INVESTIGATIONS ON SYNAPTIC INPUT AND ACTION POTENTIAL OUTPUT USING VOLTAGE IMAGING

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by
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STATEMENT

All the work described in this thesis is original and were performed between February 2004 and January 2008 under the supervision of Dr. Greg Stuart. A number of abstracts from presentations made at scientific meetings and two papers have been published as a result of the work described in this thesis:

ABSTRACTS


PAPERS


Lucy M. Palmer
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Greg Stuart, for his unwavering guidance, support and trust. My continual questioning and presence in his office did not perturb his encouragement and contagious enthusiasm. I could not have asked for a better supervisor.

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Summary

Voltage sensitive dye imaging was used to determine the electrical activity in sub-micrometer neuronal structures that have been previously inaccessible to electrophysiological investigation. The main questions investigated were the voltage response to backpropagating action potentials and synaptic input in dendritic spines located on the basal dendrites of layer 5 pyramidal neurons, where action potentials are initiated in both neocortical layer 5 pyramidal neurons and cerebellum Purkinje cells, and how action potentials propagate along layer 5 pyramidal neuron axons.

Imaging electrical activity in dendritic spines during synaptic input and backpropagating action potentials.

Most excitatory input onto neurons in the brain occurs onto dendritic spines. Although much is known about calcium dynamics in spines during backpropagating action potentials and synaptic excitation, the membrane potential changes underlying these responses are unclear. Here we investigate signalling in dendritic spines using voltage imaging in cortical pyramidal neurons. These data indicate that backpropagating action potentials fully invade dendritic spines and that during synaptic activity the peak voltage response in the spine head is on average 8 mV, and is not significantly boosted by voltage-activated channels. Comparison of the voltage in the spine head with that in the dendritic shaft indicates spine neck resistance ranges up to 500 MΩ, with an overall average value of ~200 MΩ. Simulations show that this range of spine neck resistances leads to differences in the amplitude of synaptic responses at
the soma of less than 15% (average 6%). These results indicate that while spines can compartmentalize electrical signals during synaptic stimulation, the spine neck resistance for the majority of spines is too low to act as a physical device to significantly modulate synaptic strength.

**The site of action potential initiation in layer 5 pyramidal neurons.**

Fundamental to an understanding of how neurons integrate synaptic input is the knowledge of where within a neuron this information is converted into an output signal – the action potential. While it has been known for some time that action potential initiation occurs within the axon of neurons, the precise location has remained elusive. Here we provide direct evidence using voltage-sensitive dyes that the site of action potential initiation in cortical layer 5 pyramidal neurons is ~35 μm from the axon hillock. This was the case during action potential generation under a variety of conditions, following axonal inhibition, and at different stages of development. Experiments using local application of low sodium solution and TTX, as well as an investigation of the influence of axonal length on action potential properties, provided evidence that the initial 40 μm of the axon is essential for action potential generation. To morphologically identify the relationship between the site of action potential initiation and axonal myelination, we labelled oligodendrocytes supplying processes to the proximal region of the axon. These experiments indicated that the axon initial segment was ~40 μm in length, and the first node of Ranvier was ~90 μm from the axon hillock. Experiments targeting the first node of Ranvier suggested it was not involved in action potential initiation. In
conclusion, these results indicate that in layer 5 pyramidal neurons action potentials are generated in the distal region of the axon initial segment.

**Action potential propagation in layer 5 pyramidal neurons.**

Successful propagation of action potentials along the main axon and into axon collaterals is critical for shaping the spread of excitation within cortical networks. The development of saltatory conduction was directly investigated in the axons of layer 5 pyramidal neurons using voltage imaging, illustrating that it is initially absent and develops during the first two to three weeks of age. To invoke local network signalling, the action potential must also successfully invade and propagate along axon collaterals. Although branch points have been shown theoretically and experimentally to act as sites of synaptic failure, bursts of action potentials up to 300Hz successfully invaded branch points in layer 5 pyramidal neurons, allowing efficient transfer of information within local cortical networks.

**The initiation and propagation of simple and complex spikes in cerebellar Purkinje cells**

Cerebellar Purkinje cell receives two distinct excitatory inputs which produce two different action potential outputs; the simple and complex spike. Since the interactions of these spikes are critical for motor learning, understanding the site of initiation and the characteristics of the forward- and back-propagation of these events is important for an understanding cerebellar function. Using voltage sensitive dye imaging, we illustrate that both spikes are initiated in a localised region of the axon approximately 15 μm from the axon...
hillock. Once initiated, the simple spike propagates faithfully along the axon, whereas the individual spikelets of the complex spike fail to propagate to distances greater than 80 μm from the hillock. The spikelets and initial fast component of the complex spike also fail to backpropagate to distal regions of the dendritic tree, whereas the plateau potential associated with the complex spike effectively depolarises the entire dendritic arbour.
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CHAPTER 1: GENERAL INTRODUCTION

In a single neuron, information carried by synaptic inputs sum in both time and space leading to the generation of an all-or-none action potential (AP) if threshold is reached. Neurons typically receive thousands of synaptic inputs distributed across complex dendritic trees (extending up to one millimetre from the cell body) which are integrated in complex ways. In this thesis I address two key issues relevant to information processing in single neurons: Characterisation of voltage in single spines and initiation and propagation of APs in axons. Since spines and axons are fine neuronal structures largely inaccessible to electrophysiological recording, these electrical events were investigated using voltage imaging. This introduction gives an overview of spine function and AP generation, and discusses characteristics of voltage-sensitive dye imaging.

**Historical perspective and neuron morphology**

Since the complex morphology of neurons were characterized by Santiago Ramon y Cajal, how neurons receive and process input has been a source of intense investigation and much debate. Initially, the nervous system was generally believed to function as an interconnected web of nerve fibres (the reticular theory), however the discovery principally by Cajal (1911) that neurons are discrete cells provided strong support for the alternative theory, the neuron doctrine, which states that a single neuron functions as an individual unit both anatomically and functionally (although there are exceptions: eg, gap junctions, for a review see, Bullock et al., 2005). Following the neuron doctrine,
information flow in a neuron was described by Cajal's 'law of dynamic polarization' which states that neurons are polarized, receiving signals onto their tree-like branches which then travel towards the soma and along the axon to terminate on neighbouring neurons. Both the neuron doctrine and the law of dynamic polarization provide a strong foundation for our current understanding of neuron physiology and were mostly developed from observations of neuron morphology. An individual neuron can be separated into two main morphological compartments, the dendrite and the axon, each of which receives and processes information differently. Dendrites can have very different morphologies ranging from the highly branched Purkinje cell to the simple single-branched olfactory sensory cell (for a review, see Fiala and Harris, 1999). Dendrites are the site of neuronal input, which in the case of excitatory inputs often occurs onto thorny projections called spines (Beaulieu and Colonnier, 1985). This input is channelled towards the soma along the dendritic tree and eventually sums in the axon to generate an AP. Fast transmission of the AP along the axon in most CNS neurons is achieved by ensheathment of the axon by myelinating cells called oligodendrocytes (Fig. 1.1) which forms electrical insulation for the axon.

Neuron input: The role of dendritic spines.

Dendritic spines were first described by Santiago Ramon y Cajal in 1888 when he observed that the Purkinje cell surface was "bristling with thorns (puntas) or short spines (epinas)" (see Cajal, 1911). The concept that spines are a prominent feature on the dendrites of many neurons was substantiated by EM studies in the cerebral cortex (Gray, 1959) and later in the cerebellar cortex
Figure 1.1. Neurons within the CNS are myelinated by oligodendrocytes. EM micrographs of the striatum (a) and cortex (b). Axons (A) are ensheathed by numerous wraps of myelin (M) in both brain regions. EM analysis of the striatum and cortex were performed under the guidance of Yolanda Smith (IBRO, 2005) and Tom Reese (MBL, 2006) respectively. Scale bars represent 200 nm.
Since these pioneering findings, the abundant existence of spines has been reaffirmed, with a single cortical pyramidal neuron having in excess of 14,000 spines mostly located on basal dendrites (Ballesteros-Yanez et al., 2006; Larkman, 1991). Spines are generally believed to develop from thin dendritic processes called filopodia (Daily and Smith, 1996; Ziv and Smith, 1996; for a review see Yuste and Bonhoeffer, 2004). Although there are many different spine morphologies (for a review, see Harris and Kater, 1994), spines typically consist of a bulbous spine head attached to the dendrite via a neck of varying diameter (see Fig. 1.2). A molecularly important and well-studied structure located in the head of all excitatory spines is the postsynaptic density (PSD; Fig. 1.2e). The PSD is highly enriched with different proteins including receptors, scaffolding molecules, kinases and phosphatases (Ziff, 1997), which have been implicated in stabilizing memory (Lisman and Zhabotinsky, 2001; Shen and Meyer, 1999).

Despite their prominence in the CNS and well-characterized molecular machinery, the role of spines in neuronal excitability has been a source of controversy. Initially it was believed spines were only passive extensions of the neuronal membrane, providing a docking station for the large number of synaptic inputs (Swindale, 1981). However, EM reconstructions have illustrated there is ample dendritic surface area for synaptic contact without the existence of spines (Harris and Stevens, 1988). Therefore the purpose of spines has been revised and they have more recently been shown to provide compartmentalization of ions, namely calcium. Compartmentalization of calcium during synaptic input was first shown by Muller and Connor (1991) and has subsequently been shown in numerous synapses (Denk et al., 1995; Denk et
**Figure 1.2.** Dendritic spines in layer 5 pyramidal neurons. 

a, Golgi-impregnated basal dendrite from a cortical pyramidal neuron with a high density of protruding spines. 
b, Fluorescent voltage image of spines on a layer 5 pyramidal neuron basal dendrite illustrating their differing morphologies recorded using confocal microscopy. 
c, Magnification of spine (*) in (b) illustrating how spine head and length were measured. 
d, The spine (red) can be described as an electrical circuit which is isolated from the electrical circuit of the dendrite (black) by the spine neck resistance (green). Rd = dendritic resistance, Cd = dendritic capacitance, Rn = spine neck resistance, Rs = spine head resistance, Cs = spine head capacitance. 
e, EM micrograph of a cortical synapse illustrating the presynaptic terminal filled with vesicles and the post synaptic spine. The EM micrograph was obtained under the guidance of Tom Reese (MBL, 2006).
al., 1996; Sabatini et al., 2002; Yuste and Denk, 1995). Recent studies by Bloodgood and Sabatini (2005) illustrate that the degree of calcium isolation in spines is regulated by activity. This biochemical compartmentalization may be important for synaptic plasticity, as pairing synaptic stimulation with bAPs can enhance calcium influx into spines in a non-linear manner (Yuste and Denk, 1995) which may ultimately serve to modulate synaptic strength as has been shown experimentally (Magee and Johnston, 1997; Markram et al., 1997; for a review see Dan and Poo, 2004) and theoretically (Koch and Poggio, 1983; Koch and Zador, 1993; Miller et al., 1985; Segev and Rall, 1988).

Whether spines also act as electrical compartments is currently unknown. A single spine can be represented as an electrical circuit isolated from the dendrite by the spine neck resistance (Fig. 1.2). Taking into account Ohm's law, the voltage response to a synaptic conductance on the spine head would be larger than the same conductance on the dendrite because the synaptic input on the spine head encounters impedance from the resistance in both the spine neck and dendrite, whereas synapses directly on the dendritic shaft only encounters the impedance of the dendrite. While this will boost the amplitude of the synaptic input in the spine head compared to a similar input directly onto the dendritic shaft, the increased amplitude of this synaptic input in the spine head decreases the driving force for the synaptic current. In this way the impedance of the spine neck decreases the synaptic current reaching the dendritic shaft. Whether dendritic spines act as electrical compartments which modulate the strength of synaptic input is addressed in Chapter 3.
Integration of synaptic inputs.

Excitatory input onto a single spine is integrated and transformed to ultimately result in neuronal output. The contribution of a single synaptic input to the generation of an AP is dependent on multiple factors, including the location of the synapse on the dendritic tree and the extent of dendritic integration. Since dendrites can extend for several hundred microns, according to cable theory electrotonic filtering of distal electrical events as they propagate to the soma and axon will decrease the contribution of distal inputs to AP generation, as observed in layer 5 pyramidal neurons (Nevian et al., 2007; Williams and Stuart, 2002) and CA1 hippocampal neurons (Golding et al., 2005; although see Magee and Cook, 2000). Not only does the location of an input affect individual synaptic efficacy, but it also affects the extent of dendritic integration. As illustrated by Polsky et al. (2004), simultaneous inputs onto a single dendritic branch sum either sub- or super-linearly depending on the strength of synaptic input, whereas inputs onto different branches always sum linearly. The extent of synaptic summation is also dependent on timing, with appropriately timed inputs boosted following initiation of dendritic spike (Losonczy and Magee, 2006; Williams and Stuart, 2002). The amount of synaptic integration in individual dendrites is critical to determining neuronal output, as summation of multiple synaptic inputs are required to generate enough depolarisation in the axon to reach threshold for AP generation.

Neuron output: The action potential

Since action potentials are the fundamental mechanism used to communicate between neurons in the central nervous system where they are
initiated has important consequences for the exchange of information. This issue is addressed in Chapters 4 and 6 of this thesis. Pioneering work in the 50’s suggested the AP is generated in a region between the soma and axon proper called the axon initial segment (Coombs et al., 1957a; Coombs et al., 1957b). This idea has been substantiated by more recent studies in a number of different neuronal types (Khaliq and Raman, 2006; Meeks and Mennerick, 2007; Palmer and Stuart, 2006; Shu et al., 2007). AP initiation has also been shown to occur further down the axon, possibly at the first node of Ranvier (Clark et al., 2005; Colbert and Johnston, 1996), sparking a recent controversial debate in Purkinje neurons (Khaliq and Raman, 2006). Since the groundbreaking work on the squid giant axon conducted by Hodgkin and Huxley (1952), the ionic basis of the AP has been realised. Two main voltage-dependent currents contribute to the AP: the inward current during the rising phase is due to rapidly activating (hundreds of microseconds) and inactivating (less than a millisecond) sodium channels, and repolarization is due to more slowly activating potassium channels (for a review, see Bean, 2007). Since sodium and potassium channels are the two main players in AP generation, AP initiation is likely to occur at a location where these channels are in high density (Mainen et al., 1995; Rapp et al., 1996), although Colbert and Pan (2002) suggest axon channel biophysical properties can also explain AP initiation in the axon.

Once initiated, APs propagate along the axon to postsynaptic targets (see Chapter 5). The AP can also invade the soma and dendritic tree in an active event called the backpropagating AP (bAP). bAPs were first directly recorded in layer 5 pyramidal neurons (Stuart and Sakmann, 1994) and were
shown to be supported by TTX-sensitive sodium channels which are uniformly distributed in the soma and main apical dendrite of layer 5 and CA1 pyramidal neurons (Magee and Johnston, 1995; Stuart and Sakmann, 1994). Robust bAPs have also recently been observed in layer 5 pyramidal neurons from awake rabbits (Bereshpolova et al., 2007). Whether a neuron is capable of supporting bAPs is largely dependent on neuron morphology (Vetter et al., 2001) and dendritic ion channel distribution (Magee, 1999). An example of a neuron which does not support bAPs is the Purkinje cell, which has an extensive dendritic tree (see Chapter 6) and low sodium channel density in the dendrites (Stuart and Häusser, 1994). The induction of spike-timing dependent plasticity has been shown to be dependent on bAPs (Magee and Johnston, 1997; Markram et al., 1997) and therefore successful invasion of the dendritic tree and dendritic spines is thought to be critical to learning and memory. The issue of invasion of bAPs into spines is addressed in Chapter 3.

*Measuring voltage in fine neuronal structures: voltage-sensitive dye imaging*

Neurons have complex branching morphologies, with dendritic processes that can extend up to a millimetre from the cell body, and axons that can be significantly longer. These processes are often of small diameter (sub-micrometer) and are difficult to record from using conventional recording techniques. Imaging using voltage-sensitive dyes allows changes in membrane potential to be resolved in multiple sub-micrometer structures simultaneously. Since the first optical recordings of fast membrane potential changes were performed during single action potentials in crab, lobster and squid axons (Cohen et al., 1968; Tasaki et al., 1968), voltage-sensitive dyes with increased
signal to noise have been progressively developed (Cohen et al., 1974; Grinvald et al., 1982; Gupta et al., 1981; Loew and Simpson, 1981; Ross et al., 1977). To sense a change in trans-membrane potential voltage-sensitive dyes need to contain hydrophobic elements that are embedded in the membrane. While the actual mechanism through which fast (sub-millisecond) voltage-sensitive dyes do this is unclear, work in this area suggests that for some dyes the change in fluorescence occurs in two ways: either via an "electrochromic" mechanism whereby the energy of the fluorescent dye between its ground and excited state is modulated by the trans-membrane electric field through a redistribution of electrons within the dye molecule itself (Loew, 1996; Loew and Simpson, 1981), or via a "monomer-dimer" mechanism whereby changes in the trans-membrane electric field lead to changes in dye aggregation and/or localization in the membrane (Waggoner et al., 1977). The end result is a change in membrane potential that causes a shift in the excitation or emission spectrum of the dye, leading to a change in the intensity of detected fluorescence at a particular wavelength (Loew et al., 2002) which are linearly related to membrane potential (Ross et al., 1977).

Voltage-sensitive dyes have been used to investigate numerous biological questions, with both external application (Grinvald et al., 1982; Krauthamer and Ross, 1984; Ross and Krauthamer, 1984; Salzberg et al., 1977) and internal application (Antic et al., 1999; Antic et al., 2000; Antic and Zecevic, 1995; Milojkovic et al., 2005a; Milojkovic et al., 2005b; Zecevic, 1996) of the dye. One problem with the interpretation of voltage-sensitive dye signals, however, is that they are difficult to calibrate. Voltage-sensitive dyes bind not only to the plasma membrane, but also to lipid membranes which do not
undergo a change in membrane potential during electrical signalling. For intracellular applications, non-bound dye increases the background fluorescence, causing a decrease to the signal-to-noise ratio of the voltage signal. In addition, the diameter of neuronal processes is not uniform, causing the contribution of the background fluorescence signal to the voltage-sensitive signal to be different in different compartments, leading to differences in the magnitude of the percentage change in fluorescence at different locations even if the underlying voltage change is the same. Numerous techniques can be applied to work around these problems (Djurisic et al., 2004; Kampa and Stuart, 2006), some of which have been employed in the studies contributing to this thesis and discussed in the relevant chapters.

