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Dendritic Properties of Turtle Pyramidal Neurons

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Abstract

The six-layered mammalian neocortex evolved from the three-layered paleocortex, which is retained in present-day reptiles such as the turtle. Thus the turtle offers an opportunity to examine which cellular and circuit properties are fundamental to cortical function. We characterized the dendritic properties of pyramidal neurons in different cortical regions of mature turtles, *Pseudemys scripta elegans*, using whole cell recordings and calcium imaging from the axon, soma, and dendrites in a slice preparation. The firing properties, in response to intrasomatic depolarization, resembled those previously recorded with sharp electrodes in this preparation. Somatic spikes led to active backpropagating high-amplitude dendritic action potentials and intracellular calcium ion concentration ($[Ca^{2+}]_i$) changes at all dendritic locations, suggesting that both backpropagation and dendritic voltage-gated Ca^{2+} channels are primitive traits. We found no indication that Ca^{2+} spikes could be evoked in the dendrites, but fast Na^+ spikes could be initiated there following intradendritic stimulation. Several lines of evidence indicate that fast, smaller-amplitude somatic spikes (“prepotentials”) that are easily recorded in this preparation are generated in the axon. Most synaptically activated $[Ca^{2+}]_i$ changes resulted from Ca^{2+} entry through voltage-gated channels. In some cells synaptic stimulation evoked a delayed Ca^{2+} wave due to release from internal stores following activation of metabotropic glutamate receptors. With some small differences these properties resemble those of pyramidal neurons in mammalian species. We conclude that spike backpropagation, dendritic Ca^{2+} channels, and synaptically activated Ca^{2+} release are primitive and conserved features of cortical pyramidal cells, and therefore likely fundamental to cortical function.

INTRODUCTION

The turtle cortex, or “pallium,” is an evolutionarily primitive three-layered cortical form (Northcutt 1981). It represents one of the most phylogenetically conserved examples available among present-day vertebrates (Bar et al. 2000) and therefore presents a logical foundation for studying the more elaborate six-layered cortex (Connors and Kriegstein 1986). In mammals it survives in both the piriform cortex and the hippocampal formation, often termed “archicortex” or “paleocortex.” In turtles (and other reptiles) the three-layered pallium processes not only olfactory but also other sensory information relayed via the thalamus (Desan 1984; Hall and Ebner 1970; Mulligan and Ulinski 1990). There is a broad range of evidence establishing that the more elaborate six-layered neocortex was derived from the pallium (e.g., Mulligan and Ulinski 1990; Reiner 2000). In particular, there is

evidence that projections onto the dendrites of turtle pyramidal neurons from other cortical and subcortical regions are more laminar and organized than in mammalian pyramidal neurons (Desan 1984; Hall and Ebner 1970; Mulligan and Ulinski 1990; Smith et al. 1980).

There are several clear differences between turtle and mammalian pyramidal neurons that raise questions about the universality of the conclusions that have accumulated from studies of mammalian preparations. Unlike mammalian cortical pyramidal neurons, turtle pyramids have multiple dendrites radiating in the pial direction from the soma (Fig. 1, *C* and *D*). In mammalian pyramids, the thick apical trunk has a lower spine density (and therefore little excitatory innervation), whereas in turtle pyramids, for which there is a notable absence of a thick trunk dendrite, spines are widely distributed over all the radiating branches. Furthermore, because the turtle is cold-blooded and mammals are warm-blooded, it is possible that channel properties and electrophysiological characteristics of dendrites have developed differently to accommodate their different environmental niches. It is also interesting to compare the dendritic properties of rats and turtles from the evolutionary perspective of species that diverged about 300 million years ago (Northcutt 1981).

In pioneering studies Connors and Kriegstein (1986) developed the turtle slice preparation and examined the anatomical and electrophysiological features of pyramidal neurons from the fresh water turtle *Pseudemys scripta elegans*. Their studies, which primarily used blind sharp electrode recordings from the cell body and horseradish peroxidase intracellular staining, showed that these pyramidal neurons have many electrophysiological and synaptic features in common with rat pyramidal neurons. Unfortunately, there has not been much serious study of these cells since their experiments.

In the time between those studies and the present, the emerging fields of dendritic patch recordings and calcium imaging have produced a huge store of knowledge regarding dendritic properties of neocortical pyramidal neurons. Among the most important advances since 1986 is the discovery that the apical dendrite of neocortical and hippocampal pyramidal neurons have voltage-dependent sodium channels (Spruston et al. 1995; Stuart and Sakmann 1994; Waters et al. 2003) and calcium channels (Magee and Johnston 1995; Schiller et al. 1997; Waters et al. 2003). Inositol 1,4,5-trisphosphate (IP₃)–mediated release of calcium from intracellular stores leads to propagating waves of Ca²⁺ in the dendrites of neocortical and hippocampal pyramidal neurons (Larkum et al. 2003; Nakamura et al. 1999). There are many suggestions as to the functional significance of these dendritic properties for cortical information processing (for reviews see London and Häusser 2005; Rhodes 1999) but there is still debate as to which dendritic properties are fundamental (Häusser and Mel 2003). From this perspective it is interesting to examine which properties are conserved in cortices throughout evolution (Bar et al. 2000).

