and epileptiform activity (open red triangle). In all of these cases, zero time difference occurs at approximately 100 µm, indicating initiation of the spike at approximately 50 µm. However, models of action potential propagation (see Figure 6) indicate that action potentials propagate orthodromically away from the axon initial segment more quickly than antidromically towards the cell body and that the actual point of spike initiation is about 10 µm closer to the soma.

Figure 2. Action potentials are initiated in the axon initial segment during the injection of network PSP-like conductances. A. Simultaneous apical dendritic (220 µm) and somatic whole cell recording during the injection of artificial Up states with a dynamic clamp system into the apical dendrite. B. Comparing the spikes occurring in the soma and dendrite during artificial Up states revealed that they also occurred first in the soma and then back propagated to the dendrite. C. Simultaneous whole cell recordings from the soma and axon (60 µm distant) during the injection of artificial Up states into the soma. D. Action potentials initiated first in the axon and then propagated into the soma.

Figure 3. Calculation of the initiation point of the action potential reveals an origin of approximately 46 µm from the soma. A. Schematic diagram illustrating the use of simple algebra to calculate the initiation point of action potentials based upon the relative timing of spikes initiated with depolarizing current pulses delivered to the soma or to the axon. Definitions: a: distance from the center of the soma to the initiation site. d: distance from the center of the soma to the origin of the axon. x: distance from the origin of the axon to the initiation site. L: distance between the two recording sites. t1: latency difference between the axon and somatic recorded action potentials when evoked with a somatic current pulse. t2: latency difference between axon and somatic recorded action potentials when evoked with a current pulse at the end of the axon. B. Three examples of simultaneous whole cell recordings illustrating the three possible timing relationships between cell body and axon. Multiple traces from each recording are overlain. In the
first example (1), the axonal recording is 139 $\mu$m from the cell body and the spike arrives in the soma first. In the second example (2), the axonal recording is 96 $\mu$m from the cell body and the action potential arrives in the soma and axonal recording site at about the same time. In the third example (3) the axonal recording is 67 $\mu$m from the cell body and the action potential arrives at the axonal recording site first. C. Plot of the results of the calculation of the initiation site versus the distance from the cell body of the axonal recording. The calculated initiation site is relatively consistent with an average of 46 $\mu$m.

Figure 4. Responses to the somatic and axonal injection of current pulses. Responses of the axon initial segment (50 $\mu$m from the soma) to 1.0 nA (A) and 3.5 nA (B) of current injection into the soma. Note that with large somatic current injections, the action potentials in both the soma and axon initial segment become smaller in amplitude. C and D. Response of a cell body and distal axon (440 $\mu$m) to moderate (2 nA) and intense somatic current injection (5 nA). Here, the axonal spikes do not decrease markedly in amplitude. E. Intra-axonal (151 $\mu$m from the soma) injection of a threshold (0.1) and larger (0.4 nA) current results in the initiation of only a single action potential at the beginning of the pulse. F. Expanded action potentials and dV/dt (action potentials 1, 2 and 9) as recorded from the soma and distal axon during the intense current pulse of part D. Note the fractionation of the spikes into components, which presumably are initiated in the initial segment (IS), somatodendritic (SD), and distal axon (DA) regions. Asterisk indicates an IS spike that failed to propagate down the axon.

Figure 5. Axons of layer 5 pyramidal cells of the ferret prefrontal cortex are unmyelinated for a prolonged distance. A. Silver staining of myelin (dark areas) in the 7 week old ferret prefrontal cortex shows little or no myelin present within the gray matter (light areas). B. Golgi staining, which does not stain myelinated axons, confirms that axons of layer 5 pyramidal cells are unmyelinated for prolonged (> 100 $\mu$m) distances in this 7 week old animal (see also Supplementary Figure 2). C-D. Intracellularly labeled prefrontal layer 5 pyramidal cells (green) double labeled for the presence of myelin (red) using immunohistochemistry. Myelination starts as the axon enters the corpus callosum (C), although in the large majority of in vitro cases (n=12/15) the axon is cut before myelination starts (D). Arrows in the inset in C indicate the beginning of the myelin sheath in the single labeled axon. Arrows in D point to the main axon shaft and the arrow in the upper right inset points to an axon collateral.

Figure 6. Computational model of spike initiation in a layer 5 cortical pyramidal cell. A. Computational model of a layer 5 regular spiking pyramidal cell that has the amount of axon that is typical of these neurons in vitro(Shu et al. 2006). The timing difference in the peak of the action potential (soma-axon) is plotted against the distance along the axon from its point of origin (edge of the cell body). The initiation point is in the region near 38 $\mu$m from the soma. B. Action potential initiation in the model of the layer 5 pyramidal cell with its full axon intact. Again, the initiation point is at approximately 38 $\mu$m from the cell body. C. Calculation of the local velocity of action potential propagation either when the action potential is initiated with an intrasomatic current pulse (filled circles) or with a current pulse delivered at 300 $\mu$m down the axon (open circles). Depolarization of the end of the axon results in rapid propagation of spikes in this region,
and we calculate that differences in the orthodromic and antidromic propagation speeds such as these result in an 8 µm mismatch between the estimated and actual location of action potential initiation.

Figure 7. Relationship between spike threshold and distance along the axon. The membrane potential at which the first spike was initiated in response to the injection of a depolarizing current pulse was measured at the soma (first point with SEM bars) and at varying distances along the axon. Each point represents a single neuron. The threshold for activation of an action potential is lower in the proximal 100 µm of the axon than in the more distal 200 µm of axon. There is a rough relationship between distance and threshold (dashed line), which is consistent with initiation of action potentials within the first 100 µm of the axon. Action potential threshold was defined as the membrane potential at which dV/dt passed 25 V/sec.