The strength of voltage imaging lies in its ability to measure the fast electrical signals that underlie neuronal communication both between and within neurons at high spatial and temporal resolution. In this thesis, voltage imaging was used to investigate bAP invasion and synaptic input onto spines (Chapter 3), and AP initiation and propagation in layer 5 pyramidal neurons (Chapters 4 and 5) and Purkinje cells (Chapter 6).
CHAPTER 2: GENERAL METHODS

This chapter explains the general methods that were used in the experiments described in Chapters three, four, five and six. Each of these chapters also has a separate methods section which explains further details that are unique to the particular experiments reported in the chapter.

The preparation

Wistar rats (3-5 weeks of age; unless otherwise stated) were anaesthetized by inhalation of halothane and decapitated according to procedures approved by the Animal Experimentation Ethics Committee of the Australian National University. The skull was opened while submerged in ice-cold (<4°C) artificial cerebrospinal fluid (ACSF) extracellular solution containing 125 mM NaCl, 25 mM NaHCO₃, 25 mM glucose, 3 mM KCl, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, 1 mM MgCl₂ (pH 7.4; osmolarity 300-310 mosmol l⁻¹). The brain was then removed and the required brain region (right cortical hemisphere or cerebellum) was adhered to a slicing platform and immediately submerged with the remaining ice-cold extracellular solution. Parasagittal somatosensory neocortical or cerebellum brain slices (300 μm) were obtained using a vibrating tissue slicer (Dosaka, Kyoto, Japan). Prepared slices were transferred to a holding chamber containing ACSF initially maintained at 35°C for 30 minutes and held at room temperature (~22 °C) thereafter until required.
Electrophysiology

Brain slices were placed into a recording chamber which was continuously perfused with fresh ACSF at a rate of 2 ml/min. Somatic whole-cell recordings were made from visually identified neurons using infrared differential interference contrast (DIC) optics (Stuart et al., 1993) using an Olympus BX51 or a Zeiss Axioskop microscope mounted with a PCO or Hamamatsu CCD camera, respectively. Whole-cell recordings were made with current-clamp amplifiers (Dagan, Minneapolis, MN) using the technique described below. Filamented borosilicate glass was 'pulled' by a computer-controlled electrode-puller (Sutter Instruments, USA) to create a tapered patch electrode with an approximate 1 μm tip diameter (7 – 10 MΩ). These whole-cell recording pipettes were filled with 135 mM K-gluconate, 7 mM NaCl, 10 mM HEPES, 2 mM MgCl₂, 2 mM Na₂-ATP, 0.3 mM Na₂-GTP and in most cases also included fluorescent dye (pH 7.2 adjusted with KOH; osmolarity 280 mosmol l⁻¹). The patch pipette was electrically connected with a chlorided silver wire to an amplifier headstage (0.1x) mounted on micromanipulators (Sutter Instruments, USA) and positive pressure (60 mmHg) was applied to the electrode before it was lowered into the recording chamber. In voltage-clamp mode, the voltage offset between the electrode and bath ground was corrected and the electrode was manoeuvred through the brain tissue (up to 50 μm depth) under visual guidance until it contacted the neuron of interest. The positive pressure was then released and slight negative pressure (up to 30 mmHg) was applied until an electrical seal was obtained between the electrode tip and neuron membrane of at least 3 GΩ. Sharp suction pulses were then applied until the neuronal membrane was ruptured. Whole-cell recording was established and
the resting membrane potential was maintained at ~ 60 mV (not taking into account the junction potential). The recording mode was switched to current clamp and a holding current was applied if the resting membrane potential differed greatly from -60 mV. Short current steps (10 ms; ±100 pA) were injected into the soma to allow adjustment for the voltage drop across the pipette series resistance (via the bridge balance) and high frequency filtering of the pipette capacitance (via the capacitance compensation).

Somatic current injection was typically used to evoke orthodromic action potentials (APs; 0.8 to 2.3 nA; 2 ms) and steady state pulses (100 - 400 pA; 150 - 200 ms). In experiments where electrical responses were evoked by extracellular synaptic stimulation, the extracellular stimulation pipette filled with ACSF and often including fluorescent dye (100 µM Alexa Fluor 543) to allow visualization with fluorescent microscopy, was placed in close proximity to the soma for synaptically evoked APs, in close apposition to the axon for axonal AP recordings, at distal axon locations (>150µm from hillock) for antidromic APs, or within 10µm of the imagined dendrite for subthreshold synaptic stimulation of dendritic spines.

For experiments that required visualization of the axon in neurons not filled with voltage-sensitive dye, neurons were filled via the somatic whole-cell recording pipette with a red fluorescent dye (10 µM Alexa 568; Molecular Probes, Eugene, OR) and imaged with either a CCD camera (PCO, Germany) or a confocal microscope (FV300, Olympus, Japan; or LSM 510, Zeiss, Thornwood, NY).
Voltage sensitive dye application

Voltage imaging was performed at room temperature (22 – 26 °C), unless otherwise stated, using the voltage-sensitive dye JPW1114 (Molecular Probes, USA) or JPW3028 (synthesized and kindly provided by J. P. Wuskell and L. M. Loew, University of Connecticut, Farmington, CT, USA). Recording pipettes were back filled with intracellular solution (described above) containing 1-3 mg/ml of dye, and 45 to 60 minutes allowed for the passive transfer of dye into the neuron before the patch pipette was removed. The dye-filled neuron was left undisturbed for more than 2 hours before being repatched with a dye-free pipette. Cell health was monitored by assessing changes in the somatic AP amplitude, and experiments were discontinued if the amplitude of somatic APs decreased by more than 10% during the recording procedure. On average, intracellular filling with voltage-sensitive dyes led to a small, but significant, reduction in AP amplitude by on average 3.5±1.5 % and after-hyperpolarization by on average 11.8±1.6 % (Fig. 2.1; p < 0.05, n=26). There was no significant difference between the somatic AP rise time (0.26±0.01 versus 0.26±0.01 ms) and half-amplitude width (1.23±0.06 versus 1.39±0.07 ms; p > 0.05) upon repatch (prior to the onset of fluorescent imaging) and the end of the experiment (n=26; Fig. 2.1). Furthermore, where assessed, results obtained from averages of the first and last half of the recorded trials gave similar results. Together, these data suggest there was minimal impact of photo bleaching and photo toxicity in our experiments.
Figure 2.1
Figure 2.1. The effects of voltage-sensitive dyes on cell health. a, A typical somatic AP recording before dye filling (black), upon repatch (after 1 hr filling period and ~2 hr recovery; red) and at the conclusion of the experiment (blue). b, Graph comparing the somatic AP amplitude (dark green), half-width (middle green) and rise time (light green) prior to dye filling, upon repatch and at the end of the experiment.
a

![Graph showing electrical activity with markers for Pre-filling, Repatch, and End.]

b

![Bar graph showing AP Amplitude and AP Rise Time for Pre-filling, Repatch, and End Experiment phases.]

- AP Amplitude (mV)
- AP Rise Time (ms)
- AP Half-width (ms)
Detecting Voltage Fluorescence

Fluorescence changes during electrical activity were detected using two different methods depending the degree of spatial and temporal resolution required. Experiments measuring the initiation and propagation of APs required high temporal resolution, and changes in fluorescence were detected with a back-illuminated cooled CCD camera (Red Shirt Imaging, Fairfield, CT) operating at 10 kHz (80x12 pixel resolution) using Neuroplex software (Red Shirt Imaging; Fig. 2.2a). Excitation was limited to 60ms and was achieved using either a 150 W Xenon (Optiquip, USA) or 100 W Halogen lamp (Olympus) gated by a shutter (Uniblitz Electronics, USA) and appropriate filters (excitation 520 ± 45 nm, dichroic 570 nm, emission > 610 nm). Measuring synaptic input in dendritic spines required high spatial resolution only possible with a laser scanning microscope (Fig. 2.2b). These experiments used an Olympus confocal microscope (FV300, Olympus, Japan) operating in line-scan mode (800 Hz time resolution). Excitation was achieved with a 543 nm laser (HeNe; Melles Griot) and limited to less than 150 ms per trial. Changes in fluorescence were detected with the photomultiplier tube of the confocal microscope (FV300, Olympus, Tokyo, Japan).

Data acquisition and analysis

Voltage responses were filtered at 10 kHz and digitized at 50 kHz using an ITC-16 interface (InstruTech, NY) and AxoGraph software (AxoGraph Scientific, Sydney) was used for both acquisition and analysis. To improve the signal to noise ratio, AP–evoked fluorescence signals were averaged (approximately 100 - 150 responses). Prior to averaging, individual trials were
Figure 2.2
**Figure 2.2.** Voltage-sensitive dye imaging resolution. 

**a,** Representative image of a layer 5 pyramidal neuron filled with voltage-sensitive dye captured with a cooled CCD camera operating at 10 kHz (resolution 80x12 pixels). 

**b,** Representative image of a layer 5 pyramidal neuron filled with voltage-sensitive dye excited with a 543 laser and captured with a confocal microscope. 

**c,** Example of an AP evoked by extracellular stimulation and recorded at the soma via the whole-cell recording pipette (top; timing of extracellular stimulus indicated by stimulus artefact) together with the associated voltage fluorescence signals captured by the fast CCD camera (10 kHz) at the soma for a single AP (middle) and an average of 60 trails aligned individually to the soma AP (bottom). The scaled voltage recorded at the soma is shown in red.
aligned using the AP recorded via the somatic recording pipette. This procedure removed problems due to trial-to-trial temporal jitter in the timing of AP generation. Comparison of the recorded somatic voltage with the average fluorescence change at the soma indicated that the waveform of the somatic AP could be reliably captured using voltage-sensitive dyes (CCD camera; Fig. 2.2c, also see Fig. 6.1b).

All fluorescence signals are expressed as the percentage change in light intensity divided by the resting light intensity (%ΔF/F). Fluorescence measurements were conducted on unfiltered data however for visual display CCD camera and confocal fluorescence traces are filtered at 2 kHz and 200 Hz, respectively. Fluorescence and voltage amplitudes were determined at the peak of the response. Somatic AP amplitude, rise time (10 - 90 %) and half width (full width at half height) were measured from threshold, defined as the membrane potential at which the voltage rate of rise was 50 V/s. Onset latencies were measured at half amplitude after fitting a linear regression to the rising phase centred around the region of maximum slope. Pooled data are presented as mean ± SEM. Statistical tests used a Student’s t test or ANOVA at a level of significance of 0.05.
CHAPTER 3: IMAGING ELECTRICAL ACTIVITY IN DENDRITIC SPINES DURING SYNAPTIC INPUT AND BACKPROPAGATING ACTION POTENTIALS

INTRODUCTION

Excitatory input onto neurons in the brain often occurs onto specialized projections called dendritic spines. Previous experimental work on signalling in spines is based primarily on fluorescent imaging of intracellular calcium and sodium (Rose and Konnerth, 2001; Rose et al., 1999; Sabatini et al., 2002; Sabatini and Svoboda, 2000; Yuste and Denk, 1995) (although see Nuriya et al., 2006). These studies illustrate that spines are not passive membrane projections as initially thought (Swindale, 1981), but can act as biochemical compartments (Muller and Connor, 1991; Sabatini et al., 2002; Yuste and Denk, 1995) believed to be important in learning and memory (Bliss and Collingridge, 1993). The isolation of biochemical signals in the spine head is due primarily to the spine neck acting as a barrier to ion flow (for review see Yuste et al., 2000). Whether the spine neck also acts as a barrier to electrical flow is largely unknown.

Dendritic spines receive two main electrical signals: The voltage associated with synaptic input and that mediated by active dendritic events such as backpropagating action potentials (bAPs). The interaction of these two events is thought to be important for the induction of some forms of synaptic plasticity (Golding et al., 2002; Magee and Johnston, 1997; Markram et al., 1997). Receiving synaptic input onto spines has been theorized to modulate EPSP amplitude, by either dampening or enhancing the synaptic voltage signal.
(Koch and Poggio, 1983; Koch and Zador, 1993; Miller et al., 1985; Segev and Rall, 1988). Taking into account Ohm’s law, the voltage response to a synaptic conductance on the spine head would be larger than the same conductance on the dendrite because the synaptic input on the spine head encounters impedance from the resistance in both the spine neck and dendrite, whereas synapses directly on the dendritic shaft only encounters the impedance of the dendrite. Direct experimental evidence on electrical signalling in dendritic spines is lacking, however, as their small size means they are currently inaccessible to standard electrophysiological techniques. Here we investigate electrical signalling in dendritic spines using voltage imaging in basal dendrites of cortical layer 5 pyramidal neurons. These data indicate that bAPs fully invade dendritic spines and that during synaptic activity the average voltage in the spine head is ~6 mV in amplitude and not significantly boosted by voltage-activated channels. Comparison of the voltage in the spine head with that in the dendritic shaft indicates spine neck resistance can be up to ~500 MΩ, with an average value of ~200 MΩ. Simulations show this value of spine neck resistance reduces the amplitude of synaptic responses at the soma by ~5%. These results indicate that while spines can compartmentalize electrical signals during synaptic stimulation, the spine neck resistance is in most cases too low to act as a physical device to significantly modulate the strength of synaptic input.
METHODS

Preparation

Somatosensory neocortical brain slices were prepared and maintained as described in Chapter 2. Experiments were performed at room temperature. To increase the probability of release in synaptic stimulation experiments, slices were bathed in ACSF that usually contained high calcium (3 mM CaCl₂) and in some cases low magnesium (100 µM). Since there was no difference in the synaptic responses in the different conditions, all data were pooled.

Confocal Imaging

Neurons were filled with voltage-sensitive dye (see Chapter 2) and spines were visualized on basal dendrites using confocal microscopy (open pin hole; excitation: 543 nm laser; Melles Griot). Large (~1 µm length) ‘stumpy’ spines located 37-140 µm from the soma were selected for imaging due to their large surface area (hence higher signal-to-noise ratio) and because they were stable throughout the experiment. A line-scan was positioned to transverse the spine and parent dendrite and fluorescence signals were recorded at ~800 Hz in response to bAPs, steady-state membrane potential changes (evoked by somatic current injection), and synaptic stimulation (evoked by an extracellular stimulating pipette placed in close proximity to the spine of interest). Although the voltage fluorescent amplitudes of fast electrical responses are likely to be filtered by sampling the voltage at 800 Hz, simulations show this would underestimate the reported peak amplitudes in spines by between 8 to 15 % for EPSPs (depending on the spine neck resistance) and 20 % for bAPs (data not shown). Data were not corrected for this effect.
To investigate the voltage response in spines during synaptic events, we first ensured we were recording from spines that received direct synaptic input. In these experiments, neurons were repatched with pipettes filled with the calcium-sensitive dye Oregon green BAPTA 1 (200 μM; Molecular Probes, Invitrogen) and prior to voltage imaging, calcium transients (excitation: 488 nm laser; Melles Griot) in response to synaptic stimulation were recorded at the resting membrane potential. Spines were considered to receive direct synaptic stimulation if a clearly isolated calcium response was recorded in the spine compared to the dendrite (typically 2-fold difference in ΔF/F). The probability of release was calculated for each spine by determining the proportion of trials leading to an isolated calcium transient in the spine. Voltage imaging was only performed if the synapse had a reliably high probability of release (typically > 0.8).

**Analysis**

Fluorescence images were captured using Fluorview software (Olympus, Japan) and converted to waveforms using Igor Pro (WaveMetrics, USA) for analysis in AxoGraph X (AxoGraph Scientific, Sydney). The amplitude of the optical signal is expressed as the percentage change in light intensity divided by the resting light intensity (ΔF/F). Only responses that were greater than 2.5 times the standard deviation of the noise were included in the analysis. Fluorescence amplitudes were determined at the absolute peak of the (unfiltered) signal and the difference between the spine and dendritic synaptic response was calculated at the time of the peak spine response. Due to the small signal to noise ratio, averaged responses from single spines were
summed together to create a super average where noted. Spine synaptic voltages were corrected for the impact of average probability of release of 0.87 (see Results).

**NEURON Model**

Simulations were performed with NEURON (Hines and Carnevale, 1997) using a morphologically realistic model of a cortical layer 5 pyramidal neuron (Stuart and Spruston, 1998). The passive electrical properties $R_m$, $C_m$ and $R_i$ were set to $17,000 \ \Omega\text{cm}^2$, $1 \ \mu\text{F/cm}^2$ and $105 \ \Omega\text{cm}$, respectively, based on recently published experimental data from basal dendrites of these neurons (Nevian et al., 2007). $R_m$ was halved and $C_m$ doubled in spiny compartments. In addition, we explicitly modelled a spine with a head diameter and neck length of 1 \ \mu m placed on a basal dendrite 80 \ \mu m from the soma. Spine neck resistance was varied by changing spine neck diameter over a range from 0.4 to 0.05 \ \mu m, which is consistent with previous experimental evidence in CA1 pyramidal neurons that indicates a range of spine neck diameters from 0.46 to 0.038 \ \mu m and an average neck length of 0.45 \ \mu m (Harris and Stevens, 1989). Steady-state attenuation from the soma to the base of the simulated spine located 80 \ \mu m from the soma was 5\% (Fig. 3.2a). “AMPA” EPSPs located either on the spine head or parent dendrite (as indicated) were generated by a synaptic conductance change of 500 pS (reversal potential of 0 mV) with an exponential rise and decay with time constants of 0.2 and 2 ms, respectively (Hausser and Roth, 1997), and measured either in the spine head, the adjacent dendritic shaft, or at the soma. To simulate activation of neighbouring spines additional
AMPA EPSPs were located on the same dendrite just distal to the spine. Resting membrane potential in the model was set to -75 mV.