In our new experiments we reexamined turtle pyramidal neurons using modern techniques of whole cell recording from visually identified neurons, axonal and dendritic patching including some dual recordings, and wide-field fluorescence measurements of intradendritic calcium changes. We confirmed and made more quantitative many of the conclusions of the Connors and Kriegstein papers. In addition, we found that most sodium-dependent action potentials (APs) initiate near the soma and backpropagate over the dendrites, that some sodium APs initiate in the dendrites, that prepotentials originate in the axon, that voltage-dependent calcium channels are found all over the dendrites, and that synaptically activated calcium waves can be generated in the dendrites. Although quantitative aspects of these conclusions differ in small ways from those derived from mammalian pyramids, the main observations are similar to those described in murine and rat cells. Some of these results have been reported previously in abstract form (Watanabe et al. 2004).

METHODS

Experiments were performed on cortical pyramidal neurons of mature turtles (shells 20–30 cm long), *Pseudemys scripta elegans*, obtained from Delta Biological (Vidalia, LA). Turtles were anesthetized by submersion in ice-cold water and decapitated using procedures approved by the Institutional Animal Care and Use Committee of the Marine Biological Laboratory (Woods Hole, MA). Coronal slices, 300 μm thick, were prepared by dissecting out the cortical region (Fig. 1, A and B) and embedding it in low-melting-point agarose. The block was cooled and solidified with Freez-It AntiStat spray (ITW Chemtronics), glued to the bottom of a cutting chamber, and slices were cut on a Vibratome. Recordings were made from slices submerged in a glass-bottom chamber. The chamber was superfused with oxygenated artificial cerebrospinal fluid (ACSF) at room temperature. The composition of the ACSF was (in mM): NaCl, 96.5; KCl, 2.6; CaCl_2 , 4.0; MgCl_2 , 2.0; NaHCO_3 , 31.5; and dextrose, 10; pH was 7.4 when bubbled with 95% O_2 –5% CO_2 (Connors and Kriegstein 1986).

The chamber was mounted on a stage rigidly bolted to an air table and slices were viewed with a $\times 40$ or $\times 60$ water-immersion lens (Olympus, Melville, NY) in an Olympus BX50WI microscope mounted on an X–Y translation stage. Somatic, axonal, and dendritic whole cell recordings were made using Dagan BVC-700 amplifiers and patch pipettes were pulled from 1.5-mm OD thick-walled borosilicate glass tubing (1511-M, Friedrich & Dimmock, Millville, NJ). Tight seals were made with the “blow and seal” technique using video-enhanced differential interference contrast optics to visualize the cells (Larkum et al. 2001; Sakmann and Stuart 1995). For most experiments the pipette solution contained (in mM): K-gluconate, 140; NaCl, 4; Mg-ATP, 4; Na-GTP, 0.3; HEPES, 10; and phosphocreatine, 14; pH adjusted to 7.2–7.4 with KOH (Blanton et al. 1989). This solution was supplemented with 150–200 μM bisfura-2 or 500 μM furaptra (Molecular Probes, Eugene, OR) for calcium imaging. Some electrodes were additionally supplemented with 0.2% biocytin. After recordings, slices were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer. Tissue sections were processed with the avidin-biotin-peroxidase method to reveal cell morphology. Synaptic stimulation was evoked with 100- μs pulses using a bipolar tungsten electrode that had one sharpened tip (model TM33B01KT; WPI, Sarasota, FL) about 1 mm in front of the other. Electrical traces were digitized with 16-bit resolution at intervals of 0.1 ms using an ITC-18 Computer Interface (Instrutech) under the control of custom software written in IgorPro (WaveMetrics). Data were analyzed with the same suite of programs.

APV [($\pm$)-2-amino-5-phosphonopentanoic acid], CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), MCPG [(*R,S*)- α -methyl-4-carboxy-phenylglycine], and *trans*-ACPD (1-aminocyclopentane-*trans*-1,3-di-carboxylic acid) were obtained from Sigma-RBI (St. Louis, MO). All other chemicals were obtained from Fisher Scientific (Piscataway, NJ).

Time-dependent intracellular calcium ion concentration ($[\text{Ca}^{2+}]_i$) measurements from different regions of the pyramidal neuron were made as previously described (Larkum et al. 2003; Lasser-Ross et al. 1991). Briefly, changes in Ca^{2+} indicator fluorescence were detected with a Photometrics (Tucson, AZ) Quantix 57 cooled charge-coupled detector camera, operated in the frame-transfer mode. Custom software controlled readout parameters and synchronization with electrical recordings. A second custom program was used to analyze the data. Typical readout rates were 30 frames s^{-1} . Fluorescence changes of bis-fura-2 and furaptra were measured with single-wavelength excitation (382 ± 10 nm) and emission >455 nm. $[\text{Ca}^{2+}]_i$ changes are expressed as $-\Delta F/F$, where F is the fluorescence intensity when the cell is at rest and ΔF is the change in fluorescence during activity. Corrections were made for indicator bleaching during trials by subtracting the signal

measured under the same conditions when the cell was not stimulated. To measure the resting fluorescence (F) accurately we subtracted the background fluorescence of the slice from the cell image. This background level was estimated by measuring the fluorescence of an equivalent position in the slice that contained no indicator-injected neurons.