Figure 8. Spikes occurring in vivo have properties consistent with initiation in the axon. A. Overlay of action potentials recorded in vitro in the soma of layer 5 pyramidal cells following their initiation through the delivery of a large current pulse in the apical dendrite (280 µm from the soma). Note the smooth rise at the foot of the action potential. B. Action potentials that initiate in the axon (as revealed by simultaneous axonal and somatic whole cell recordings) exhibit a prominent “kink” at the initiation point. C. Action potentials recorded in the cat primary visual cortex in vivo during visual stimulation also exhibit a prominent kink. D. Phase plot of the dV/dt versus membrane potential for an action potential that initiates in the axon shows a rapid slope at the initiation point of the spike (raw spike is shown in inset). E. Histograms of the distribution of the slopes of the phase plots (at dV/dt= 15 mV/ms) for spikes initiated in the axon (blue), initiated in the apical dendrite (green) and as recorded in vivo in the cat primary visual cortex during visual stimulation (red) or in vivo in the ferret prefrontal cortex during the generation of Up states (purple). Note the similarity between the distributions recorded in vivo and the spikes initiated in the axon in vitro. Slopes were measured as the average slopes of three adjacent points in the phase plot of dV/dt versus Vm at the criteria level of 15 mV/ms for each action potential.

Figure 9. Spikes are initiated in the axon and propagate through the cell and apical dendrite during epileptiform activity. A. Simultaneous whole cell and dendritic (86 µm) recording during the generation of epileptiform activity induced by the block of GABA_A ionophores with the bath application of picrotoxin (50 µM). B. Simultaneous whole cell recording from the soma and axon (47 µm from the soma) during the generation of epileptiform activity. C. Simultaneous whole cell recording from the cell body and more distal axon (133 µm) during the generation of epileptiform activity. Note that the more distal axon generates several spikes during the initial PDS that do not occur in the soma. D. Simultaneous whole cell somatic and extracellular axonal recording, demonstrating that during epileptiform bursts, axonal spikes may become smaller than background noise levels in extracellular recordings. Same cell as recorded in part C.

Figure 10. Action potentials maintain their height in the distal axon better than the proximal axon and soma during epileptiform activity. A. Simultaneous recording of an
epileptiform burst in the soma and axon (50 µm from the soma). Note that during the epileptiform burst spikes decrease dramatically in amplitude in the soma, but also decrease to some degree in the axon initial segment. B. Simultaneous recording of an epileptiform burst in the soma and distal axon (440 µm from the soma). Note that although the amplitude of spikes in the soma is dramatically affected by the epileptiform burst, the amplitude of spikes in the distal axon is not strongly affected.
Figure 2

A  Simultaneous Apical Dendrite - Somatic Recording
  dendrite 220 µm current
  Vm
  soma

C  Simultaneous Axon - Somatic Recording
  axon 60 µm
  Vm
  soma

B  soma dendrite

D  axon soma

1 nA
50 mV
500 ms
1 ms
Figure 6

A

Slice neuron: 6 collaterals, 23 subcollaterals
total axon length = 19.3 mm

Initiation point: 38 μm

Latency difference (μs)

Distance along axon (μm)

B

Full neuron: 8 collaterals, 154 subcollaterals
total axon length = 66.7 mm

Initiation point: 38 μm

Latency difference (μs)

Distance along axon (μm)

C

Initiated at axon bleb
antidromic propagation

Initiated at IS, antidromic to soma
orthodromic down axon

Velocity (m/s)

Distance along the axon (μm)
Figure 7

Spike recorded at Axon Bleb
DC injection to Axon Bleb

Spike threshold (mV)

Axonal recording distance from soma (µm)
Figure 8

A. In vitro, somatic spike, dendrite initiated

B. In vitro, somatic spike, axon initiated

C. In vivo

D. Phase Plot

E. Probability of phase slope at spike threshold (ms⁻¹)

- In vitro-soma dendrite initiated
- In vivo ferret spontaneous spikes
- In vivo cat visual stimuli induced spikes
- In vitro-soma axon initiated
Figure 10

A
Axon Initial Segment (50 μm)

Soma

B
Distal Axon (440 μm)

Soma
Supplementary Figures

Properties of action potential initiation in neocortical pyramidal cells: evidence from whole cell axon recordings

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Figure 1. Distribution of interspike intervals during natural and artificial Up states. A. Interspike interval distribution for natural Up states for simultaneous somatic and axonal and somatic and apical dendritic recordings (n=637 spikes in 7 neurons). B. Comparison of the interspike interval distributions for spontaneous Up states as recorded from the soma and axon, versus artificial Up states in response to the injection of a noisy conductance (n=1072 spikes in 5 neurons).
Figure 2. Even in adult, layer 5 pyramidal cells of the ferret prefrontal cortex have a long unmyelinated segment of the main axon. A. Comparison of the length of the main axon of layer 5 pyramidal cells in Golgi stained sections of ferret prefrontal and primary visual cortices in young (7 week) and adult (15 month) old animals. Since Golgi does not stain myelinated axons, we assume that the point at which the axon is no longer stained corresponds with the beginning of myelination. B. Pictures of Golgi labeled pyramidal cells in 7 week old prefrontal cortex and primary visual cortex. C. Same as B, except in a 15 month old animal. Black arrow heads indicate the course of the main axon. White arrow heads indicate axon collaterals.