**RESULTS**

*Somatically evoked backpropagating action potentials invade spines without loss of voltage*

We first investigated to what extent bAPs invade dendritic spines. Despite their linear response to membrane potential (Ross et al., 1977), voltage-sensitive dye signals cannot be easily calibrated (discussed in detail in Chapter 1). To allow comparison of voltage signals generated in spines and adjacent dendrites fluorescence signals must be normalized to a signal of known voltage. In the experiments shown in Figure 3.1, bAPs were normalised to the response to steady-state hyperpolarization as voltage changes in the spine and adjacent dendrite are effectively isopotential during steady-state unless spine neck resistance is extremely high (greater than 10 GΩ; data not shown). A number of previous studies indicate that neck resistances of this magnitude are very unlikely (Bloodgood and Sabatini, 2005; Denk et al., 1996; Harris and Stevens, 1989). The fluorescence signal in individual spines and parent dendrite (Fig. 3.1a) in response to somatic steady-state hyperpolarization (Fig. 3.1b) and bAPs (Fig. 3.1c) were interleaved and averaged. The response to bAPs was then normalised to the response to steady-state hyperpolarization (Fig. 3.1d). The normalized response in spines and the adjacent parent dendrite during bAPs did not significantly differ in amplitude (Fig. 3.1e; 2.87±0.20 and 2.84±0.16, respectively; n=26), illustrating that bAPs successfully invade dendritic spines without voltage attenuation. These results are in agreement with a recently published study using second harmonic imaging (Nuriya et al., 2006). There was also no significant difference between the rise-time (spine: 1.69±0.15 compared to dendrite: 1.69±0.18 ms) and half-width (spine:
Figure 3.1. Back-propagating action potentials successfully invade spines. 

Layer 5 pyramidal neuron filled with VSD (left) and magnified view of the region outlined by the box showing the line-scan (dashed line) transversing a spine and its parent dendrite (right) located 96 μm from the soma. 

b, Steady-state hyperpolarization recorded at the soma (black) in response to somatic current injection (-300 pA; 150 ms; middle) and the corresponding changes in fluorescence recorded in the spine (red) and dendrite (grey) shown in (a). Exponential curves were fitted to the fluorescence traces and amplitudes were calculated at steady state (120 - 150 ms; average of 160 trails). 

c, Fluorescence change recorded in the spine (red) and parent dendrite (grey) shown in (a) in response to somatically evoked action potentials (black; average of 160 individually aligned trials; 2 ms somatic current pulse; second from the top). 

d, Comparison of the action potential fluorescence signal in the spine (red) and dendrite (grey) shown in (c) after normalization by the steady-state response at each location. 

e, The normalized action potential fluorescence signal in the spine (red) and parent dendrite (grey) did not significantly differ (n = 26) illustrating that action potentials successfully invade dendritic spines without voltage attenuation. Error bars are mean ± s.e.m.
3.08±0.28 compared to dendrite: 2.90±0.21 ms) of bAP responses in the spine and parent dendrite (p>0.05). These data indicate that the spine and parent dendrite are effectively isopotential during bAPs. In subsequent experiments the fluorescence change during bAPs, rather than somatic hyperpolarizations, was used to normalize fluorescent signals in spines and parent dendrites during synaptic input.

*Synaptic fluorescence signals in spines and parent dendrites were converted to absolute voltage using the recorded bAP amplitude and the known dendritic location*

Based on the amplitude of the somatic steady-state hyperpolarization, fluorescent signals in spines during bAPs were converted to absolute voltage. Simulations in NEURON illustrate that steady-state hyperpolarization attenuates by approximately 5 % as it propagates from the soma to a spine located 80 µm from the soma (Fig. 3.2a). Therefore, in the example illustrated in Fig. 3.2b, 5% steady state attenuation of the hyperpolarization pulse recorded in the soma (-40 mV) means the fluorescence response in the spine is approximately -38 mV. According to the amplitude of the fluorescence change (ΔF/F) in the spine during steady-state hyperpolarization, in this example 1% ΔF/F equates to approximately 13 mV. Therefore the amplitude of the spine bAP (4.4% ΔF/F) is approximately 57 mV. This procedure was used to convert bAP fluorescence to absolute voltage, and the data was fitted with an exponential curve (Fig. 3.2c; n=55). This “calibration curve” was used in subsequent experiments to convert spine and parent dendrite fluorescent signals into absolute voltage during synaptic input.
Figure 3.2
**Figure 3.2.** Determining backpropagating action potential amplitude using steady-state hyperpolarization. *a,* Simulated hyperpolarizing somatic current pulses recorded simultaneously at the soma (black) and in a basal dendrite at a distance of 80 μm from the soma (grey). At this distance the somatic voltage has attenuated by ~5%. *b,* Steady-state response to hyperpolarizing somatic current injection (200 ms) recorded at the soma (top) and in a dendritic spine (middle, 93 μm from the soma) interleaved with action potentials generated by brief somatic current injection and recorded in the same spine (bottom, red). Based on 5% steady-state voltage attenuation for the soma (amplitude 40 mV) to the spine shown we estimate the amplitude of the steady-state fluorescence response in the spine to be -38 mV. From this we estimate the amplitude of the action potential response in the spine to be ~57 mV (bottom). All traces are averages of 160 trials. *c,* Plot of action potential amplitude in basal dendrites at different distances from the soma. The data set is fitted with a single exponential, which is used in subsequent experiments to convert spine and parent dendrite fluorescent signals into absolute voltage during synaptic input.
The voltage response in spines and parent dendrites during synaptic stimulation

Although all active spines had isolated calcium signals in response to synaptic stimulation, and a measurable voltage fluorescence change during bAPs (average: 5.7±0.2% ΔF/F; n=68), only 19% of spines had a clear synaptic voltage response (13 out of 68). Figure 3.3a shows the average fluorescent voltage response to bAPs and synaptic stimulation in spines (top) and the adjacent dendrite (bottom; n=13). Synaptic fluorescence signals in spines and parent dendrites were converted to absolute voltage based on the amplitude of the fluorescent response to the bAP at that dendritic location (see Fig. 3.2c), which enabled the synaptic response in the spine and dendrite to be directly compared (Fig. 3.3b). Although care was taken to use minimal stimulation the average somatic EPSP in these experiments was substantial (5.3±0.6 mV), indicating that multiple synapses were stimulated. This background depolarisation sums with both the spine and dendrite synaptic response. To isolate the voltage response in active spines the dendritic synaptic response was subtracted from the spine synaptic response. Following this procedure the isolated spine synaptic response was on average 11.1±2.7 mV (Fig. 3.3c; n=13). The amplitude of this average spine voltage response is influenced by the release probability. For example, if the probability of release is 0.6 then 40% of trials included in the average would represent failures, reducing the amplitude of the spine response by 40%. The probability of release was calculated for each spine and was on average 0.87 (n=13; Fig. 3.4a,b). This high average probability of release is clearly visualized in Figure 3.4c where a large average spine signal during the first EPSP (same data as in Fig. 3.3) is followed by synaptic depression during the second EPSP (average of 13
Figure 3.3. Voltage response in spines during synaptic stimulation. 

a, Average voltage fluorescence responses during somatic APs (thin traces) and synaptic input (thick traces) for 13 active spines (red, top) and parent dendrites (grey; bottom).

b, Synaptic fluorescence signals were converted to voltage using the calculated amplitude of the bAP (see Fig. 5.3), allowing the synaptic response in the spine (red) and dendrite (grey) shown in (a) to be directly compared. The difference in the average amplitude of the spine and dendrite response (black line) represents the isolated spine synaptic response.

c, The isolated spine response was determined by subtracting the average dendritic response from the average spine response. The asterisk indicates the peak of the spine response. Arrows indicate timing of synaptic stimulation.
EPSP

Dendrite 4

J 0.5 %AF/F

10 ms

Difference

Spine

Dendrite

EPSP

5 mV

10 ms

EPSP

2 mV

1 ms
Figure 3.4
Figure 3.4. Determining active spines via calcium imaging. **a,** Dendritic spine on a basal dendrite of a layer 5 pyramidal neuron filled with voltage sensitive dye and a calcium indicator (left) and the average calcium response to paired pulse synaptic stimulation (right; average of 20 responses; arrows indicate timing of stimulus). **b,** The average voltage recorded by the somatic whole-cell recording pipette (top) and associated calcium transients (bottom) in response to synaptic stimulation in the spine (green) and dendrite (grey) shown in (a). This spine had a probability of release of 1. **c,** Average fluorescence voltage signal in 13 active spines (red) and parent dendrites (grey) in response to paired pulse synaptic stimulation. The larger amplitude of the synaptic response during the first EPSP illustrates that the active spine response has high release probability. **d,** The amplitude of the AP (black) and EPSP (coloured) in the first half of trials (middle) did not drastically differ with the amplitude during the second half of trials (bottom; average of 65 trials; arrows indicate timing of stimulus). **e,** The amplitude of the spine synaptic response normalized to the amplitude of the AP (EPSP/AP) did not significantly differ when all trials (red), the first-half (blue) and the second-half (orange) were averaged for all spines with a synaptic voltage response (n=13). Error bars are mean ± s.e.m.
spines). The probability of release did not significantly change during the experiment as there was no significant difference in the normalized synaptic fluorescence response in the spine during the first half of trials (0.61±0.06% ΔF/F) compared to the second half of trials (0.55±0.06% ΔF/F; Fig. 3.4d,e; p>0.05; n=13). Taking into account the average release probability of 0.87, we estimate the synaptic response in this subset of spines to be on average approximately 13 mV in amplitude.

**Contribution of voltage-activated channels to the spine synaptic signal**

To test for the possible contribution of VACs to synaptic responses in spines, active spines were sequentially stimulated at rest (Fig. 3.5a) and during steady-state hyperpolarization (Fig. 3.5b). Hyperpolarization will increase the driving force for current flow through glutamatergic receptors, boosting the size of the primarily AMPA-mediated voltage response at the hyperpolarized potential. Conversely, hyperpolarization would be expected to decrease the likelihood that EPSPs activate voltage-activated sodium and calcium channels, reducing their potential contribution to the synaptic response. These effects would be expected to lead to opposite changes in the amplitude of the synaptic response during hyperpolarization. Comparison of the spine response at the resting membrane potential (average: 21.1±3.8 mV; n=13) with that at a hyperpolarized membrane potential (average: 36.6±2.8 mV; n=13) illustrates that the spine response is significantly increased by hyperpolarization (Fig. 3.5c). Subtracting the dendritic depolarisation from the spine synaptic response highlights this significant increase in the synaptic response during hyperpolarization (average at rest: 13.2±2.2 mV Vs. hyperpolarization average:
Figure 3.5
Figure 3.5. Voltage-activated channels do not boost synaptic responses in spines. Average (n=13) synaptic response recorded at the soma (top) and in spines (bottom) at rest (a; -72 mV at the soma) and during hyperpolarizing current injection (b; -106 mV). c, Comparison of the average synaptic fluorescence response in spines at rest (EPSP, red) and hyperpolarized membrane potentials (hEPSP, blue). d, Histogram of the average difference between the spine and dendritic synaptic response at the hyperpolarized membrane potential (blue), at rest (red), and the expected response at the resting membrane potential (red striped) assuming the increase in the response at hyperpolarized membrane potentials is entirely due to the increased driving force during hyperpolarization (n = 13). Error bars are mean ± s.e.m.
19.2±2.2, p<0.05, n=13). Based on the assumption that this difference is due to the change in driving force alone, the expected amplitude of the spine synaptic response at the resting membrane potential can be calculated from the response observed at the hyperpolarized membrane potential (assuming 5% steady-state voltage attenuation of the somatic hyperpolarization in these experiments: -26.9±1.6 mV). This analysis revealed that there was no significant difference between the measured voltage difference between the spine and dendrite during synaptic responses at rest and that predicted from the change in driving force (average predicted: 14.1±1.6 mV, Fig. 3.5d; p>0.05; n=13). These data indicate that VACs are unlikely to substantially contribute to the synaptic voltage response in dendritic spines. Consistent with this idea, there was no significant difference between the rise-time (2.2±0.7 vs 2.2±0.5 ms) or half-width (3.0±0.5 vs 3.4±0.3 ms) of spine responses at rest and hyperpolarized membrane potentials. Furthermore, subthreshold steady-state depolarisation (average: 9.7±0.7 mV; n=8) did not measurably increase the amplitude of synaptic spine responses, again consistent with an effect of driving force rather than VACs in modulating the synaptic voltage response in spines.

Hyperpolarization reveals spine synaptic voltage responses in a minority of spines

As indicated above, clearly identifiable synaptic responses were observed in 19% of spines at resting membrane potentials, with a difference in the peak amplitude of synaptic responses in the spine head compared to the parent dendrite of approximately 13 mV. Only taking into account spines where clear synaptic responses at the resting membrane potential were observed
Figure 3.6. Hyperpolarization reveals spine synaptic voltage responses in a minority (30%) of spines. a-c, Example of the somatic voltage response (top) at rest (red) and after steady-state hyperpolarizing current injection (black) during paired-pulse synaptic stimulation, together with the fluorescent spine response at rest (red, middle) and at the hyperpolarized potential (black, bottom). In a minority of spines (5/32) a synaptic voltage response was recorded both at rest and during hyperpolarization, as in a (arrows; 83 μm from the soma). In 30% of the remaining spines (8/27) a synaptic voltage response was observed only after hyperpolarization, as in b (arrow; 122 μm from the soma). These data indicate that at least 30% of spines have synaptic voltage responses that are apparently below detection threshold at the resting membrane potential. Hyperpolarization increases the driving force for these responses, boosting their amplitude above detection threshold. The remaining 70% of spines (19/27) did not have measurable synaptic voltage responses at rest or hyperpolarized potentials, as in c (39 μm from the soma). These spines either have a voltage response that is too small to be measured using our technique, have for some technical reason stopped being stimulated, or lack AMPA receptors (are effectively “silent”).
overestimates the amplitude of the average synaptic response in spines as it is based on data from spines with the largest responses. Analysis of experiments where hyperpolarization increased the amplitude of synaptic spine responses indicated that ~30% (8 out of 27) of spines which did not have a clear spine synaptic response at the resting membrane potential had a response at the hyperpolarized membrane potential (Fig. 3.6). These data indicate that at least 30% of the 81% of spines that did not show a response at the resting membrane potential were not “silent” (Isaac et al., 1995; Liao et al., 1995) (that is, did not lack AMPA receptors). The remaining spines did not have a measurable synaptic response at rest or during hyperpolarization (Fig. 3.6c).

Determining spine neck resistance

As just mentioned, clearly identifiable synaptic responses were observed in 19% of spines at resting membrane potentials, giving an isolated peak spine response of ~13 mV (Fig. 3.7a, top; same data as in Fig. 3.3). When spines with no clear synaptic response at the resting membrane potential were averaged the difference between the spine and dendritic response was negligible (Fig. 3.7a, middle). Analysis of experiments where hyperpolarization increased the amplitude of synaptic spine responses indicated that 30% (8 out of 27) of spines which did not have a clear spine synaptic response at the resting membrane potential had a response at the hyperpolarized membrane potential (Fig. 3.6). These data indicate that approximately a third of the 81% of spines that did not show a response at the resting membrane potential had synaptic responses that were presumably too small to be detected. We therefore generated a new average consisting of all spines where there was a clear
**Figure 3.7.** Spine neck resistance has minimum impact on synaptic strength. 

**a,** Super average of the synaptic voltage response in spines (coloured) and parent dendrites (grey) for spines with a discernable synaptic response at rest (red; top; n = 13), spines with an isolated calcium transient but no discernable synaptic response at rest (blue; middle; n=55), and spines with a discernable synaptic response at rest summed together with 30% of the response from spines where there was no synaptic voltage response (green; bottom; see text). The difference between the spine and dendrite synaptic voltage responses for each super average is noted.

**b,** Simulation of the experimentally observed voltage changes in spines and parent dendrites during synaptic stimulation for a spine located 80 μm from the soma assuming a synaptic conductance of 500 pS. As noted, the spine neck resistances required to reproduce the experimental voltage transients were 514 MΩ, 8 MΩ, and 204 MΩ.

**c,** Simulation of EPSP recorded at the soma in response to synaptic input (500 pS) onto spines located 80 μm from the soma for the range of observed spine neck resistances (red: 514 MΩ; green: 204 MΩ; blue: 8 MΩ). For comparison the response during direct synaptic input onto the parent dendrite is also shown (black).

**d,** Plot of spine neck resistance versus somatic EPSP amplitude for the range of observed spine neck resistances in our study. These data suggest that modulation of spine neck resistance over the physiological range does not significantly influence synaptic strength (< 15% change). Coloured dots as in (c).
[Diagram with labels and measurements]

- Spine
- Dendrite

- 13 mV
- 5 mV
- 6 mV
- 50 μV
- 20 ms
- 200 400 600

- Neck Resistance (MΩ)
- Soma EPSP (%)
response at the resting membrane potential together with 30% of the response from spines where there was no clear response at the resting membrane potential (Fig. 3.7a, bottom). This new average spine response, which is likely to be the most accurate estimate of the average spine synaptic voltage response, had an isolated spine synaptic voltage of 6.2 mV (Fig. 3.7a, bottom).