RESULTS

Somatic recordings

We made whole cell recordings from the somata of >150 pyramidal neurons. These cells were identified by their position in the cortex (mostly between regions D1 and D2, Fig. 1B) and their firing pattern in response to sustained depolarization (Connors and Kriegstein 1986). This identification was confirmed in a subset of these cells by observing the pattern of dendritic arborization following the development of the biocytin stain (Fig. 1C). In response to sustained depolarization the cells first fired single spikes at the start of the pulse and, then with increasing current, fired a train of spikes that accommodated during the step. The time constant could be estimated from these recordings but signal averaging was often required because of the large amount of spontaneous synaptic activity. We measured membrane properties for a subset of these cells: spike height, 93 ± 12 mV ($n = 43$); spike width, 2.2 ± 0.5 ms ($n = 51$); membrane time constant (τ_m), 82 ± 30 ms ($n = 23$); and input resistance (R_N), 270 ± 160 M Ω ($n = 11$). These values differed from those of Connors and Kriegstein (1986), probably because of the whole cell recording conditions (e.g., Li et al. 2004; Major et al. 1994; Staley et al. 1992). The large variation in input resistance may reflect the variety of different neuron morphologies found in the turtle cortex (Desan 1984).

Dendritic recordings

Because the dendrites of turtle pyramidal neurons arborize widely in all directions it was harder to see and follow these processes than it was for rat pyramidal neurons, which have one main apical dendrite that invariably runs perpendicular to the cortical surface. Nevertheless, we made 29 dendritic whole cell recordings; most of these were from locations >200 μ m from the soma. In five cells we successfully made dual recordings with one electrode in the soma and the second in the dendrites. For these cells we could accurately estimate the distance of the dendritic electrode from the soma. For the isolated dendritic recordings we could sometimes determine the exact distance from the soma by using the fluorescence that accumulated after some time in the cell body. In most cases, however, the intradendritically injected fluorescent indicator did not spread all the way to the soma. In these cases, we made an estimate based on the probable location of the cell body, which usually lies within an approximately 50- μ m band of cells reminiscent of the mammalian hippocampus (thick blue line, Fig. 1B).

An important issue for cortical pyramidal neurons is whether spikes initiate in the soma or dendrites and whether they actively backpropagate over the dendrites. For all cells with dual recordings we found that following moderate depolarization to either the soma or dendrites the spike was first recorded at the somatic electrode (Fig. 2A, *inset*, and 2C), indicating spike initiation in that region (Stuart and Sakmann 1994). To determine whether the spikes actively backpropagated over the dendrites we plotted the peak amplitude from resting potential as a function of the distance from the soma (Fig. 2D). For each cell we selected trials at close-to-threshold depolarization to minimize errors due to bridge imbalance and/or the contribution of the depolarizing potential, although measurements in trials with stronger pulses indicated that spike amplitude was relatively insensitive to this parameter. Cells with either single dendritic electrodes or dual recordings are included in the plots. Although there is greater scatter in the points than that in plots from most mammalian cortical preparations it is clear that the spike amplitude decrements with distance, but is still substantial at

locations $>350\ \mu\text{m}$ from the soma. Again, this result is similar to that in mammalian cortical neurons (Stuart and Sakmann 1994). Part of the variability can probably be ascribed to inaccuracies in estimating the distance to the soma.

This pattern of slow amplitude decrement with distance closely resembles the pattern in mammalian cortical neurons where active backpropagation has been clearly established. However, it is possible that the pattern in turtle pyramids could reflect passive spread, especially since the spikes are slower than those in warm-blooded species and therefore experience less capacitative filtering. There are two arguments against purely passive backpropagation. First, in several cells where we made dual somatic and dendritic recordings we found that successive spikes in a train increased in amplitude and became narrower at the dendritic site, whereas the amplitude at the somatic site decreased or stayed the same (Fig. 2C). This pattern is impossible with passive dendrites. Second, quantitative simulations using the NEURON modeling environment (Hines 1998) suggest that the profile of measured dendritic amplitudes does not match the passive profile. To set up this simulation we used dendritic morphology reconstructed from a biocytin-filled pyramidal neuron and inserted the waveform of an electrically recorded AP into the somatic compartment (see Supplemental Fig. S11 for more details). No active channels were placed anywhere in the cell and the specific membrane capacitance (C_m) and resistance (R_m) were estimated from the time constant and input impedance determined earlier. We calculated the amplitude decrement using several different assumptions for the intracellular resistivity (R_i) based on the measurements of Stuart and Spruston (1998) for rat neocortical pyramidal neurons. These are conservative assumptions since no fast activating K conductances were included, which might be expected to further reduce spike amplitude in the dendrites. Representative curves are plotted in Fig. 2D. There are two main differences between the measured and modeled spike amplitudes. First, the modeled amplitude drops more sharply in the proximal dendrites compared with the drop in the distal dendrites. This drop results from the greater capacitative filtering in the proximal region where the spike rise time is faster. In contrast, the measured decrement is more gradual, consistent with the pattern in mammalian cortical neurons (e.g., Stuart and Sakmann 1994). Second, the amplitude of the modeled spikes in the distal dendrites was much lower than the measured amplitudes in that region. Together with the reversal in dendritic amplitudes noted earlier, this strongly suggests that spike backpropagation in turtle pyramids is active over a significant part of the dendrites. Since there was some variation in propagation in our results, it seems possible that among the variety of pyramidal neurons found in the turtle cortex (Desan 1984) there are some with less active dendrites.