The electrical compartmentalization described above results from differences in spine neck resistance between different spines. It has long been speculated that modulation of spine neck resistance could act to regulate synaptic strength (Rall, 1970). This arises as modulation of spine neck resistance alters the amplitude of the spine synaptic response, influencing the driving force for synaptic current flow. To calculate spine neck resistance and its impact on the amplitude of the synaptic response at the soma we simulated the range of observed spine and parent dendrite synaptic responses using a morphologically realistic model of a layer 5 pyramidal neuron (see Methods). We chose a synaptic conductance of 500 pS, which gave a local dendritic EPSP amplitude of approximately 1.5 mV at the dendritic location of the spines in our study, consistent with recent observations (Nevian et al., 2007). Background depolarisation due to activation of neighbouring spines was simulated by including the appropriate level of synaptic input on the same dendrite. These simulations revealed spine neck resistances could be as high as ~500 MΩ (Fig. 3.7b, top), with the best estimate of the average synaptic spine response (Fig. 3.7b, bottom) associated with a spine neck resistance of ~200 MΩ. Furthermore, simulations indicate that an isolated spine response of 6.2 mV is associated with a peak voltage response in the spine head during
synaptic input of on average \( \sim 8 \text{ mV} \). We next performed simulations to investigate how this range of spine neck resistances impacts on the somatic EPSP amplitude (Fig. 3.7c). These simulations showed that modulation of spine neck resistance over the range observed in our experiments would lead to changes in the somatic EPSP amplitude of less than 15% (neck resistance: 500 M\( \Omega \)), with a spine neck resistance of \( \sim 200 \text{ M}\Omega \) leading to a reduction in the amplitude of the somatic EPSP by only 6%. 
DISCUSSION

Spines as electrical compartments

While it is clear that spines can act as biochemical compartments (Muller and Connor, 1991; Yuste and Denk, 1995), the extent to which they also compartmentalize electrical signals is unknown. We provide the first direct estimates of the voltage experienced by dendritic spines during synaptic activation, which was on average ~8 mV. It is clear that during synaptic input dendritic spines compartmentalize electrical signals potentially influencing the extent of concurrent NMDA receptor activation and secondary messenger activation. Further, we show that bAPs effectively invade dendritic spines without voltage loss (see also Nuriya et al., 2006), which is presumably critical for their role as a retrograde messenger during spike-timing dependent synaptic plasticity (Koester and Sakmann, 1998; Magee and Johnston, 1997; Markram et al., 1997).

Contribution of voltage-activated channels to the spine synaptic signal

In addition to containing glutamatergic receptors (AMPA and NMDA), spines are thought to contain various types of voltage-activated channels (VACs), including calcium channels (Bloodgood and Sabatini, 2007; Sabatini and Svoboda, 2000) and sodium channels (Araya et al., 2007; Tsay and Yuste, 2002), as well as calcium-activated potassium channels (Bloodgood and Sabatini, 2007; Faber et al., 2005; Ngo-Anh et al., 2005). A number of theoretical studies have proposed that activation of voltage-activated sodium channels in spines could boost the synaptic response (Miller et al., 1985; Perkel and Perkel, 1985; Segev and Rall, 1988), with recent experimental evidence...
supporting this idea (Araya et al., 2007). In contrast, we observe that the synaptic voltage change in dendritic spines is not significantly boosted by VACs. The absence of a significant contribution of VACs to spine synaptic responses is not surprising given that the largest isolated responses in spines where there was a clear synaptic voltage response was on average ~13 mV, which is unlikely to be sufficient to activate VACs from the resting membrane potential. Furthermore, our findings are consistent with theoretical studies (Miller et al., 1985; Perkel and Perkel, 1985; Segev and Rail, 1988) that indicate that regenerative responses in spines require densities of VACs more than 100-fold higher than experimental estimates in dendrites of cortical pyramidal neurons (Stuart and Sakmann, 1994).

Spine neck resistance estimates compared with previous studies

By simulating our range of spine and parent dendrite voltage responses we provide evidence that spine neck resistance ranges up to ~500 MΩ, with an overall average value of ~200 MΩ. These estimates are similar to those based on anatomical reconstruction (Harris and Stevens, 1989), (CA1 pyramidal neurons: 0.9 - 411 MΩ; cerebellar Purkinje cells: 2.6 - 80 MΩ), and also similar to, albeit somewhat higher than, spine neck resistance estimates calculated by dye diffusion (Svoboda et al., 1996) (CA1 pyramidal neurons: 4 - 50 MΩ; although see (Bloodgood and Sabatini, 2005)). We show that this range of spine neck resistances leads to changes in somatic EPSP amplitudes of less than 15% (typically closer to 5%), indicating that modulation of spine neck resistance is unlikely to play an important role in regulating synaptic strength. It should be noted that these experiments were performed on large stumpy
spines, and it is possible that further electrical compartmentalization may be achieved in spines with different spine neck geometry (Crick, 1982; Koch and Poggio, 1983), however reconstructions suggest that even small spines necks are not sufficiently restrictive to pose a significant barrier to synaptic currents (Koch and Zador, 1993).

In conclusion, these data indicate that bAPs fully invade dendritic spines and that during synaptic activity the peak voltage in the spine head is on average 8 mV, and is not significantly boosted by voltage-activated channels. Comparison of the voltage in the spine head with that in the dendritic shaft indicates spine neck resistances up to ~500 MΩ (average ~200 MΩ). These data indicate that while spines can compartmentalize electrical signals during synaptic stimulation, the spine neck resistance is likely to be too low in most cases to act as a physical device to significantly modulate the strength of synaptic input.
CHAPTER 4: THE SITE OF ACTION POTENTIAL INITIATION IN LAYER 5 PYRAMIDAL NEURONS

INTRODUCTION

Action potentials (APs) are the fundamental electrical signal used by the nervous system to relay information. In single neurons the probability of AP generation is largely determined by the summation of synaptic inputs in a process called synaptic integration. Given that neurons usually receive thousands of synaptic inputs distributed across complex dendritic trees (extending up to one millimetre from the cell body), an essential step to understanding synaptic integration is the knowledge of where within a neuron APs are generated. Pioneering work in spinal motoneurons in the 50's suggested the AP is initiated in the axon initial segment or possibly the first nodes of Ranvier (Coombs et al., 1957; Fatt, 1957; Fuortes et al., 1957). Subsequent studies have supported this idea in other neuronal types, with direct evidence for an axonal site of AP initiation coming from simultaneous axonal and somatic recordings in the mid 1990s (Colbert and Johnston, 1996; Stuart and Häusser, 1994; Stuart et al., 1997).

Based on the assumption that the axon initial segment contains a high density of voltage-activated sodium channels, theoretical studies predict AP initiation will occur in the axon initial segment (Dodge and Cooley, 1973; Mainen et al., 1995). Experimental evidence for a high density of sodium channels in the axon initial segment is contradictory, however. Antibody staining illustrates clustering of sodium channels in the axon initial segment in many neuronal types (Boiko et al., 2003; Inda et al., 2006; Jenkins and Bennett, 2001;
Kordeli et al., 1995; Meeks and Mennerick, 2007; Van Wart et al., 2007; Wollner and Catterall, 1986; Zhou et al., 1998). Conversely, patch experiments by Colbert and Johnston (1996) detect little or no difference in the density of sodium channels in the axon initial segment compared to that found at the soma and suggest that differences in sodium channel properties may underlie AP initiation in the absence of a high sodium channel density (Colbert and Pan, 2002). Consistent with a low density of sodium channels in the axon initial segment, there is evidence that AP initiation can occur at the first node of Ranvier in some neuronal types (Clark et al., 2005; Colbert and Johnston, 1996; Gogan et al., 1983). In contrast, recent experimental evidence has revealed the axon initial segment as the primary site of AP initiation in a variety of neuronal types (Khaliq and Raman, 2006; Meeks and Mennerick, 2007; Palmer and Stuart, 2006; Shu et al., 2007). Consistent with these data, Kole et al. (2008) have recently shown that earlier patch estimates underestimated the sodium channel density in the axon initial segment due to anchoring of sodium channels to the actin cytoskeleton. Furthermore, these recent data show that the axon initial segment sodium channel density is indeed significantly higher than that found in the soma and dendrites.

To localise the site of AP initiation based on small differences in AP latency one would ideally need to record simultaneously from multiple axonal sites. Here, the site of AP initiation in cortical layer 5 pyramidal neurons was addressed using the intracellular application of voltage-sensitive dye: a method which allows the simultaneous monitoring of membrane potential at multiple sites. The results provide direct experimental evidence that in layer 5 pyramidal neurons AP initiation occurs in the distal region of the axon initial segment.
METHODS

The site of AP initiation was investigated using multiple techniques, with the general methods outlined in Chapter 2. All other techniques specific to this chapter are outlined below. For voltage imaging, fluorescence changes during APs were detected with a back-illuminated cooled CCD camera. To maximize the signal to noise ratio we used regions of interest (ROIs) of 3 pixels length (~10 μm) and 2 pixel width (~6 μm), and typically averaged 100 - 150 responses.

To test the influence of reducing sodium current on AP generation at different axonal sites, a low sodium extracellular solution (125 mM C5H14NO.Cl, 25 mM glucose, 25 mM NaHCO3, 3 mM KCl, 1.25 mM NaH2PO4, 2 mM CaCl2, 1 mM MgCl2 and 5 μM Alexa 568; pH 7.4; osmolarity 300-310 mosmol l⁻¹) or extracellular solution containing tetrodotoxin (TTX; 10 μM) was pressure ejected (5-15 PSI) from a standard patch pipette to visually identified regions of the axon using a Picospritzer II (General Valve, USA). In experiments where axons were severed at varying lengths (21 – 968 μm), neurons were selected at random and the length of axon determined at the end of the experiments after loading with a fluorescent dye (10 μM Alexa 568) via the somatic recording pipette using a confocal microscope (FV300, Olympus, Japan; or LSM 510, Zeiss, Thornwood, NY). Heterogeneity of axon lengths was a consequence of the brain slicing procedure. AP characteristics (threshold, amplitude, rise time) were determined from the first AP generated more than 50 ms after the onset of a 1 second long somatic current injection (150 – 600 pA).

Oligodendrocytes were identified by their characteristic spherical cell body using DIC imaging and filled with the red fluorescent dye Alexa Fluor 568
(10 μM). Myelinating processes were visualized using a confocal microscope (LSM 510, Zeiss, Thornwood, NY) and layer 5 pyramidal neurons with cell bodies immediately above (closer to the pia) the myelinating processes of the filled oligodendrocyte were selected and patched with patch pipettes containing the green fluorescent dye Oregon Green 488 (150 μM; Molecular Probes, Eugene, OR). In experiments testing the effect of GABA application to the axon initial segment, an extracellular solution containing GABA (100 μM) was pressure ejected (5 ms; 12 PSI) from a standard patch pipette using a Picospritzer II (General Valve, USA). APs were evoked by brief somatic current injection (1 - 5 nA; 2 ms) in the absence (control) or just after (20 – 50 ms) GABA application, alternating between control and GABA in blocks of approximately 30 trails.

In all experiments, the onset latency of the AP was measured at half amplitude after fitting a linear regression to the rising phase centred around the region of maximum slope. Similar results were obtained when using onset latency based on the time to reach 10% of peak amplitude. Voltage imaging and TTX experiments were performed at room temperature (~22 °C) unless otherwise stated, whereas the low sodium, severed axon and extracellular experiments were performed at near physiological temperatures (~34 °C).
RESULTS

Determination of the site of AP initiation with voltage-sensitive dyes

Firstly, the temporal resolution of the camera-based system used to detect fluorescent signals with voltage-sensitive dyes was determined. To test this an AP-like light response was generated by driving a light-emitting diode (LED) with an AP voltage command within its linear range. The onset of the AP command was then shifted in 20 μs steps and the LED output recorded using a photodiode sampling at 100 kHz, or our camera-based system sampling at 10 kHz (Fig. 4.1a). Despite a 10-fold lower temporal resolution, the camera-based detection system could reliably resolve changes in AP-light response onset with a resolution of less than 20 μs (Fig. 4.1b). The ability to detect time differences less than the sample interval is because the AP rise time is slow enough that there are multiple sample points on the rising phase. Any detection system that can describe the rising phase of an event can resolve time differences significantly less than the sample interval. These results are also important for interpreting the data presented in Chapters 5 and 6.

Next, membrane potential was recorded from cortical layer 5 pyramidal neurons filled with voltage-sensitive dye. Simultaneously sampling the fluorescent change at high temporal resolution (10 kHz) during somatically-evoked APs in the axon, soma and proximal apical dendrite (Fig. 4.2a) showed that consistent with previous findings (Stuart et al., 1997; Stuart and Sakmann, 1994) APs are initiated in the axon of layer 5 pyramidal neurons and backpropagate into the soma and apical dendrite (Fig. 4.2b,c).

To identify the site of AP initiation in the axon, we simultaneously recorded the fluorescence signal in 10 μm regions along the axon during AP
Figure 4.1
Figure 4.1. Precision of onset latency measurement and CCD resolution. a, Examples of action potential light responses with different onset latencies, generated by driving a light-emitting diode with an AP voltage command within its linear range and detected with either a photodiode (PD) sampling at 100 kHz (top) or our camera-based system sampling at 10 kHz (bottom). b, Plot of the onset latency of “AP” light responses detected with the PD versus the camera system.
Figure 4.2. Action potentials are initiated in the axon in layer 5 pyramidal neurons. a. Image of a layer 5 pyramidal neuron filled with voltage sensitive dye (resolution 80x12). Regions of interest (ROI) at the soma (blue), apical dendrite (green) and axon (red) are indicated. b, Example of the average voltage fluorescence responses (100 trials) to a somatically evoked AP (top) recorded in the axon (red), soma (blue) and proximal apical dendrite (green) for the ROIs indicated in (a). The onset of the fluorescence responses in the middle panel are magnified for clarity in the bottom panel. c, Plot of the average onset latencies for the fluorescence traces measured at the different neuronal locations as noted (n=9). Axon hillock = 0µm. Error bars are SEM.
30 mV

Soma (V)

Axon

Soma

Dendrite

Soma

Dendrite

Axon

200

1 ms

200 μs

Axon

Soma

Dendrite

Axon

Soma

Dendrite

C

Onset Latency (μs)

-80 -60 -40 -20 0 20 40

Distance (μm)
Chapter 4

generation (Fig. 4.3a). We then compared the onset latency of axonal fluorescence changes during APs with the fluorescence change recorded at the soma (Fig. 4.3b). By definition the site in the axon with the shortest onset latency is the site of AP initiation. On average, the shortest onset latency was 35.6 ± 2.3 μm from the axon hillock (Fig. 4.3c; n=34). Similar results were obtained in experiments where APs were evoked by synaptic stimulation (35.0 ± 3.8 μm; n=7) and at close to physiological temperatures (~34°C; 35.0 ± 5.8 μm; n=3; data not shown). Together, these data indicate that APs in layer 5 pyramidal neurons are initiated in a localised region of the axon approximately 35 μm from the axon hillock.

These results contrast with that observed when APs were evoked by antidromic stimulation via extracellular stimulation near the border of layer 6 and the white matter (Fig. 4.3c; n=6). During antidromic APs, the site in the axon with the shortest onset latency was always close to the site of the extracellular stimulation pipette, hundreds of micrometers from the soma, consistent with a site of AP initiation in the distal axon (Fig. 4.3c). These findings indicate that our voltage fluorescence recordings are sensitive enough to detect differences in the site of AP initiation during orthodromic and antidromic stimulation.

Mapping the site of AP initiation using extracellular recording

Given that voltage-sensitive dyes have a low signal to noise ratio, the site of AP initiation was also investigated using other techniques. In the first set of experiments, extracellular voltage recordings were obtained from different locations along the axon. To avoid potential complications due to myelination
Figure 4.3
Figure 4.3. Imaging AP initiation with voltage-sensitive dyes. a, Left, High magnification image of the axon of a layer 5 pyramidal neuron filled with VSD. Right, Average fluorescence change (ΔF/F) of 130 individually aligned APs recorded at the indicated axonal locations (black). APs were evoked by somatic current injection (red). b, Onset latency of axonal fluorescence signals relative to the somatic response (0 μm) plotted against distance along the axon for the data in (a). Site of AP initiation is indicated by a red arrow. c, Average onset latency of axonal fluorescence signals relative to the somatic response plotted against axon distance for orthodromic (black; n=34) and antidromic (green; n=6) APs. Data for orthodromic APs was pooled from experiments where APs were evoked by brief somatic current injection (n=27) and synaptic stimulation (n=7).
Voltage

Soma

20-30

40-50

60-70

80-90

100-110

120-130

140-150

160-170

180-190

_10.1 \%

500 \mu s

Axon Distance (\mu m)

Onset Latency (\mu s)

Orthodromic

Antidromic
these experiments were conducted on both mature (3-5 week old; n = 11) and immature (14 day old; n = 9) rats. Based on visual inspection using infrared differential interference contrast optics, myelination of axons of rat somatosensory layer 5 cortical pyramidal neurons occurs around 3 weeks of age. The axons of layer 5 pyramidal neurons were imaged using either a CCD camera or a confocal microscope after filling neurons with a fluorescent dye (Alexa 568) included in the somatic recording pipette. Extracellular voltage responses were recorded sequentially at different distances along the axon in response to APs evoked by somatic current pulses (Fig. 4.4a). The onset of the extracellular voltage response at different axonal locations was compared to the intracellular somatic voltage response, and indicated that AP onset latency was shortest at distances around 40 µm from the axon hillock (Fig. 4.4b,c). Similar findings were made during recordings from myelinated and non-myelinated axons, suggesting that myelination did not influence our ability to detect differences in AP onset (Fig. 4.4c). These results are consistent with the results using voltage imaging, and indicate that AP initiation occurs approximately 30 to 40 µm from the axon hillock.

**Mapping the site of AP initiation using low sodium applications**

To test the role of different axon regions in AP initiation, the impact of locally reducing inward current flow through voltage-activated sodium channels was investigated. This was achieved by brief application of a low sodium extracellular solution to different regions of the axon, which should allow a rapid and local reduction in sodium current with immediate recovery. The rationale was that if AP initiation occurs in the proximal region of the axon, as the voltage
Figure 4.4
**Figure 4.4.** Extracellular recordings of AP initiation.  

a, Left, Confocal image of a mature (p21-35) layer 5 pyramidal neuron filled with Alexa 568. Right, Normalised extracellular voltage responses recorded at the indicated locations (average of 20 to 50 trials). APs were evoked by somatic current injection (red).

b, The onset latency of the extracellular response relative to the somatic AP plotted against distance from the axon hillock (0 μm) for the data in (a). S=soma.

c, Average onset latency of extracellular voltage responses relative to the somatic AP for 11 myelinated (closed circles) and 9 non-myelinated (open circles) axons plotted against distance from the axon hillock. Data in (b) and (c) were fitted with a function \( f(x) = a \cdot \text{abs}(x-b)+c+d \cdot x \).
imaging experiments suggest, reducing sodium current in this region would be expected to significantly influence AP initiation. Focal application of low sodium extracellular solution to the proximal axon led to a significant increase in AP threshold, as well as a decrease in the amplitude of the somatic AP (Fig. 4.5a). These effects were greatest during applications 20 µm from the hillock and decreased back to control levels at axonal sites more than 60 µm from the soma (Fig. 4.5b,c). On average, low sodium applications 20 µm from the hillock increased AP threshold by 4.5 ± 1.0 mV and decreased AP amplitude by 8.8 ± 1.5 mV (n=8; p < 0.05). Low sodium applications also slightly increased AP rise time, with the greatest effect during applications to the axon hillock (53 ± 9 µs; n=8; p < 0.05). Our finding of an increase in AP threshold during low sodium applications to the axon initial segment is consistent with the idea that this region is responsible for AP initiation.

Brief applications of low sodium solution also reduced the amplitude of the voltage response to a just subthreshold somatic current pulse in a distance dependent manner, with the greatest effect during applications 20 µm from the axon hillock (Fig. 4.5d,e). This finding is consistent with previous work indicating that the persistent sodium conductance (Inap) is located in the axon of layer 5 pyramidal neurons (Astman et al., 2006; Stuart and Sakmann, 1995). A reduction in axonal Inap would be expected to lead to an observed increase in AP threshold at the soma, as larger somatic current pulses will be required to compensate for the loss of axonal depolarisation mediated by Inap. While this effect can account to some extent for the observed increase in somatic AP threshold during low sodium applications to the axon initial segment, the reduction in amplitude of subthreshold voltage pulses was significantly smaller
Figure 4.5. Impact of local reductions in sodium current on AP and subthreshold response properties. a, Example of APs evoked by somatic current injection during brief (20 ms) low sodium application to the axon initial segment (20 μm from the axon hillock; blue) compared with control APs recorded pre and post low sodium application (grey). Low sodium extracellular solution applied to the axon initial segment led to significant increases in both AP threshold and the amplitude of the somatic current step required to reach threshold. Average shift in somatic AP threshold (b) and amplitude (c) relative to control during brief application of low sodium solution (blue) or normal extracellular solution (open circles) to the axon. S represents the soma, and the axon hillock is 0 μm. d, An example of the subthreshold voltage response during low sodium application to the axon initial segment compared with control (no solution application; dashed). Note a decrease in subthreshold voltage during low sodium application. e, Difference in somatic voltage response to a just subthreshold current pulse relative to control during application of low sodium solution to the soma, 20 μm and 40 μm from the axon hillock. f, Somatic APs evoked by somatic current injection during brief (5 - 10ms) focal application of TTX (10 μM) to the axon initial segment (red) compared with a control AP recorded before TTX application (grey).
than the observed increase in somatic AP threshold (0.94 ± 0.2 mV versus 4.5 ± 1.0 mV; 20 μm from the axon hillock; Fig. 4.5e).

On average, focal application of the sodium channel antagonist tetrodotoxin (TTX; 10 μM, 5 ms) 20 μm from the hillock increased AP threshold by 14.6 ± 4.5 mV, increased rise time by 137 ± 45 μs, and decreased AP amplitude by 28.2 ± 6.0 mV (n=6; p < 0.05; Fig. 4.5f). While these findings are consistent with the results obtained using voltage imaging, the high affinity of TTX for sodium channels is likely to reduce the spatial resolution of these experiments in comparison to those using low sodium applications, which may explain the larger impact of TTX on somatic AP properties. Together, these findings indicate that locally reducing current flow through voltage-activated sodium channels has the greatest impact on AP properties when applied to proximal axonal sites, illustrating that the site of AP initiation resides in the proximal region of the axon.

Influence of axon length on AP properties

If the region of axon responsible for AP initiation was severed during the slicing procedure, one would expect AP properties such as threshold and amplitude to be altered. The minimum length of axon required to generate APs with “normal” properties should therefore give an indication of the site in the axon where APs are initiated. To investigate this issue, somatic whole-cell recordings were made from layer 5 pyramidal neurons with axons randomly severed at lengths ranging from 21 to 968 μm (n=108) during the brain slicing procedure (Fig. 4.6a). The temporal derivative of membrane voltage (dV/dt) can be used to dissociate the individual somatic AP components which result from
Figure 4.6
Figure 4.6. Impact of axonal length on AP properties.  

a, Confocal images of layer 5 pyramidal neurons (Alexa 568) with axons of the indicated lengths.  
Note the characteristic bulbous end of the severed axons.  

b, Double differentiation of the somatic AP voltage in the neurons illustrated in (a).  
All neurons exhibited two distinct components, presumably resulting from the separate charging of the initial segment and the soma.  
The voltage change required to reach AP threshold (c) and AP amplitude (d) measured from threshold in neurons with different axon lengths (21 to 968 μm; n=108).  
APs were evoked by somatic current injection.
the sequential activation of the axon initial segment and somato-dendritic compartment (Coombs et al., 1957). Two distinct components of the double derivative of the somatic AP waveform were observed in all layer 5 pyramidal neurons irrespective of the axonal length (Fig. 4.6b). This finding suggests that the generation of APs even in neurons with very short axons follows a similar sequence.

Both the voltage change required to reach somatic AP threshold and AP amplitude were similar in neurons with axonal lengths ranging from 25 to nearly 1000 µm (Fig. 4.6c,d). Furthermore, there was no statistically significant correlation between axonal length and AP rise time (92.6 ± 2 µs; \( p=0.08 \)), half width (395 ± 9 µs; \( p=0.58 \)), or the resting membrane potential (-66 ± 0.48 mV; \( p=0.39 \); data not shown). These observations indicate that neurons with very short axons (~30 µm) contain the appropriate machinery to produce normal APs, and that more distal regions of the axon are not required for AP generation.

Anatomical identification of the site of AP initiation

To determine how the site of AP initiation in the proximal axon relates to axon myelination, sequential whole-cell recordings were made from oligodendrocytes and layer 5 pyramidal neurons using patch pipettes filled with different fluorescent dyes (Alexa 568 and Oregon Green 488) and subsequently imaged using confocal microscopy. The aim of these experiments was to label oligodendrocytes supplying the first region of myelin to layer 5 pyramidal neuron axons, thereby defining the length of the axon initial segment as well as the location of the first node of Ranvier, which by definition will be at the end of the
Figure 4.7
Figure 4.7. Morphological identification of the axon initial segment.  

a, Two examples of confocal microscope images illustrating the extensive myelinating processes projecting from a single oligodendrocyte cell body (arrow). Scale bar represents 10 μm.  

b, Two examples of confocal microscope images of oligodendrocytes and associated processes (red; Alex 568) myelinating the proximal axon of a layer 5 pyramidal neuron (yellow; Oregon Green 488). In all images, the oligodendrocyte cell body can be identified as a red sphere (arrow). Scale bar represents 10 μm.  

c, Pooled data (n=6) of the average distance from the axon hillock to the start of the first myelin process (~40 μm), the length of the first myelin process (~50 μm), and the distance from the axon hillock to the end of the first myelin process (~90 μm), indicating the distance to the first node of Ranvier.
first myelinated segment. Oligodendrocytes were identified anatomically as having spherical cell bodies with an average diameter of 7.8 ± 0.6 μm located in layer 5 and 6, and were electrically inexcitable. Each oligodendrocyte had multiple myelin processes of differing lengths and widths that ensheathed the axons of several neurons (Fig. 4.7a). On 6 occasions layer 5 pyramidal neurons receiving myelination from one of these oligodendrocyte processes were imaged (Fig. 4.7b). The average distance of the myelin to the axon hillock was 39.9 ± 5.2 μm and the average length of this myelin process was 50.2 ± 9.6 μm (n=6). These results indicate that the length of the unmyelinated axon initial segment region is approximately 40 μm, the length of the first region of myelination is approximately 50 μm, and therefore the first node of Ranvier occurs approximately 90 μm from the soma (Fig. 4.7c).

It is very likely that these oligodendrocyte processes are indeed the first regions of myelination of layer 5 pyramidal neuron axons as oligodendrocyte processes shorter than 40 μm were never observed. In addition, the oligodendrocyte process that ensheathed the proximal region of the layer 5 pyramidal neuron axon had a larger diameter (1.5 ± 0.2 μm) than other oligodendrocyte processes (1.0 ± 0.1 μm). Combining this anatomical data on the length of the axon initial segment (40 μm) with our observed site of AP initiation ~35 μm from the axon hillock indicates that the site of AP generation in layer 5 pyramidal neurons is in the distal region of the axon initial segment.
Role of the first node of Ranvier

The original studies on the origin of AP initiation suggested that initiation may occur at the first node of Ranvier (Coombs et al., 1957), with more recent studies supporting this idea (Clark et al., 2005; Colbert and Johnston, 1996; Gogan et al., 1983). The anatomical data presented above suggests that this site will be approximately 90 μm from the axon hillock. As it is known that branch points commonly arise from nodes of Ranvier (Fraher and Kaar, 1984; Sloper and Powell, 1979), we searched for the first axonal collateral process of layer 5 pyramidal neuron axons after filling neurons with a fluorescent dye (Alexa 568) using confocal microscopy (Fig. 4.8a). On average this collateral process was 105 ± 8 μm (n=6) from the axon hillock, consistent with the idea that it represented the first node of Ranvier. Following identification of the putative first node of Ranvier, its potential role in AP initiation was investigated using voltage imaging (as in Fig. 4.3), extracellular recording (as in Fig. 4.4), and brief applications of low sodium extracellular solution and TTX (as in Fig. 4.5). APs were evoked by somatic current injection and the onset of the AP response at the first axon collateral was compared to that recorded in the axon initial segment (40 μm from the soma). In all cases the onset of the extracellularly recorded response at the first axon collateral occurred significantly later than the response recorded in the axon initial segment of the same neuron (Fig. 4.8b; axon initial segment: -144 ± 13 μs versus -78 ±17 μs at the first collateral, relative to the somatic AP; n=7; p < 0.05). Similarly, the onset latency of the AP voltage fluorescence at the first axon collateral occurred significantly later than the response recorded in the axon initial segment of the same neuron (Fig. 4.8c; axon initial segment: -150 ± 10 μs versus +20 ± 20 μs
Figure 4.8
Figure 4.8. Role of the first node of Ranvier in AP initiation. a, Confocal image of a layer 5 pyramidal neuron axon (Alexa 568) with initial segment (IS) and collateral branches (arrows) indicated. b, Normalised extracellular voltage responses during somatically evoked APs recorded at the initial segment 40 µm from the axon hillock (red; average of 110 trails) and at the first axon branch point (blue; 127 µm from the axon hillock; average of 110 trails). The onset of the extracellular voltage response recorded in the initial segment (IS) precedes that recorded at the first branch point. c, The normalized AP voltage fluorescence in the initial segment (IS; red; 35 µm from the hillock) also preceded that observed at the first node of Ranvier fluorescence (blue; 95 µm from the hillock; average of 140 somatically evoked APs). These data suggest that AP initiation does not occur at the first node of Ranvier. d, APs evoked by somatic current injection during application of low sodium solution to the initial segment (IS; red), soma (black) and first axon collateral (blue). Focal application of low sodium extracellular solution significantly influenced AP threshold only when applied to the axon initial segment.
at the first collateral, relative to the somatic AP; n=13; p < 0.05). The role of the first node of Ranvier in AP initiation was further investigated with focal applications of low sodium extracellular solution to the first axon branch point (n=5). Somatic AP threshold was unaffected by low sodium applications to the first branch point (-1.7 ± 0.2 mV; n=5; p = 0.53), whereas similar applications to the axon initial segment of the same neurons caused a statistically significant shift in AP threshold (Fig. 4.8d; +5.1 ± 0.8 mV; n=5; p < 0.05). Consistent with this result, focal applications of TTX (10 µM) targeted to the first branch point did not affect somatic AP threshold (+0.8 ± 1 mV; n=6; p = 0.94). Together, these data indicate that the first node of Ranvier is not the site of AP initiation in layer 5 pyramidal neurons.

**Impact of axonal inhibition**

The axon initial segment of layer 5 pyramidal neurons receives GABAergic inputs from chandelier cells (Freund et al., 1983; Peters et al., 1982). To test whether activation of these inhibitory inputs alters the site of AP initiation local applications of GABA (100 µM) was applied to the axon initial segment. These GABA applications inhibited AP firing, leading to a significant increase in the amount of somatic current required to initiate APs (Fig. 4.9a; control: 1.24 ± 0.01 nA vs. GABA: 2.75 ± 0.05 nA; p <0.05), however there was no significant effect on the site of AP initiation (Fig. 4.9b,c; n=9). These data illustrate that the site of AP initiation is robust even under conditions of axonal inhibition.
Figure 4.9
Figure 4.9. The effect of inhibition on AP initiation. a, Left, AP evoked by somatic current injection during control (no GABA). Right, focal application of GABA (100 µM) to the initial segment inhibited AP generation, which could be re-established by increasing the amplitude of the somatic current pulse. b, Example of the onset latency of axonal VSD signals relative to the somatic response (0 µm) plotted against distance from the axon hillock during GABA application to the initial segment (filled circles) and control (open circles). c, Average onset latency of axonal VSD signals relative to that at the soma plotted against distance from the axon hillock during GABA application to the initial segment (n=9).
Development of action potential initiation

Despite their immature state, AP initiation in p5 neurons occurred at a similar distance from the axon hillock as in p14 and p21-28 preparations (Fig. 4.10). These results further illustrate the robustness of AP initiation in the proximal region of the axon, and show that this site is conserved across development. Furthermore, as the axons of layer 5 pyramidal neurons are not myelinated at p5 and p14, these data indicate that the site of AP initiation in layer 5 pyramidal neurons does not depend on myelination and presumably instead depends on ion channel properties and distribution.
Figure 4.10
Figure 4.10. Development of AP initiation. Average onset latency of axonal fluorescent signals relative to the somatic response (0 µm) plotted against distance from the axon hillock for p5 (green), p14 (blue) and p21-28 (red) layer 5 pyramidal neurons. APs were evoked by brief (2ms) somatic current injection.
DISCUSSION

The discovery of electrical conduction in nerves by Galvani in the 18th century beckoned the question where within nerve cells electrical impulses, or APs, are generated. This question is fundamental to an understanding of how neurons integrate synaptic inputs. In this study, we provide direct evidence that APs in layer 5 pyramidal neurons are initiated ~35 µm from the axon hillock, at the distal axon initial segment. This was observed during AP generation under a variety of conditions, following axonal inhibition, and at different stages of development.

Using voltage-sensitive dyes to assess the site of AP initiation has advantages and disadvantages. The main advantage is that it allows voltage to be imaged simultaneously at multiple locations in the axon with high temporal (10 kHz) and spatial (~10 x 7 µm; 3 x 2 pixels) resolution. Disadvantages included a low signal to noise ratio (hence the need to average multiple trials), and the fact that voltage-sensitive dyes can be toxic, although not very apparent in our experiments (see Chapter 2). For these reasons it was necessary to support the results obtained with voltage imaging using alternative methods. Firstly, the onset of the extracellularly recorded AP at different sites along the axon was compared with the somatic AP in both p21-35 (myelinated) and p14 (unmyeliated) rats (Fig. 4.4). These results were consistent with the results using voltage imaging, and indicate that AP initiation occurs approximately 30 to 40 µm from the axon hillock. Additionally, the impact of reducing current flow through axonal voltage-activated sodium channels using brief (20 ms) applications of either a low-sodium external solution or TTX was investigated (Fig. 4.5). The reasoning here was that if the axon initial segment is responsible
for AP initiation then one would expect that reducing current flow through sodium channels at this location should lead to significant changes in AP threshold. Indeed, this was observed, with the greatest change in AP threshold during low-sodium and TTX applications occurring in the proximal axon. Furthermore, a reduction of active sodium channels in the proximal axon would decrease the AP amplitude at the initiation site which subsequentially compromises the amplitude of the somatic AP. Presumably, the greatest effect of low sodium applications was observed at 20 μm, rather than at the site of AP initiation ~35 μm from the axon hillock, as the maximum effect on AP threshold occurs when current flow over the entire axon initial segment is compromised. Given that the axon initial segment is 40 μm long, it is not surprising that low-sodium applications at its mid-point (20 μm) have the greatest effect. While these experiments cannot be used to accurately pinpoint the site of AP initiation, they provide strong support for the notion that AP initiation occurs in the axon initial segment. Finally, the impact of axonal length on AP properties was investigated (Fig. 4.6). As with the low sodium and TTX experiments, these data indicated that the machinery necessary for AP initiation is contained within the axon initial segment, adding further weight to the conclusions from the voltage-sensitive dye experiments.

Comparisons with previous studies

A recent study in rat cerebellar Purkinje neurons provided evidence that AP initiation can occur at the first node of Ranvier (Clark et al., 2005) (but see Khaliq and Raman, 2006). Based on intracellular filling of the oligodendrocyte that provided the first myelinated segment to the axon of layer 5 pyramidal
neurons, the anatomical location of the site of AP initiation is the distal region of the axon initial segment. In addition, the possibility that APs are initiated at the first node of Ranvier was directly addressed using extracellular recording and low sodium or TTX applications at the first axon branch point (Fig. 4.8), which has previously been identified to often occur at the first node of Ranvier (Sloper and Powell, 1979). Consistent with the idea that this site represents the first node of Ranvier, the average distance from the axon hillock to the first branch point (105 μm) was similar to the average distance from the axon hillock to the end of the first myelinated axon process (90 μm). These data indicated that the first node of Ranvier is not the site of AP initiation in layer 5 pyramidal neurons. The observation that the axon initial segment is the site of AP initiation in cortical pyramidal neurons is in agreement with recent axonal patch recordings from rats (Kole et al, 2007) and ferrets (Shu et al., 2007). Finally, APs were also initiated in the axon initial segment following axonal inhibition (Fig. 4.9), and at different stages of development (Fig. 4.10). Together, these experiments indicate that the site of AP initiation in cortical layer 5 pyramidal neurons is robust under a variety of conditions and during development.