Dendritic spike initiation

In both neocortical and hippocampal pyramidal neurons from the rat, brief, intense intradendritic stimulation can initiate Na^+ -dependent APs in the dendrites (Gasparini et al. 2004; Golding and Spruston 1998; Larkum et al. 2001) even though modest synaptic or dendritic stimulation usually initiates spikes in the axosomatic region (Richardson et al. 1987; Spruston et al. 1995; Stuart and Sakmann 1994). We tested whether similar dendritic spikes could be evoked in turtle dendrites. Figure 3A shows an experiment where an excitatory postsynaptic potential (EPSP)-like brief current injection in a dendrite at a point $250\ \mu\text{m}$ from the soma evoked an all-or-none response when the peak current reached $450\ \text{pA}$. Figure 3A3 shows the peak voltage response as a function of the injection current, clearly revealing the threshold for this event. Similar responses were detected in experiments in 11 of 17 tested cells. These recordings and plots closely resemble those made from dendrites of hippocampal pyramidal neurons (Gasparini et al. 2004) where dual somatic and

¹The online version of this article contains supplemental data.

dendritic recordings clearly established that the spikes initiated in the dendrites. The few dual recordings we made did not reveal such events. However, related observations strongly argue that under some circumstances local spikes were initiated in the dendrites. In the dual-electrode recordings where EPSP-like dendritic current injection failed to initiate a dendritic spike, the backpropagating AP came 30 – 40 ms after the start of current injection (Fig. 3C). However, spikes resembling local Na⁺ spikes were typically detected <5 ms after the start of current injection (Fig. 3A1). Moreover, in several dendritic recordings where an early spike could be detected (Fig. 3B), a second spike could be evoked that initiated about 30 – 40 ms after the first action potential resembling the backpropagating AP. These later spikes rose abruptly from the falling phase of the driving potential and had about the same amplitude as that of the earlier spikes. If this is the typical delay for a dendritic subthreshold potential to reach the soma and initiate a spike it is unlikely that the spikes initiated with a delay of only a few milliseconds (Fig. 3A1 and others) could be evoked in the soma by electrotonically spreading dendritic potentials.

In several cells ($n = 5$) we tried to evoke dendritic Ca²⁺ spikes using either sustained intradendritic depolarization or trains of high-frequency backpropagating APs. Both of these protocols successfully evoked Ca²⁺ spikes in rat hippocampal and neocortical pyramidal neurons (Golding et al. 1999; Larkum et al. 1999a; Tsubokawa and Ross 1997; Williams and Stuart 2000). In our experiments Ca²⁺ spikes were never evoked even though the dendrites have Ca²⁺ channels all along their length (see following text) and it was known that Ca²⁺ spikes could be evoked if K⁺ channels are blocked (Connors and Kriegstein 1986). Although there are many possible explanations for the failure to generate dendritic Ca²⁺ spikes (including differences in distribution and types of dendritic Ca²⁺ channels), it should be noted that the dendritic morphology of turtle pyramids lacks a vital aspect for the generation of Ca²⁺ spikes compared with mammalian cells. In mammalian neurons the tuft dendrites converge on a dendritic location (the major apical branch point), representing a smaller current sink than that of the cell body (Rhodes and Gray 1994; Rhodes and Llinás 2001).

Prepotentials

Connors and Kriegstein (1986) found that many somatically recorded APs had shoulders on their rising phase. They also found smaller spikes, which they called “prepotentials,” analogous to the spikes recorded in mammalian pyramidal neurons in vivo (Spencer and Kandel 1961). We saw similar potentials in whole cell recordings. Based on spike shape and the shape of the potential derivative (dV/dt) we divided the spikes into four categories (Supplemental Fig. 2). Isolated prepotentials were a small but significant fraction of these events. Connors and Kriegstein (1986) suggested a dendritic origin for these events, although they did not completely rule out an axonal origin (see DISCUSSION).

Several lines of evidence using dual soma-dendritic recordings, dual soma-axon recordings, and calcium imaging suggest that the prepotentials are axonal spikes that fail to invade the soma and are not dendritic spikes. First, in all examples where we had a dual soma-dendritic recording and where we recorded prepotentials in the soma (e.g., Figs. 2C and 4, E and F) the corresponding dendritic potential was slower and smaller than the somatic potential. Second, in all cases where we imaged [Ca²⁺]_i changes in the dendrites corresponding to large-amplitude somatic spikes (see following text) we did not see a [Ca²⁺]_i change in any dendrite corresponding to a prepotential caused by a local dendritic spike (data not shown). It is possible that spikes initiated in dendrites from which we did not record or image, but the lack of any examples argues against an active dendritic event. We estimated the likelihood of our missing a local dendritic spike from our measurements of dendritic branching patterns in the biocytin fills and the number of dendritic recordings. We found an average of 7.2 ± 3.4 dendritic branches in 17 cells and we made 30 dendritic recordings

without observing a local spike. Therefore the probability that we always missed the right dendrite is approximately $0.01 \approx \{[1 - (1/7.2)]^{30}\}$, assuming all neurons are similar. Third, these prepotentials were the remnants of local spikes and not EPSPs because spontaneous EPSPs (e.g., Fig. 4G), even those with the fastest rise times at the soma, were slower and smaller than the prepotentials or the shoulder potentials (Fig. 4, *H* and *I*).