In conclusion, while it has long been recognized that APs are initiated in the axon, exactly where in the axon has been the subject of much debate. Together, the data presented in this Chapter indicate that APs in cortical layer 5 pyramidal neurons are initiated at the distal end of the axon initial segment approximately 35 μm from the soma. These findings have important implications for understanding how single neurons translate synaptic inputs into an output signal – the action potential.
CHAPTER 5: ACTION POTENTIAL PROPAGATION IN LAYER 5 PYRAMIDAL NEURON AXONS

INTRODUCTION

Communication between neurons is achieved by the generation and propagation of a large all-or-none voltage response, the action potential (AP). Summation of synaptic input results in the initiation of APs in the distal end of the axon initial segment in layer 5 pyramidal neurons (Palmer and Stuart, 2006; see Chapter 4), which then forward propagates along the axon to postsynaptic targets. The reliability and timing of AP propagation is essential for shaping the spread of excitation in cortical neural networks, with many learning processes requiring precise timing of inputs in the order of milliseconds (Bi and Poo, 1998; Markram et al., 1997). To speed propagation, most axons in the CNS are ensheathed by specialized myelinating cells, called oligodendrocytes, which increase the resistance and decrease the capacitance of the axon. Early experimental (Bishop and Levick, 1956; Huxley and Stampfli, 1949) and theoretical (Stampfli, 1954) studies illustrate that APs propagate along the axon by ‘jumping over’ the myelinated regions to consecutive nodes of Ranvier in a process called ‘saltatory conduction’. More recent experiments have shown that nodes of Ranvier support saltatory conduction by containing very high densities of voltage-sensitive sodium channels (Boiko et al., 2003; Rasband et al., 1999), which act to actively regenerate the AP waveform as it propagates along the axon.

To facilitate network signalling, neurons typically have an extensive axon arborization which consists of a main axon branch with many collateral
branches extending from branch points. Therefore, APs must not only propagate down the main axon branch, but must also invade branch points to successfully excite synaptically connected neurons. Early reports by Goldstein and Rall (1974) suggest that, depending on the geometric change, AP propagation can either increase, decrease, remain unchanged or fail to propagate through a branch point. For example, an abrupt increase in axon diameter (as can occur at branch points) causes a decrease in both velocity and peak amplitude of the AP, and can lead to failure of AP propagation (Goldfinger, 2000; Zhou and Chiu, 2001). Experimental measurements of AP propagation in axons of neurons in the brain has been largely limited to calcium imaging at synapses (Geiger and Jonas, 2000; Sabatini and Regehr, 1996) and in axons (Callewaert et al., 1996; Cox et al., 2000), with more recent work using direct electrophysiological recordings (Khaliq and Raman, 2005; Monsivais et al., 2005). Reports of the success of AP propagation along axons of cortical neurons is conflicting, with some studies reporting small variability of axon terminal invasion (Cox et al., 2000; Mackenzie and Murphy, 1998; Mackenzie et al., 1996), whereas others report high failure rates (Frenguelli and Malinow, 1996). In the study reporting high failure rates, it is unclear whether AP failure occurs at the branch point or another location along the axon.

In addition, neurons typically have multiple AP firing modes (Bean, 2007) which convey information that also needs to be successfully transmitted to postsynaptic targets. Evidence for successful propagation of high frequency AP bursts along the axon is contradictory, however. Experimental studies in peripheral nerves from numerous species including the lobster (Grossman et al., 1979a), frog (Stoney, 1990) and cat (Coleman et al., 2003) have indicated
that high frequency AP firing fails to invade axon branches, whereas Cox et al (2000) used calcium imaging to illustrate that high frequency APs (100Hz) are able to successfully invade axon varicosities in rat neocortical neurons.

This study uses voltage-sensitive dye imaging to visualize AP propagation along the main axon branch and into axon collaterals in layer 5 pyramidal neurons. In addition, we tested whether branch points impose a significant electrical barrier to propagating APs during high frequency AP bursts.
METHODS

Parasaggital cortical slices were obtained from Wistar rats and layer 5 pyramidal neurons were filled with the voltage-sensitive dye JPW3028 (3 mg/ml) by the whole-cell recording pipette (as described in Chapter 2). The axon was visualized and changes in fluorescence ($\Delta F/F$) were recorded by a cooled CCD camera operating at 10 kHz as described in Chapter 2. Fluorescence responses to somatically evoked APs (~1nA somatic current injection) were imaged in 10 μm regions of interest at sequential locations along the main axon and proximal axon collaterals. Axon collaterals were clearly identified as branches extending off the main axon. AP propagation was recorded at different developmental ages: postnatal day 21-28 (P21-28), postnatal day 14 (P14) and postnatal day 5 (P5). Imaging propagation of single APs along the axon was performed at room temperature (~22 C), whereas experiments using AP bursts were performed at near physiological temperature (~33 C).
RESULTS

Saltatory Conduction and development of action potential propagation

Early investigations of AP propagation in myelinated axons indicated propagation occurs in a "saltatory" manner, progressing along the axon by jumping between active nodes of Ranvier (Bishop and Levick, 1956; Huxley and Stampfli, 1949). A form of saltatory conduction was observed during orthodromic conduction of APs from the site of initiation along the axon in P21-28 axons, which was represented by reoccurring shortenings in onset latency of fluorescence signals at distant axon locations (Fig. 5.1a,c). These reoccurring shortenings in AP onset latency were also observed in preparations from P14 rats (n=5; data not shown), but were absent in P5 preparations (Fig. 5.1b,c; n=8). The axonal sites of shortest onset latency were associated with axonal branch points in P21-28 neurons (Fig. 5.1c, arrows), which are thought to be sites of nodes of Ranvier (Fraher and Kaar, 1984; Sloper and Powell, 1979).

Action potentials decrease in width in the myelinated axon

Analysis of the AP as it propagates along the axon illustrated that the AP waveform was dependent on axonal location. As illustrated in Figure 5.2, the AP waveform recorded at the soma and initial segment (35 μm) were wider than the AP recorded at axonal distances greater than approximately 50 μm from the hillock. This trend is clearly illustrated in Figure 5.2d, where the AP waveform becomes progressively briefer at distal axonal locations. On average, APs had the greatest width 20-30 μm from the hillock, at the site of AP potential initiation (see Chapter 4). These data are similar to those reported by Kole et al (2007).
Figure 5.1. Imaging AP propagation along the axon with voltage-sensitive dyes. 

a, Left, Fluorescent image of a P21-28 layer 5 pyramidal neuron. Arrows indicate the location of axon collaterals. Right, Averaged and normalised fluorescence signal (ΔF/F) measured at the indicated locations along the axon (average 100 trials). Note that the fluorescence signal at the first branch point, \sim 115 \mu m from the hillock, occurs before the fluorescence signal recorded in the preceding axon region (75 \mu m from the hillock). 

b, Left, Fluorescent image of a P5 layer 5 pyramidal neuron. Arrows indicate the location of axon collaterals. Right, Averaged and normalised fluorescence signal (ΔF/F) measured at the indicated locations along the axon (average 140 trials). Note the sequential propagation of the fluorescence signal along the axon. 

c, Onset latency of axonal fluorescence signals relative to the somatic response (0 \mu m) plotted against distance from the axon hillock for the P5 and P21-28 layer 5 pyramidal neurons shown in (a) and (b). Arrows indicate the location of axonal branch points (red P21-28; green P5). Note the correlation between branch point location and occurrence of a 'dip' in the onset latency for P21-28 axons.
Figure 5.2
Figure 5.2. APs have shorter widths in the myelinated axon. a, Image of layer 5 pyramidal neuron filled with voltage-sensitive dye. b, Average normalized fluorescence change ($\Delta F/F$) in response to 220 individually aligned somatic APs recorded at the axonal locations shown in (a). The somatic AP response recorded by the whole-cell recoding pipette is shown in black (top). c, Overlay of the normalized AP fluorescence signals illustrated in (b). Note the narrower AP fluorescence recorded at 115 µm (green) and 165 µm (blue) compared with the initial segment (35 µm; red). d, AP half-width is greatest in the axon initial segment (red line) and decreases with distance along the axon. e, AP rise time (10-90%) does not alter significantly with distance along the axon.
In contrast, AP rise time remained relatively uniform at the different axonal distances (Fig. 5.2e).

**Developmental differences**

When comparing axons from rats of different ages (Fig. 5.3), AP waveform showed developmental differences at different axonal locations. Figure 5.3a shows individual examples of the fluorescence response to a single AP in the initial segment (35 μm from the hillock) and the axon proper (135 μm from the hillock) in a P21, P14 and P5 axon. On average, the width of the AP fluorescence signal in the axon proper was significantly narrower than the AP in the axon initial segment in P21 axons (by 54%) and P14 axons (by 25%), whereas there was no significant decrease in AP width along the axon in P5 axons. This developmental narrowing of the axonal AP is clearly evident when AP waveforms recorded at different axonal locations are compared during development. As illustrated in Fig. 5.3b and plotted in Fig. 5.3c, the AP in the initial segment (35 μm from the hillock) is significantly wider in P5 axons (3.8±1.0 ms) and P14 axons (2.6±0.4 ms) than in P21 axons (1.6±0.1 ms), whereas by P14 the AP width in the distal axon is similar to P21 axons (0.6±0.2 ms and 0.9±0.1 ms, respectively), and significantly narrower than in P5 axons (2.8±0.6 ms). There are no such developmental effects on AP rise-time, although Figure 5.3d suggest that the AP in the axon initial segment (35 μm from the hillock) is slower in P5 axons (1.0±0.2 ms) than P14 axons (0.6±0.1 ms) and P21 axons (0.7±0.1 ms; Fig. 5.3d). These results indicate that not only does the AP waveform differ between axonal regions but the extent of this difference changes developmentally.
Figure 5.3. Axonal AP propagation during development. a, Individual examples of layer 5 pyramidal neuron axons filled with voltage-sensitive dye (left) and overlayed AP fluorescence signals (right) recorded from the initial segment (dark colour; 35±5 µm from the hillock) and the main axon branch (light colour; 135±5 µm from the hillock) in P21 (red), P14 (blue) and P5 (green) animals. The axonal regions of interest are indicated by 10 µm boxes on the respective images. b, Super average of normalized AP fluorescent signals recorded at the indicated axonal locations in cells from P21 (red; n=24), P14 (blue; n=5) and P5 (green; n=8) animals. The width (c) and rise time (d) of the somatically evoked AP at different distances along the axon depended on the developmental age. The red bar represents the length of the initial segment. All fluorescence traces are an average of greater than 100 individually aligned somatically evoked APs. Error bars represent SEM.
response to the first AP in the burst (P21). One way to get around this is to compare peak amplitude at different distances. Comparing the amplitudes of the first AP, the second and third APs in this 100 Hz burst (Fig 5.4b; p = 0.01). There was also a significant difference between the normalized rise and fall times of the first, second, and third APs in this 100 Hz burst (Fig 5.4c; p = 0.04).
**Action potential bursts invade axon collaterals**

One problem with the interpretation of voltage-sensitive dye signals is that they are difficult to calibrate (discussed in Chapter 1). Although patch recordings illustrate the faithful propagation of single APs along the proximal region of the main axon without loss of amplitude (Khaliq and Raman, 2005; Meeks et al., 2005; Raastad and Shepherd, 2003), the voltage fluorescence response to the first AP in the burst (which represents a single AP) shown in Figure 5.4a has varying amplitudes, preventing the direct comparison of the AP peak at different locations. One way to get around this problem is to compare relative changes in voltage-sensitive dye signals in response to different stimuli at the same location (Djurisic et al., 2004; Kampa and Stuart, 2006). Assuming single APs propagate faithfully, the first AP in a burst can be used as a yardstick for determining whether subsequent APs in the burst successfully propagate along axons and invade collateral branches. Comparing the amplitude of the first AP with the second and third AP in a 200 Hz burst (Fig. 5.4b), there was no significant difference in the amplitude of successive APs in axon collaterals (located 117±14 μm from the hillock) and the axon branch point (n = 5; p > 0.05). There was also no significant difference between the normalized first, second and third AP amplitude in a 300 Hz burst (Fig. 5.4c; n = 4; p > 0.05).
Figure 5.4
Figure 5.4. AP bursts successfully invade axon collaterals. a, An example illustrating the propagation of a 200 Hz burst of APs along an axon and into two axon collaterals. Left, layer 5 pyramidal neuron filled with voltage sensitive dye illustrating collateral branches extending from the main axon. Boxed regions are colour coded to areas where voltage fluorescence (ΔF/F) were recorded at various locations along the axon, collaterals and background in response to a 200Hz AP burst evoked by somatic current injection (grey). The colour of the distance noted in the traces correspond to recordings from the soma (grey), axon (blue), collaterals (green) and background (black). There was no significant difference in the amplitude of the second and third AP compared to the first at any location along the main axon (blue) or in the collaterals (green) during a 200 Hz (b) and 300 Hz (c) AP burst, indicating AP bursts up to 300 Hz are able to successfully propagate along the main axon and invade collateral branches.
DISCUSSION

Historically, AP propagation has been a source of intrigue (for a review see Stampfli, 1954) and it was not until experiments by Huxley and Stampfli (1949) and later experiments by Bishop and Levick (1956) that saltatory conduction of APs along axons was clearly illustrated (but see Laporte, 1951). Using voltage imaging, we also clearly demonstrate saltatory conduction, with shortenings in onset latency occurring at the site of axon branch points in P21-28 axons. Since branch points represent nodes of Ranvier (Fraher and Kaar, 1984; Sloper and Powell, 1979), these data suggest APs propagate along myelinated axons by jumping between nodes. In contrast, APs propagated along P5 axons with uniform velocity irrespective of the presence of branch points. This developmental difference in axonal AP propagation is presumably due to different ion channel expression during development. Analysis of the developmental expression of sodium channels in the optic nerve illustrates that the node of Ranvier (Boiko et al., 2001) and initial segment (Boiko et al., 2003) contain the ‘mature’ sodium channel (Nav1.6) by P14, which was able to support saltatory conduction. Prior to this however, and before myelination, P5 axons contain a relatively uniform distribution of a different sodium channel (Nav1.2) (Rasband et al., 1999). These developmental differences would influence the speed of AP conduction, and although saltatory conduction would propagate at a considerably faster velocity (Brill et al., 1977), the uniform conduction of the broader APs in P5 axons may be developmentally important for successful AP propagation into the axonal arbor. In addition, numerous experiments illustrate that increased AP width causes an increase in synaptic strength (Byrne and Kandel, 1996; Geiger and Jonas, 2000; Jackson, 1993,
Kole, 2007 #76), which may be important for the establishment of networks in early development.

_Invasion into branch points_

While the main axon branch provides a communication cable between neurons, most local signalling is conducted via collateral branches. Depending on the diameter difference of the main axon and the daughter branch (see Goldstein and Rall, 1974) the branch point can represent an electrical obstacle to the propagating AP due to a mismatch in input impedance (Joyner et al., 1980; Zhou and Chiu, 2001). Cox et al. (2000) illustrate that single APs are able to successfully invade axon collaterals in layer 2/3 cortical neurons, however, successful invasion of more complex firing patterns is unknown. Previous experiments have shown that AP failure is dependent on prior axonal activity (Luscher et al., 1994a; Luscher and Shiner, 1990), with the propagation of the APs in Purkinje cells decreasing at frequencies above 200 Hz (Khaliq and Raman, 2005; Monsivais et al., 2005). These AP failures at high frequencies may be due to accumulation of extracellular potassium or intracellular calcium (Grossman et al., 1979b; Luscher et al., 1994b) or due to cumulative sodium channel inactivation (Golding, 2003). Our experiments illustrate that high-frequency bursts (up to 300 Hz) successfully propagate along the main axon of layer 5 pyramidal neurons, and invade proximal regions of the axon collaterals (Fig. 5.4).

The extensive axon arboration in layer 5 pyramidal neurons enables the synchronous excitation of synaptically connected neurons. Successful propagation of APs along the axon and into branch points is critical in shaping
the spread of excitation within networks. This study directly investigated the development of saltatory conduction, illustrating that P21-28 axons are able to support the non-uniform conduction of APs along axons, which involves the AP jumping between successive nodes of Ranvier. To invoke local network signalling, the AP must also successfully invade and propagate along axon collaterals. Although branch points have been shown theoretically and experimentally to act as sites of AP failure, bursts of APs up to 300Hz were found to successfully invade branch points in layer 5 pyramidal neurons, allowing efficient transfer of information within in local cortical networks.
Chapter 6: The Initiation and Propagation of Simple and Complex Spikes in Cerebellar Purkinje Cells

Introduction

Purkinje cells are the primary integrative and output neurons of the cerebellar cortex, transmitting inhibitory signals to the deep cerebellar nuclei. Each Purkinje cell receives excitatory input from two different sources; parallel fibers extend from granule cells to make thousands of dendritic synapses, which contrasts with the single climbing fiber input from the inferior olivary complex. Subsequently, Purkinje cells produce two main outputs: Simple spikes, which occur spontaneously (Hausser and Clark, 1997) and can exceed 100 Hz in response to excitation from parallel fibers (Thach, 1968), and complex spikes, which are elicited by climbing fiber excitation and consist of an initial large spike followed by a series of smaller ‘spikelets’ (Eccles et al., 1967; Llinas and Sugimori, 1980b).

The initiation of simple spikes has created some controversy recently, with the initial report of initiation at the first node of Ranvier (Clark et al., 2005) being contested by a subsequent study suggesting initiation in the axon initial segment (Khaliq and Raman, 2005). Where the AP is initiated is important in ultimately determining the extent of synaptic integration and the timing of neuronal output. Both the initial segment and nodes of Ranvier are sites of high sodium channel density in Purkinje cells (Jenkins and Bennett, 2001; Komada and Soriano, 2002; Pan et al., 2006) making them ideal candidates as regions of low threshold for action potential (AP) generation. Here we investigate where
the simple and complex spike are initiated, as well as investigate how they propagate along the axon to postsynaptic targets.

The interaction of the simple and complex spikes are critical for motor learning (Gilbert and Thach, 1977). Although simple spikes are typically silenced by complex spikes (Ebner and Bloedel, 1981; Konnerth et al., 1992; Sato et al., 1992), complex spikes are unaffected by simple spike firing (Shin et al., 2007). The complex spike contributes both to the generation of movements and to the gradual, long-term improvement of these movements (Kitazawa et al., 1998). This information is crucial for motor activity and might be conveyed by individual spikelets of the complex spike (Eccles et al., 1967), which can attain extremely high rates (>500 Hz). However, in vivo studies have suggested that spikelet propagation along the axon is variable (Campbell and Hesslow, 1986; Ito and Simpson, 1971), and direct recordings from Purkinje cell axons indicate that not all spikelets successfully propagate to the distal axon (Khaliq and Raman, 2005; Monsivais et al., 2005). If spikelets are not transmitted to postsynaptic neurons, it begs the question of the purpose of spikelets, and their possible role in signalling in the proximal axon and somatodendritic region.

Since the Purkinje cell is the sole output of the cerebellar cortex, understanding AP initiation and propagation in these neurons is critical to an overall understanding of cerebellum function. This study uses voltage-sensitive dye imaging to directly measure simple and complex spike initiation and to record their propagation along the proximal axon to distances up to 150 μm from the axon hillock.
METHODS

Cerebellar slices were prepared from 21-28 day old Wistar rats and Purkinje cells located at the slice surface were targeted for filing with voltage-sensitive dye (JPW 3028) according to the procedure outlined in Chapter 2. After filling and upon repatch, simple spikes usually occurred spontaneously (n=3) but in one case were initiated by brief (~1 nA; 2 ms) current injection through the somatic whole-cell recording pipette. Since the results were the same, all simple spike data was pooled. Complex spikes were initiated by an extracellular stimulating pipette (7 MΩ tip-resistance; ~1 μm tip diameter) placed in the white matter at least 50 μm from the recorded cell. Fluorescence changes during APs were detected with a back-illuminated cooled CCD camera (see Chapter 2) sampling at 10 kHz.

To maximize the signal-to-noise ratio we used regions of interest (ROIs) of 3 pixels length (~10 μm) and 2 pixel width (~6 μm) in both the axon and dendrite. The onset latency of fluorescent signals was measured at half amplitude after fitting a linear regression to the rising phase centred around the region of maximum slope. All experiments were performed at room temperature (22° C).
RESULTS

Purkinje cell characteristics

The Purkinje cell is a morphologically elaborate neuron which is characterised by an extensive dendritic arbour with multiple thin branchlets extending from the main branches (Fig. 6.1a). Excitatory input onto the dendrite by either parallel fibers or climbing fibers evokes the generation of a simple or complex spike, respectively, which can be accurately captured using voltage-sensitive dyes (Fig. 6.1b). Purkinje cells typically exhibit continuous firing of simple spikes (Fig. 6.1c), which are uniformly small (60-70 mV in peak amplitude at the soma) and brief (0.51±0.09 ms; Fig. 6.1d). In contrast, the complex spike differs considerably between different cells and is characterised by an initial large fast component (~100 mV in peak amplitude at the soma) followed by a prolonged depolarisation containing multiple ripples (spikelets) of varying amplitudes and frequencies (Fig. 6.1e).

Determination of the site of AP initiation with voltage sensitive dyes

Comparison of the onset latency of the fluorescence signals recorded at various locations in the axon with the somatic signal during simple spikes (Fig. 6.2a-c) illustrates that the site along the axon with the shortest onset latency, which by definition is the site of AP initiation, occurs on average 22.5±7.5 μm from the hillock (Fig. 6.2d; n=4). Despite their dramatically different waveforms, the first spike in the complex spike is initiated at approximately the same axonal site, 15±0 μm from the axon hillock (Fig. 6.3; n=4). This initiation site is also very similar to the location where the AP in layer 5 pyramidal neurons is initiated (35±2 μm; for details see Chapter 4; Fig. 6.4a). Despite the obvious
Figure 6.1. Purkinje cell characteristics. a, Purkinje cell filled with voltage-sensitive dye. Note the extensive dendritic arbour. b, An overlay of the normalized simple spike voltage (black) and fluorescence (red) recorded in the soma clearly demonstrates that voltage imaging accurately tracks membrane potential during an AP. Purkinje cells fire two types of action potentials; simple spikes evoked by parallel fibers (c and d) and complex spikes evoked by stimulation of the climbing fiber (e). Purkinje cells are spontaneously active (c), maintaining constant firing at ~20 Hz. d, Examples of somatic whole-cell recordings of simple spikes recorded in two different cells, illustrating the APs are uniformly brief and small (~70 mV peak amplitude). In contrast, the complex spike is a large (~100 mV peak amplitude) prolonged depolarisation which displays different waveforms, as shown by the complex spikes recorded in different Purkinje cells in (e).
Figure 6.2
Figure 6.2. Initiation of the simple spike occurs in the proximal axon approximately 15 μm from the hillock. a, An example of a Purkinje cell filled with voltage-sensitive dye highlighting the soma and axon. b, Fluorescence traces (bottom) in response to a simple spike (top; boxed region) recorded at the colour-corresponding axon locations shown in (a). The fluorescence trace in the proximal axon (red; 25μm from the hillock) precedes the soma and all other axon locations. c, The onset latency of the simple spike fluorescence response relative to the somatic response plotted against distance from the axon hillock for the neuron shown in (a). Coloured points represent the onset latencies of the fluorescence trace recorded at the axonal distances depicted in (a) and (b). d, Average onset latency of the simple spike at different axonal distances relative to the soma (n=4).
Figure 6.3
Figure 6.3. Initiation of the first spike in the complex spike occurs in the proximal axon approximately 15 μm from the hillock. a, An example of a Purkinje cell filled with voltage sensitive dye highlighting the soma and axon. b, Fluorescence traces (bottom) of the first spike in the complex spike (top; boxed region) recorded at the colour-corresponding axon locations shown in (a). The fluorescence trace in the proximal axon (red; 25μm from the hillock) precedes the soma and all other axon locations. c, The onset latency of the first spike in the complex spike relative to the somatic response plotted against distance from the axon hillock for the neuron shown in (a). Coloured points represent the onset latencies of the fluorescence trace recorded at the axonal distances depicted in (a) and (b). d, Average onset latency of the complex spike at different axonal distances relative to the soma (n=4).
morphological and physiological differences, this data indicates that the AP is initiated in the distal end of the axon initial segment which is \( \sim 20 \) \( \mu \)m long in Purkinje cells (Clark et al., 2005) and \( \sim 40 \) \( \mu \)m in length in layer 5 pyramidal neurons (Palmer and Stuart, 2006).

*Forward- and back- propagation of the simple and complex spike*

Once initiated, simple and complex spikes both forward-propagate along the axon to postsynaptic targets, and back-propagate into the soma-dendritic region (albeit to different spatial extents, see Llinas and Sugimori, 1980a; Stuart and Häusser, 1994). As illustrated in Fig. 6.4a, the simple spike took significantly longer to backpropagate from the site of initiation into the soma than the complex spike (184±16 \( \mu \)s versus 40±13 \( \mu \)s; \( p < 0.05 \)). This is presumably due to pre-charging of the somatic and dendritic capacitance during the large climbing fiber input. In contrast, the speed of forward propagation along the axon of the simple and complex spike from the site of initiation was not significantly different (39±3 cm/s and 38±7 cm/s, respectively; \( p > 0.05 \); Fig 6.4a).

Both the simple (Fig. 6.4b) and complex spike (Fig. 6.4c) change their waveform as they propagate from the initial segment along the axon. At distal axonal locations, the simple spike is only slightly modified whereas the complex spike becomes drastically simplified consisting only of the initial large-amplitude fast component. The reduced complexity of the complex spike at distal locations is due to the failure of spikelets to propagate along the axon (Fig. 6.5). Figure 6.5a illustrates an example of a Purkinje cell filled with voltage-sensitive dye and the corresponding average complex spike recorded in the soma and
Figure 6.4
Figure 6.4. Forward and back propagation of simple and complex spikes. a, Average onset latency of the simple spike (SS; blue; $n=4$) and complex spike (CS; red; $n=4$) compared with APs in layer 5 pyramidal neurons (L5; black; $n=44$) at different axonal distances relative to the soma. There is a significant difference in the backpropagation of APs from the site of initiation into the soma during simple and complex spike generation (indicated by an asterick), however there is no difference in the velocity of forward propagation of the simple and complex spike along the axon. The length of the initial segment in the Purkinje cell and pyramidal neuron are illustrated by a red/blue and black line, respectively. The average normalized simple spike (b) and complex spike (c) response recorded at the soma (whole-cell recording pipette) and indicated axon locations (fluorescence) ($n = 4$). Note the differences in AP waveform.
Distance (μm)

Onset Latency (μs)

- Distance (μm)
  - 0
  - 50
  - 100

Onset Latency (μs)

- L5
- CS
- SS

b Simple Spike

- Soma
- 15μm
- 115μm
- 175μm

1 ms

1 ms

Complex Spike

- Soma
- 15μm
- 115μm
- 175μm
Figure 6.5. The complex spike spikelets fail to propagate to the distal axon. a, An example of a Purkinje cell filled with voltage sensitive dye highlighting the soma and axon. b, The complex spike response recorded at the soma (whole-cell recording pipette) and indicated axon locations (fluorescence) for the Purkinje cell shown in (a). c, Spikelet amplitude as a proportion of the 1st response in the complex spike at different axonal distances for the complex spike shown in (b). The first component of the complex spike faithfully propagates along the axon, however the spikelets become progressively smaller in amplitude at more distal axonal locations and eventually undetectable at distances greater than 120 μm.
various locations along the axon as indicated (Fig. 6.5b). The complex spike has pronounced spikelets that are discernable at proximal axonal locations (15 μm and 45 μm) but are significantly reduced in amplitude at more distal axonal locations. This is clearly illustrated when the spikelets are normalized to the amplitude of the large-amplitude first spike of the complex spike and plotted versus axonal distance from the axon hillock (Fig. 6.5c).

**Simple and complex spike invasion of the dendritic tree**

Once initiated in the axon, both the simple and complex spike backpropagate into the soma and, to differing extents, the dendritic tree (Llinas and Sugimori, 1980a; Stuart and Häusser, 1994). The spatial extent of this dendritic depolarization during simple and complex spikes was measured in Purkinje cells filled with voltage-sensitive dye. At all measured locations, the complex spike was considerably larger than the simple spike (n=2). In the example shown in Figure 6.6, at dendritic distances of 20 and 40 μm from the soma the complex spike is 1.8-fold and 2.1-fold larger than the simple spike, respectively, and at distances greater than 40 μm from the soma, the simple spike is negligible. As previously described by Stuart and Hauser (1994) and Llinas and Sugimori (1980a) and illustrated in Figure 6.6b, neither simple spikes nor the spikelets that make up complex spikes backpropagate significantly into the dendritic tree.
Figure 6.6
Figure 6.6. Simple and complex spikes differentially backpropagate into the dendritic tree. a, Purkinje cell filled with voltage-sensitive dye. Note the extensive dendritic tree. b, Direct comparison of the voltage fluorescent traces in response to simple spikes (blue) and complex spikes (red) recorded in the axon (1), soma (2), main dendrite branch (3-5) and secondary branchlets (6) at the specified distances from the soma in 10 μm regions of interest numerically highlighted in (a). At all recording sites, the complex spike is larger in amplitude than the simple spike. The simple spike and the first spike of the complex spike are not detected at distal dendritic locations and in the branchlets.
**DISCUSSION**

*Action potential initiation and comparisons with previous studies*

Early work by Eccles (1955) illustrated that the AP recorded in the soma is comprised of two components: a smaller initial segment (IS) spike and a larger somato-dendritic (SD) spike. The IS spike always preceded the SD spike independent of orthodromic or antidromic stimulation, leading to the hypothesis that APs are initiated in the axon. Subsequent studies have analysed the site in the axon where APs are initiated, illustrating the initial segment as the site of initiation in CA3 neurons (Meeks and Mennerick, 2007) and layer 5 pyramidal neurons in rats (Palmer and Stuart, 2006, see Chapter 4) and ferrets (Shu et al., 2007). Prior to this study, the site of simple spike initiation in Purkinje cells was controversial with both the initial segment (Khaliq and Raman, 2006) and the first node of Ranvier (Clark et al., 2005) being reported as the site of initiation. In contrast to Clark et al (2005), we observed initiation of APs in the axon initial segment during simple (and complex) spikes. The most likely explanation for this discrepancy is differences in the recording conditions and techniques employed. With respect to recording conditions our experiments were recorded at room temperature, whereas those of Clark et al. (2005) were at physiological temperatures. At the technical level voltage-sensitive dye imaging has an advantage over the single-site axonal electrophysiological recording technique used by Clark et al. (2005) as it enables simultaneous measurement of the axonal AP waveform from multiple sites in the axon of the same cell. In addition, while the extracellular recording technique used by Clark et al. (2005) has higher time resolution it suffers from the problem that it is both indirect and complex to interpret.
Our finding that both the simple spike and the first component of the complex spike are initiated in the proximal axon supports findings in other neuronal cell types showing that the axon initial segment is the site of AP initiation. The conservation of the AP initiation site between neurons is somewhat surprising considering the extremely different cell morphologies. Purkinje cells have an extensive dendritic tree that lacks voltage-activated sodium channels (Stuart and Häusser, 1994) and is likely to exert a significantly greater electrical load on the soma than is the case in layer 5 pyramidal neurons (Bekkers and Hausser, 2007). Despite these obvious morphological and physiological differences, the AP is initiated in the distal end of the initial segment in all cell types investigated (discussed in detail in the Discussion).

*Simple spike and complex spike axonal propagation*

Our findings illustrate that within a single Purkinje cell the same axonal location can produce two very different spike waveforms. These differences in simple and complex spike waveforms were maintained in the proximal axon but the simple and complex spike progressively converged to the same waveform at more distal axonal locations. The similarity of the waveforms at distal axon locations is presumably due to the failure of spikelet propagation from the site of initiation or an increased failure rate for active regeneration of spikelets at distal locations (although see Khaliq and Raman, 2005 and Monsivais et al., 2005). The difference between the propagation of the last spikelet reported in our studies and those in the literature is probably due to different recording temperatures and the averaging procedure in our studies may dampen the spikelet amplitudes if there is considerable trial-to-trial jitter. It is clear that more
experiments are required to determine the mechanisms behind the reported differences in the axonal propagation of the simple and complex spike, which may be important for the control of motor activity.

*Back propagation of simple and complex spikes into the dendritic tree*

Once initiated, simple spikes and the spikelets that make up the complex spike backpropagate into the dendritic tree, although poorly (Stuart and Häusser, 1994). It has previously been suggested that the extensive branching of the Purkinje cell dendritic tree favours the propagation of slow potentials, but strongly attenuates fast voltage transients (Vetter et al., 2001). This was clearly shown in our experiments where the slow somatic plateau depolarisation was evident in both the main dendritic branch and branchlets, whereas the first spike was only recorded at proximal dendritic locations and did not invade the distal dendritic tree. The plateau depolarisation and spikelets during the complex spike are thought to be caused by the large synaptic depolarisation associated with climbing fibre input together with dendritic calcium spikes (Llinas and Sugimori, 1979; Llinas and Sugimori, 1980a; Midtgaard et al., 1993; Miyakawa et al., 1992) or sodium currents (Callaway and Ross, 1997), whereas simple spikes and the fast initial response in complex spikes are generated purely by sodium channels (Llinas and Sugimori, 1980a; Stuart and Häusser, 1994). Along with morphological effects, this can explain the rapid failure of simple spikes and the fast response of the complex spike to invade the dendritic tree, as the density of sodium channels in the Purkinje cell dendrite declines rapidly with increasing distance from the soma (Stuart and Häusser, 1994).
Together, these data indicate that both the simple and complex spike are initiated in a localised region of the axon approximately 20 µm from the axon hillock, at the distal end of the axon initial segment. The reliability of action potential propagation along the axon is important in determining the effectiveness of network signalling and once initiated, the simple spike and first component in the complex spike propagate faithfully along the axon, whereas the individual spikelets of the complex spike can fail to propagate to distances greater than 80 µm from the hillock. The spikelets and first component of the complex spike also fail to backpropagate to distal regions of the dendritic tree whereas the plateau depolarisation underlying the complex spike depolarises large regions of the dendritic arbour. Since the interaction of these spikes are critical for motor learning, the site of initiation and the characteristics of the forward- and back-propagation of these spike waveforms is critical to understanding cerebellar cortex functioning.
CHAPTER 7: GENERAL DISCUSSION

Cortical neurons receive thousands of excitatory inputs typically onto dendritic spines which then integrate to generate a single all-or-none output, the action potential (AP). This thesis investigated the voltage response at the site of synaptic input and the generation and propagation of the axonal output. As discussed in Chapter 3, we illustrate that the isolated spine synaptic voltage is on average 6 mV in amplitude which equated to a spine neck resistance of approximately 200 MΩ. Although spine neck resistance caused measurable isolation of synaptic voltage in the spine head, this was considered to be inconsequential to influence the synaptic weight of the spine. Chapters 4 and 5 illustrate that the AP is initiated at the distal end of the initial segment and propagates faithfully along the axon main branch and into collaterals in layer 5 pyramidal neurons. Chapter 6 addresses AP initiation in the Purkinje cell and illustrates that both the simple spike and complex spike are initiated in the initial segment. This chapter is separated into four main topics to discuss the overall findings presented in this thesis including the function of dendritic spines, the importance of action potential initiation and propagation, the use of voltage-sensitive dyes and future questions resulting from this research are highlighted.

THE FUNCTION OF DENDRITIC SPINES

Why does synaptic input occur onto spines?