More significantly, in three cells we recorded APs and prepotentials with electrodes on both the axon and soma (Fig. 4, *A–C*). In each of these cases, whether we looked at isolated prepotentials or shoulders on somatically recorded spikes, we found that the corresponding potential on the axon had a faster rise time and larger amplitude than those of the potential at the somatic electrode. This comparison shows that the direction of propagation was from the axon to the soma; the events did not originate in the dendrites. This result strongly suggests that the prepotentials are axonal events that do not successfully fire the soma; spikes with shoulders are axonal events that do invade the soma.

Calcium imaging of electrical activity

Following intrasomatically evoked APs, transient changes in bis-fura-2 fluorescence were detected at all locations where the dendrites were in focus (Fig. 5A; $n > 30$). In some cells calcium transients were also detected from the distal dendrites (some $>350 \mu\text{m}$ from the soma) when these processes were imaged and spikes were evoked with intradendritic pipettes in that region (Fig. 5B). There were no detectable fluorescence changes following subthreshold depolarizations. Since Ca^{2+} does not diffuse far from the site of entry in the membrane these observations indicate that voltage-dependent calcium channels are widely distributed in the dendrites of turtle pyramidal neurons and that there is little Ca^{2+} entry through low-threshold channels. These conclusions are similar to those made about hippocampal (e.g., Nakamura et al. 2002) and neocortical (e.g., Larkum et al. 2003) pyramidal neurons of the rat. In three cells we used the low-affinity indicator fura-2 to estimate the recovery time of a spike-evoked transient (Fig. 5C). The time constant was about 100 ms near the soma and slightly faster in the dendrites, similar to values determined in mammalian hippocampal and neocortical pyramidal neurons (Helmchen et al. 1996), a surprising result since these measurements were made at room temperature and membrane pumps are generally temperature sensitive.

Synaptically activated calcium changes and calcium waves

We looked for postsynaptic $[\text{Ca}^{2+}]_i$ changes following synaptic activation. Stimulation was evoked with the tungsten electrode positioned in the dendritic region with the tip usually within $40 \mu\text{m}$ of the dendrites. In some experiments we used one to five stimuli at 100 Hz to generate a postsynaptic response. If the summing EPSPs were large enough to evoke an AP, a brief, widespread transient was detected similar to the transients when spikes were evoked intrasomatically. When the electrical response was subthreshold, little or no $[\text{Ca}^{2+}]_i$ increase could be detected in the dendrites (data not shown). Similarly, no $[\text{Ca}^{2+}]_i$ changes were detected corresponding to spontaneous EPSPs (Fig. 4G). For many experiments we extended the stimulation protocol to give 50 pulses at 100 Hz. In this case, we could usually detect a local $[\text{Ca}^{2+}]_i$ increase in the dendrites near the stimulation electrode. This increase was synchronous with the stimulation train (Fig. 6). To examine the source of this increase we did a pharmacological profile ($n = 2$; Fig. 6B). Application of $100 \mu\text{M}$ APV and/or 1 mM MCPG had no detectable effect on the Ca^{2+} signal in the dendrites. [The slow increase with time during this application (Fig. 6B) probably results from additional indicator loading or possibly a slight tissue movement.] Therefore this synchronous increase does not result from entry through *N*-methyl-D-aspartate-receptor channels or activation of metabotropic glutamate receptors (mGluRs). Most of the increase could be blocked with $10 \mu\text{M}$ CNQX, suggesting that it was due to entry through voltage-gated Ca^{2+} channels opened by the

synaptic potentials, which were also reduced by CNQX. The addition of a bolus of tetrodotoxin ($\sim 1 \mu\text{M}$ final concentration) completely blocked the synaptic potential but left a small residual postsynaptic $[\text{Ca}^{2+}]_i$ increase. The origin of this residual increase was not examined but may have resulted from direct stimulation of the dendrite.

In ten cells we found a $[\text{Ca}^{2+}]_i$ increase that was not synchronous with the synaptic train (Fig. 7). In fact, the increases occurred at different times in different dendritic locations. Plotting the changes using a “linescan” display (Nakamura et al. 2000) showed that these $[\text{Ca}^{2+}]_i$ changes spread as a wave in the dendrites from an initiation point close to the stimulation electrode. These waves resembled those detected in hippocampal and neocortical pyramidal neurons following similar stimulation protocols (Larkum et al. 2003; Nakamura et al. 2002; Power and Sah 2002). In those experiments we established that the waves were due to Ca^{2+} release from internal stores following the mobilization of IP_3 by activation of mGluRs. These turtle waves probably resulted from the same mechanism since they could be blocked by the mGluR antagonist MCPG (1 mM; $n = 3$ with reversal following washout in one cell). However, there were some quantitative differences between the waves in turtle pyramids and in hippocampal pyramids. The amplitude of the $[\text{Ca}^{2+}]_i$ increase during hippocampal waves usually reached several micromoles, much higher than the changes evoked by a burst of APs (Nakamura et al. 1999). The peak wave amplitude in turtle cells was usually of a magnitude comparable to the changes evoked by a burst of spikes. The waves in turtle pyramidal neurons were usually confined to a length of $\lesssim 50 \mu\text{m}$ in the dendrites, whereas the waves could extend over a greater length (but not all of the dendrites) in hippocampal cells. A third difference was that the waves in hippocampal neurons were found in the aspiny primary apical dendritic shaft, whereas the waves in turtle pyramidal neurons occurred in spiny regions (of necessity since all the dendritic regions had spines in these cells).