The purpose of dendritic spines has attracted considerable attention since their morphology was first described by Cajal over 100 years ago. During the past century, three hypotheses have emerged to explain the function of
spines: (1) spines serve to connect dendrites and axons, (2) spines act as biochemical compartments, and (3) spines provide an electrically isolated domain. Although there is ample dendritic surface area for synaptic contact without the existence of spines (Harris and Stevens, 1988), and the importance of calcium sequestering in spines is well described (Denk et al., 1995; Denk et al., 1996; Muller and Connor, 1991; Sabatini et al., 2002; Yuste and Denk, 1995, although see Goldberg et al., 2003), whether spines also compartmentalize voltage has remained unknown. For the first time we provide direct evidence that the synaptic response in the spine was measurably larger than the dendrite, demonstrating that dendritic spines electrically isolate synaptic events. Although this attenuation of the synaptic input from the spine head to the dendrite was minimal and did not significantly affect the synaptic weight of the spine, why would any attenuation of synaptic input (albeit small) be desirable?

In 1952, Chang observed that due to their long thin necks, input onto dendritic spines would attenuate the effect of the synapse on the cell (Chang, 1952). He argued that this attenuation would result in an increased number of inputs required to reach threshold for AP generation. In addressing this, our measured range of spine neck resistances does not have a considerable effect on the overall excitability of a neuron as detailed below. Since the threshold for AP initiation in our experiments is ~14 mV and single unitary EPSPs in layer 5 pyramidal neurons are ~200 µV in amplitude at the soma (Nevian et al., 2007), simple arithmetic illustrates that ~70 spines need to be simultaneously activated to reach threshold in the initial segment for AP initiation (assuming linear synaptic integration). The largest average spine synaptic response (13 mV)
required a spine neck resistance of 500 MΩ which led to an approximate 15% decrease in the EPSP amplitude recorded at the soma. Therefore, even the largest physiologically measured spine neck resistance will only slightly alter neuron excitability (requiring ~80 simultaneously activated synapses).

Since synaptic input onto a spine does not significantly affect the synaptic weight of the spine or the number of active synapses required to reach threshold for AP generation, is the spine neck of any functional relevance to the synapse? Firstly, it is possible that the spine neck ensures linear summation of synaptic effects by reducing the coupling between neighbouring synapses. However, higher spine neck resistances and greater voltage attenuation than we report would be required to linearize synaptic inputs (see Araya et al., 2006a). Secondly, it is possible that electrical compartmentalization may ensure synaptic democracy, where spines located at different dendritic locations have the same synaptic efficacy (as determined by the EPSP at the soma). In essence, this would require synaptic input in the distal dendrite to result in a larger voltage response than proximal inputs, as has been reported by Iansek and Redman (1973) and more recently by Williams and Stuart (2002) and Nevian et al. (2007). However, there was no such trend observed in our data (see Chapter 3). Thirdly, perhaps electrical compartmentalization in spines is important for synaptic plasticity. The NMDA receptor has been reported as a coincidence detector important in spike-timing-dependent plasticity as the voltage-dependent magnesium block inside the channel pore is only removed when presynaptic glutamate release is coincident with postsynaptic depolarisation (Mayer et al., 1984; Nowak et al., 1984). The spine neck may serve to isolate the synaptic input from the parent dendrite and neighbouring
synapses, leading to the activation of NMDA receptors only in the active spine thus ensuring localized plasticity. Although electrical compartmentalisation was minimal in our experiments, it is possible that even this small amount of compartmentalisation provides sufficient depolarisation to activate NMDA receptors on a single spine. Finally, although unlikely, it is possible that the reported electrical compartmentalization measured in the spine head is purely a physiologically-irrelevant consequence of the spine morphology required for housing the molecular machinery important for chemical compartmentalization.

Since input onto spines alone does not modulate synaptic strength, neurons may use other mechanisms by which synaptic strength can be altered including the recruitment or removal of ion channels from the postsynaptic density (Carroll et al., 1999; Liao et al., 2001), increased channel conductance (Benke et al., 1998; Sobczyk and Svoboda, 2007), or the enhancement of molecular signalling cascades (Sanes and Lichtman, 1999).

Comparison of spine neck resistance measurements with other studies

The hypothesis that spine necks might influence synaptic input was extended by Rall (1970) who proposed that “...the spine stem resistance could be an important variable which might be used physiologically to change the relative weights of synaptic inputs from different afferent sources”. Although our studies show that the measured range of spine neck resistances does not significantly influence synaptic weight of the spine, it is possible that other spines illustrate greater electrical compartmentalization. By simulating our range of spine and parent dendrite voltage responses we provide evidence that spine neck resistance ranges up to 500 MΩ, with an overall average value of ~200
MΩ. These estimates are similar to those based on anatomical reconstruction (CA1 pyramidal neurons: 0.9 - 411 MΩ; cerebellar Purkinje cells: 2.6 - 80 MΩ; Harris and Stevens, 1989) and also similar to, albeit somewhat higher than, spine neck resistance estimates calculated by dye diffusion (CA1 pyramidal neurons: 4 - 50 MΩ; Svoboda et al., 1996). In contrast, other imaging studies suggest spine neck resistances greater than a giga-Ohm (Bloodgood and Sabatini, 2005, and Araya et al., 2006b). The difference in the spine neck resistances presented in this thesis and those reported in the literature may be due to a number of reasons. Firstly, the experiments presented in this thesis were performed on large stumpy spines and it is possible that further electrical compartmentalization may be achieved in spines with different spine neck geometry (Crick, 1982; Koch and Poggio, 1983). Thin filapodia-like spines, which were inaccessible to voltage imaging due to their small surface area, have greater electrical compartmentalization as reported by Araya et al. (2006b) and consequently may exert greater influence on synaptic strength. This is contested in reconstructions by Koch (1993) which suggest that even small spines necks are not sufficiently restrictive to pose a significant barrier to synaptic currents. Secondly, the imaged spines were located on basal dendrites where most synaptic input occurs in pyramidal neurons (Larkman, 1991) and it is possible that the voltage synaptic response could be different in spines located on different dendrites (e.g., apical, oblique) or in different neurons. Since the spine synaptic input encounters impedance from both the spine neck and dendrite, smaller dendrites would be expected to have higher input resistances and consequently larger spine responses than thicker dendrites.
The purpose of voltage-activated channels in spines

Although many modelling studies simulate the synaptic voltage response in spines with active conductances (Miller et al., 1985; Perkel and Perkel, 1985; Segev and Rall, 1988), our results illustrate that there was not a significant influence of activation of voltage-activated channels (VACs) on the synaptic voltage response in spines. Since spines have been shown to contain VACs (Araya et al., 2007; Bloodgood and Sabatini, 2007; Sabatini and Svoboda, 2000; Yuste and Denk, 1995), if they do not act to boost the synaptic input, then one must question what is their purpose? VACs have been shown to be active during backpropagating APs (bAPs) leading to the suggestion that spines actively aid the invasion of bAPs into the often highly branched dendritic tree (Tsay and Yuste, 2002). Furthermore, spine VACs are activated during other large voltage signals such as during a dendritic spike (Golding et al., 2002; Schiller et al., 2000; Schiller et al., 1997) and may play a critical role in inducing plasticity during these physiological events.

Clinical importance of understanding spine physiology

Alterations in spine physiology and morphology is associated with a number of human diseases. During the course of Alzheimer’s disease, there is a dramatic synapse and dendritic spine loss that correlates with the degree of cognitive impairment (Spires and Hyman, 2004). Reduced dendritic spine density has also been reported in golgi-impregnated cortical neurons from patients with schizophrenia (Garey et al., 1998; Glantz and Lewis, 2000) and a 50% decrease in spine density has been reported in the elderly (Jacobs et al., 1997). Furthermore, spine density and morphological changes have been
reported in Down syndrome (Marin-Padilla, 1976; Suetsugu and Mehraein, 1980) and fragile X syndrome (Comery et al., 1997; Irwin et al., 2001). Due to their prevalent mutations in numerous mental disorders and diseases, understanding spine function during electrical activity is critical and of upmost importance.

THE IMPORTANCE OF ACTION POTENTIAL INITIATION AND PROPAGATION

Why are action potentials initiated in the initial segment?

Since the pioneering work by Eccles (1955) subsequent studies have analysed the site of AP generation illustrating the initial segment as the site of initiation in CA3 neurons (Meeks and Mennerick, 2007), Purkinje cells (Chapter 6 and Khaliq and Raman, 2006, although see Clark et al., 2005) and layer 5 pyramidal neurons in rats (Chapter 4 and Palmer and Stuart, 2006) and ferrets (Shu et al., 2007a). The conservation of the AP initiation site between neurons is somewhat surprising considering the extremely different cell morphologies and axon characteristics. Hippocampal CA3 neurons are unmyelinated (Meeks et al., 2005) and Purkinje cells are known to have a short initial segment (Clark et al., 2005), whereas pyramidal neurons have an initial segment 40 μm in length (Palmer and Stuart, 2006). In addition, Purkinje cells have an extensive dendritic tree that lacks voltage-activated sodium channels (Stuart and Häusser, 1994) and is likely to exert a significantly greater electrical load on the soma than is the case in layer 5 pyramidal neurons (Bekkers and Hausser, 2007). AP initiation in the distal axon initial segment is consistent with theoretical studies, which predict that under conditions of a highly excitable axon initial segment its distal end is the favoured site for AP initiation (Mainen et
Historically, the lower AP threshold in the axon has been attributed to a high density of voltage-activated channels in the axon initial segment. Support for this idea came from early electronmicroscope (EM) work which showed that the axon initial segment has an electron dense undercoating that resembles that observed at nodes of Ranvier (Palay et al., 1968; Sloper and Powell, 1979). More recent studies using sodium channel and associated protein stainings provide evidence for a high density of sodium channels in the axon initial segment of many neuronal types (Boiko et al., 2003; Inda et al., 2006; Jenkins and Bennett, 2001; Kole et al., 2008; Komada and Soriano, 2002; Kordeli et al., 1995; Meeks and Mennerick, 2007; Van Wart et al., 2007; Wollner and Catterall, 1986; Zhou et al., 1998). Although patch recordings contradict these results suggesting that the axon initial segment contains a similar density of sodium channels to the soma (Colbert and Johnston, 1996; Colbert and Pan, 2002), these experiments presumably underestimate the density of sodium channels in the axon initial segment due to an inability to detach sodium channels in the axon initial segment from the cytoskeleton (Kole et al., 2008).

The importance of action potential initiation in the initial segment

Initiation of APs in the axon initial segment rather than more distal axonal sites such as the first nodes of Ranvier would have several advantages. Firstly, initiation in the initial segment would increase the probability of robust AP backpropagation, while at the same time reducing the distance excitatory synaptic inputs need to propagate to influence AP initiation. This may be important for reducing cable filtering of synaptic events, thereby allowing their temporal integration to influence AP initiation. Additionally, the central location
of the axon, which usually emerges from the soma of neurons, is ideally positioned to respond to all synaptic inputs a neuron receives. While the soma could also serve this purpose, its large surface area, as well as its proximity to large dendritic branches, makes it an electrically unfavourable location. This disadvantage could be surmounted if the density of somatic sodium channels was sufficiently high, but due to the large number of sodium channels this would require this is an energetically unfavourable solution. Furthermore, it is important to note that the axon initial segment receives direct inhibitory synaptic input from the GABAergic chandelier cell (Buhl et al., 1994; DeFelipe et al., 1985; reviewed in Howard et al., 2005). These inhibitory synapses are located directly at the site of AP initiation and so are ideally placed to inhibit or modulate the generation of APs. Finally, high densities of voltage-sensitive channels in the axon initial segment may not only serve to initiate APs, but also modulate the resulting AP waveform. Recent studies by Kole et al (2007) and Shu et al (2007b) illustrate that the axon has a high density of potassium channels (specifically Kv1), which act to modulate axonal AP width. Inactivation of axonal Kv1 channels, for example during subthreshold depolarisation, leads to AP broadening and an increase in synaptic strength (Kole et al., 2007).

**Action potential propagation along the axon**

An extensive axon arboration enables the synchronous excitation of synaptically connected neurons. Therefore, rapid propagation of APs along the axon is critical in shaping the spread of excitation within networks and invoking postsynaptic plasticity (for a review see Dan and Poo, 2004). Since saltatory conduction increases the speed of AP propagation (Brill et al., 1977), this mode
of axonal propagation demonstrated in Chapter 5 would facilitate synchronous firing between connected neurons. However, to invoke local network signalling, the AP must not only successfully propagate along the main axon branch, but it must also invade branch points. Although branch points have been shown theoretically to act as sites of AP failure (Goldfinger, 2000; Zhou and Chiu, 2001), bursts of APs up to 300Hz were found to successfully invade branch points in layer 5 pyramidal neurons. However, these results do not indicate the successful transfer of information within local cortical networks as failure may subsequently occur during collateral propagation or at the site of synaptic transmission. Failure of high frequency APs to successfully propagate along axons (as seen in Purkinje cells by Khaliq and Raman, 2005, and Monsivais et al., 2005) is not necessarily unfavourable as it may provide a mechanism for preventing hyperexcitability such as during epileptic seizures (Prince and Connors, 1986; Topolnik et al., 2003).

VOLTAGE-SENSITIVE DYE IMAGING

Limitations of the voltage sensitive dye imaging technique

The strength of voltage imaging lies in its ability to measure fast electrical signals at high spatial resolution. Unlike the commonly used calcium-sensitive dyes, the response of voltage-sensitive dyes is not limited by the slow response of the bound chemical to changes in voltage. Despite this advantage, voltage imaging can be problematic and hence is generally not the preferred technique to measure electrical events. Notably, voltage signals are extremely fast and proportionally fast fluorescence detection methods are therefore required. There is a trade off between optimising temporal resolution and spatial
resolution, as imaging at high temporal resolution (e.g., 10 kHz) means less photons are captured per image. Increased spatial resolution during fast sampling was achieved in our studies by performing most experiments at room temperature. The effects of lower temperatures have been shown to increase input resistance, produce larger and wider APs and increase excitability in CA1 neurons (Thompson et al., 1985) and neocortical pyramidal neurons (Volgushev et al., 2000). Although decreasing temperature did not affect the overall conclusions in Chapters 4 and 5 as AP initiation still occurred in the initial segment and successfully propagated along the axon during physiological temperatures, the effects of temperature may have greater effect on spine voltage due to a slowing of the channel kinetics involved in the response (Chapter 6).

Additionally, voltage-sensitive dyes are lipophilic and embed in the neuronal membrane (Loew, 1996) which requires long wholecell patch recordings during filling. Using the described methods, neuron health was not severely compromised by the internal application of voltage-sensitive dyes (see Chapter 2). The main limitation during voltage imaging was the small signal to noise ratio of voltage-sensitive dyes. The experiments in this thesis investigated electrical events in submicron neuronal structures which compound the already small voltage fluorescence changes. To improve the signal, over a hundred events were individually aligned and averaged for each experiment which masked any biological inconsistencies between trials. Therefore, unfortunately it was not possible to detect trial-to-trial variability in the synaptic response or AP initiation.
The future of voltage imaging

With the use of voltage imaging, previously inaccessible questions about processing in fine neuronal structures were addressed in this thesis. With the development of more sensitive voltage sensitive dyes and improvements in detection technology, further increases in both the sensitivity and spatial resolution of voltage imaging in single neurons is possible. The use of genetically coded voltage sensors (Ataka and Pieribone, 2002; Chanda et al., 2005; Guerrero et al., 2002; Sakai et al., 2001; Siegel and Isacoff, 1997) or improvements in the excitation and detection of voltage dyes by laser confocal microscopy (Bullen et al., 1997; Bullen and Saggau, 1999), or a variant of two-photon microscopy using second-harmonic generation (Araya et al., 2006b; Dombeck et al., 2004; Dombeck et al., 2005; Millard et al., 2004; Millard et al., 2005; Nuriya et al., 2006) could offer improved resolution to explore the electrical events underlying single neuronal computation.

CONCLUSIONS AND FUTURE DIRECTIONS

There are two main results presented in this thesis. Firstly, dendritic spines electrically compartmentalize synaptic input but do not function to control synaptic strength (Chapter 3). Secondly, action potentials are initiated in the distal end of the initial segment in layer 5 pyramidal neurons (Chapter 4) and Purkinje cells (Chapter 6) and propagate faithfully along the axon in layer 5 pyramidal neurons (Chapter 5). These findings highlight the complexity of the different voltage signals used by neurons to communicate and promote further questions to be asked.
The successful invasion of AP bursts into branch points (Chapter 5) beckons the question whether the high frequency APs continue to propagate along collateral branches and invade the axon terminal. Failure of a burst to transmit to postsynaptic targets could be due to either failure of AP propagation along the collateral branch or failure at the presynaptic terminal. Potential future experiments could use voltage imaging to record the propagation of a burst of APs along collateral branches, therefore either promoting or dismissing failure of high frequency AP propagation along the collateral as the cause of frequency-dependent depression in the postsynaptic response. Future experiments addressing the function of spines could investigate the voltage changes in a single spine in response to the induction of synaptic plasticity. Previous experiments have analysed synaptic plasticity via calcium responses in the spine or dendrite (Bloodgood and Sabatini, 2005; Magee and Johnston, 1997; Nevian and Sakmann, 2006) or using somatic and dendritic electrical recordings (Debanne et al., 1998; Letzkus et al., 2007; Markram et al., 1997). These techniques are limited as calcium imaging may not reflect the actual voltage response as other ions (for example sodium) are also involved in synaptic signalling. Furthermore, electrical recordings from non-synaptic sites may not capture the direct response of the potentiated spine. Measuring the voltage directly at the site of plasticity is of critical importance for understanding learning and memory. Additionally, further experiments could address the voltage response in spines during dendritic spikes, to test whether these physiological processes activate VACs in the spine and modify synaptic strength.
In summary, it is clear that spines are more than simple passive compartments. Although they do not influence synaptic strength, the spine neck resistance provides slight electrical compartmentalization with measurable voltage attenuation from the spine to the dendrite. The synaptic response then propagates along the dendrite and integrates with other synaptic inputs to ultimately reach threshold for AP generation. Despite different AP waveforms and neuron morphology, APs are initiated in the distal end of the initial segment in all APs investigated (layer 5 pyramidal neuron AP, the Purkinje cell simple spike and complex spike). Once initiated, the AP propagates faithfully along the main axon and into branch points. Overall, this thesis directly demonstrates Cajal's 'law of dynamic polarization' by investigating the synaptic input and the action potential output of neurons.
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