In hippocampal pyramidal neurons we could evoke large-amplitude dendritic Ca^{2+} waves by adding $20 \mu\text{M}$ *trans*-ACPD or $3 \mu\text{M}$ carbachol (CCh) to the bath and then intrasomatically evoking several spikes (Nakamura et al. 2000). We performed similar experiments on turtle pyramids. When the slices were superfused with $30 \mu\text{M}$ *trans*-ACPD we could barely detect a change in the response to a train of backpropagating APs. When the concentration was increased to $100 \mu\text{M}$ a clear change was detected (Fig. 8), although it was still much smaller than the change in similar experiments on rat hippocampal pyramidal neurons (Nakamura et al. 2000). Similarly, we detected no response to $50 \mu\text{M}$ CCh on turtle pyramids, >15 -fold the effective concentration in hippocampal experiments. To control for the possibility that our reagents were ineffective, we tested the same compounds on rat hippocampal experiments on the same day as the turtle experiments. In these experiments large Ca^{2+} waves were evoked by backpropagating spikes, similar to those we previously reported ($n = 2$; Nakamura et al. 2000). Therefore turtle pyramidal neurons are less sensitive to metabotropic agonists than hippocampal pyramids.

DISCUSSION

The main conclusion of this study is that the basic electrophysiological properties of turtle pyramidal neuron dendrites are similar to those of neocortical pyramidal neurons from murine and rat preparations. Importantly, these include active, decremental Na^+ -channel-dependent spike backpropagation accompanied with Ca^{2+} influx, somatic spike initiation following weak dendritic stimulation, and dendritic spike initiation following strong dendritic stimulation. These properties were observed in acute slices recorded at room temperature. It is possible that measurements in vivo or in semi-intact preparations might be slightly different but we did not test these conditions.

Most forms of calcium signaling were similar to signaling in neocortical cells. Voltage-gated Ca^{2+} entry was detected everywhere APs propagated and delayed calcium release waves could be evoked following synaptic stimulation. Local, rapid, synaptically activated $[\text{Ca}^{2+}]_i$ changes were primarily through voltage-gated channels, although we did not have the spatial resolution to examine changes in isolated spines. This contrasts with results on hippocampal pyramidal neurons (e.g., Nakamura et al. 2002) where APV clearly reduced the synaptically activated $[\text{Ca}^{2+}]_i$ increase in the oblique dendrites. The difference may result from the lack of oblique dendrites in turtle pyramidal neurons, although spines are prominent on the main dendrites of these cells (Kriegstein and Connors 1986).

A notable difference is that turtle pyramidal neurons lack broad Ca^{2+} spikes in the distal regions of their dendrites, which suggests that this is a more recent specialization of mammalian neocortical and hippocampal pyramidal neurons. This property corresponds to the apical tuft branches of neo-cortical pyramidal neurons (i.e., the arborization extending from the main bifurcation), where Ca^{2+} spikes also fail to initiate due to local current injection (Larkum and Zhu 2002; Rhodes and Llinás 2001). The most obvious consequence of the lack of dendritic Ca^{2+} spikes in reptilian pyramidal neurons is that dendritic input does not switch the somatic firing pattern from regularly spiking to bursting as in the neocortex (Larkum and Zhu 2002). It has been suggested that the Ca^{2+} spike in neocortical pyramidal neurons serves to associate feedback inputs arriving at the tuft with feedforward inputs in the basal regions (Larkum et al. 1999b, 2007), which clearly is not possible in the turtle cortex. Both these similarities and differences have implications for understanding and accurately modeling the behavior of the three-layered cortical network (Nenadic et al. 2002).

Prepotentials

Fast prepotentials have been seen in other preparations and have often been interpreted as dendritically initiated spikes that do not propagate fully to the soma. Spencer and Kandel (1961) famously made this interpretation about prepotentials recorded in vivo in the hippocampus of anesthetized cats. Their main argument against an axonal origin for these potentials was that they were not seen when the cells were stimulated antidromically, unlike in the earlier work on motoneurons (Coombs et al. 1957). Similar events have since been seen with somatic recordings in CA1 pyramidal neurons (Ariav et al. 2003), neocortical pyramidal neurons (Crochet et al. 2004; Milojkovic et al. 2005; Nevian et al. 2007), and under certain conditions in mitral cells of the olfactory bulb (Chen et al. 2002). In some of these studies, whole cell patch recordings at the soma and either dendritic stimulation or/and dendritic recordings were used to show a dendritic origin (however, see Schmitz et al. 2001 for evidence of axonal initiation).

Connors and Kriegstein (1986) reported fast prepotentials in turtle pyramids using sharp electrode recordings and stimulation at the soma. They found that these events had a lower threshold than that of the large-amplitude spikes recorded in the soma, suggesting that they were different events. Since they could be initiated by distal synaptic stimulation they suggested that they probably had a dendritic origin, but they did not completely rule out axonal initiation. Collision experiments with antidromic spikes suggested that the prepotentials could orthodromically invade the axon, although there was some ambiguity in these experiments. Our experiments clearly showed that these events originated in the axon.

Why are prepotentials more prevalent in turtle pyramidal neurons than in mammalian cells? One possibility is that the lower temperature and channel properties make spike initiation and somatic invasion more problematic. We have no direct evidence relevant to this explanation. Another possibility is that the highly branched dendritic structure of turtle pyramids makes it more difficult for spikes to invade the soma because the branches contribute to the somatic load. This is similar to the argument of Vetter et al. (2001) who

found that the failure of spikes to invade Purkinje cell dendrites (although they do invade the soma) could be explained entirely by the highly branched dendritic morphology of these cells. The difference, of course, is that once spikes in turtle pyramidal neurons make it past the soma they then backpropagate over the entire dendritic tree as found in the anatomically distinct mammalian pyramids.

Conclusions

The similarity between turtle and rat pyramidal neuron dendritic properties emphasizes the fundamental nature of spike backpropagation and dendritic calcium signaling in cortical pyramidal function. They have been conserved over widely divergent evolutionary pathways in animals with different developmental and ecological niches. Presumably their importance relates to the same properties that have attracted attention in studies of rat neurons, i.e., synaptic plasticity, enzyme mobilization, and gene activation (Holthoff et al. 2006; London and Häusser 2005; Stuart et al. 1999).

Many details still need to be determined. It is not clear whether the properties and distributions of ligand-gated and voltage-gated channels are the same in turtle and mammalian pyramids. It is not known whether synaptic inputs from different sources are integrated in the same way in the dendrites. Nevertheless, these findings encourage the continued use of the turtle cortical slice preparation in seeking the characteristics that define the fundamental functional properties of cortical cells and circuits across a wide range of vertebrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1.

Dendritic structure of turtle pyramidal neurons. *A*: schematic diagram of a typical coronal cortical slice positioned over a photograph of the dissected turtle brain. *B*: diagram of a cortical slice in more detail. The picture of the pyramidal neuron shows the region from which most recordings were made. The thick blue line is the region containing most cell bodies. The main regions of the dorsal cortex are labeled from medial (M) to lateral (L) including the dorsal-medial region (DM) and two dorsal subregions (D1 and D2) from which most of the recordings were made. *C* and *D*: outline of turtle and rat L5 pyramidal neurons determined from biocytin fills. Note that that most of the dendrites from the turtle cell are single branches that extend from the somatic region. The L5 neuron has a major apical branch with oblique branches, an apical tuft, and basal dendrites.

FIG. 2.

Most action potentials (APs) initiate in the somatic region of turtle pyramidal neurons and actively backpropagate over the dendrites. *A*: dual-patch recordings (*inset*) with a dendritic recording (red pipette) was made 350 μm from the soma (black pipette). A 1-s current pulse (1 nA) was injected into the dendrite that elicited a train of 4 APs, all of which propagated back into the dendrite. *Inset*: shows that the AP was recorded earlier at the somatic pipette (scale: 10 mV, 10 ms). *B*: the same current pulse injected at the somatic electrode elicited APs that also started first at the soma (see *C1* and *C2* for enlargements). Small “prepotential”-like spikes (arrowheads) were also recorded by the somatic pipette but were not visible at the dendritic electrode. *C*, *insets* from *B*, *1*: the amplitude of the first few dendritic spikes clearly grew, whereas the somatic spike amplitudes decreased or stayed about the same. *2*: the size and duration of the dendritic spikes in a train varied considerably. One dendritic spike (red arrow) is clearly narrower than the somatic spike even though the spikes were evoked by depolarization in the cell body. *D*: the amplitude of the backpropagating AP (measured from resting potential) recorded at different locations in the dendrites of different neurons using dendritic current injection. The open circles represent dual recordings and closed circles, single dendritic recordings. The dotted trace shows the best exponential fit to the data; the solid traces show the calculated amplitude decrement assuming passive backpropagation and 3 different assumptions for R_i (see text and Supplemental Fig. S1 for further details).

FIG. 3.

Fast spikes can be initiated in the dendrites. *A*: in response to a series of excitatory postsynaptic current-like current injections (*A2*; 2-ms rise time, 8-ms fall time), the dendritic potential became regenerative when the current was >450 pA. *A1*: patch electrode was 250 μ m from the soma. *A3*: plot of peak voltage vs. peak injected current showing the onset of the regenerative response at 450 pA. *B*: in some cells a similar current injection showed a fast AP with a second spike arising on the falling phase of the graded response. *C*: paired recording with similar dendritic current injection (electrode 150 μ m from the cell body) did not give an early spike, but the second spike was almost synchronous with the somatic spike evoked from electrotonic spread of the dendritic potential to the somatic region.

FIG. 4.

The origin of the prepotentials is in the axon. *A*: schematic diagram of dual-patch recording from the soma (black) and axon (blue). *B*: recording from axon (blue) and soma (black) during depolarizing current to the soma that evoked APs. *C*: magnified traces showing the regions of recording in *B* in dashed boxes labeled with numbers 1–3. In each case, the prepotential and AP at the axonal recording site preceded the signal at the somatic recording site. *D–F*: experiments similar to *A–C* with dual-patch recordings at the soma (black) and dendrite (red, 350 μm from the soma). The fast rising potentials were seen only at the somatic electrode. *G*: spontaneous excitatory postsynaptic potentials (EPSPs) recorded simultaneously from another neuron with pipettes on the soma and a dendrite 250 μm away. Note that some sharp EPSPs are clearly faster at the soma and others are faster at the dendritic location (*inset*; scale bars, 10 ms and 5 mV). In contrast, the slower potential changes are similar at both locations. *H*: histogram showing the amplitude distribution of the EPSPs (red), isolated prepotentials (as in *C3*, striped), and shoulder potentials (as in *C2*, blue) as recorded from the somata of different neurons. The selected EPSPs were a biased sample of events that appeared to have the fastest rise times. *I*: 10–90% rise-time distributions of EPSPs and prepotentials included in *H*. Even with the sample bias the prepotentials rose much faster than the EPSPs.

FIG. 5.

Somatically evoked Na^+ -dependent spikes evoke fast calcium transients at all locations in the soma and dendrites. *A1*: fluorescence image of the somatic region of a bis-fura-2-filled pyramidal neuron. Two dendrites are in focus. *A2*: pseudocolor image of the fractional fluorescence change ($\Delta F/F$), corresponding to changes in intracellular calcium ion concentration ($\Delta[\text{Ca}^{2+}]_i$), resulting from the somatically evoked spikes shown in *A3*. The image reflects the change between rest and the time at the end of the spikes (indicated by the red arrow). *B*: spike-evoked calcium transients in the distal dendrites. *B1*: image of the bis-fura-2-filled dendrite 300 μm from the soma. The line of small yellow regions indicates the “line” from which the “linescan” image in *B2* was taken. *B2*: bis-fura-2 fluorescence changes in response to subthreshold and supra-threshold intradendritic stimulation pulses. The pseudocolor image shows the time dependence of the changes at each location. There was a clear increase at each location along the dendrite in response to the spike and no detectable change to the subthreshold pulse. *B3*: the same dendritic region with 3 regions of interest marked. *B4*: spike-evoked transients in these 3 regions. The timescale applies to both *B2* and *B4*. *C*: rapid recovery of a calcium transient in a pyramidal neuron filled with

500 μ M furaptra, a low-affinity indicator. The recovery time constant was about 100 ms in the marked dendritic region.

FIG. 6.

Synaptically activated $[\text{Ca}^{2+}]_i$ changes in the dendrites are not blocked by $(\pm)$ -2-amino-5-phosphonopentanoic acid (APV) or (R,S) - α -methyl-4-carboxyphenylglycine (MCPG), but are reduced by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). **A:** image of bis-fura-2-filled turtle pyramidal neuron showing the region of interest (ROI) and the position of the stimulating electrode. A patch electrode on the soma was used to fill the neuron and record the synaptic potentials evoked at 100 Hz for 0.5 s. The cell was hyperpolarized to -90 mV to prevent spiking. The fluorescence change and the synaptic potential are shown at 3 times indicated in the plot below. **B:** the peak fluorescence change and peak voltage response are shown for all trials during the experiment. APV ($200 \mu\text{M}$) and MCPG (1 mM) had no clear effect on the fluorescence response. The addition of $10 \mu\text{M}$ CNQX reduced the synaptic potential and the fluorescence change. Tetrodotoxin ($\sim 1 \mu\text{M}$) blocked the remaining synaptic potential but did not completely eliminate the fluorescence change.

FIG. 7.

Repetitive synaptic stimulation evokes calcium waves in the dendrites. *A* and *C*: image of a bis-fura-2-filled pyramidal neuron showing one dendrite in focus, the patch electrode on the soma, and the stimulating electrode positioned near the dendrite about 50 μm from the soma. In *A*, a “line” of pixels is indicated along the dendrite and through the soma. In *C*, 3 ROIs are marked. In response to 50 pulses (100- μs duration) at 100 Hz a subthreshold train of EPSPs was recorded with the somatic electrode (*bottom trace* in *D*). Simultaneously, calcium transients were recorded in the dendrites. The responses in the 3 regions (*top traces* in *D*) clearly occurred at different times. The more refined presentation in *B* shows that the $[\text{Ca}^{2+}]_i$ change spread as a wave that initiated in the dendrites at a point close to the stimulating electrode and then spread toward but not into the soma.

FIG. 8.

1-Aminocyclopentane-*trans*-1,3-dicarboxylic acid (*trans*-ACPD) weakly enhances the calcium transients evoked by backpropagating action potentials. The fluorescence images show a bis-fura-2-filled pyramidal neuron with a patch electrode on the soma. Similar to the previous figures, the pixels and boxes indicate the locations for the “linescan” image and the ROIs. In “Control” conditions (normal artificial cerebrospinal fluid solution), a train of 5 APs at 30-ms intervals evoked transient $[Ca^{2+}]_i$ changes at all the visible dendritic and somatic locations. When 100 μ M *trans*-ACPD was added to the bath the amplitude and time course of the fluorescence changes increased slightly. These changes were reversed on washout.