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Advances in Understanding Cortical Function Through Combined Voltage-Sensitive Dye Imaging, Whole-Cell Recordings, and Analysis of Cellular Morphology

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Abstract: Voltage-sensitive dyes can be used to image cortical network function with millisecond temporal resolution and with a horizontal spatial resolution of approximately 50 μm. This imaging technique can be combined with whole-cell patch-clamp

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measurement of membrane potential followed by the anatomical analysis of neuronal morphology. Together, such experiments reveal the relationship of activity recorded in individual identified neurons with the spatiotemporally resolved ensemble dynamics of a cortical region. Application of these techniques to the rodent barrel cortex has advanced our understanding of the synaptic mechanisms underlying sensory responses to simple whisker stimuli.

Keywords: axonal and dendritic morphology, sensory processing, voltage-sensitive dye imaging, whole-cell patch-clamp recording

I. INTRODUCTION

Understanding the organizing principles and functions of the neocortex is likely to lead to insight concerning the mechanisms of sensory perception and behavior. Such complex mental processes are likely to result from the interactions of many neurons distributed throughout cortical and subcortical areas. A variety of different approaches have been taken to analyze large-scale brain activity. Electrophysiological techniques were among the first to be applied to analyze brain function. Recent technological advances in electrophysiology allow simultaneous extracellular recordings of action potential activity from hundreds of individual neurons located in multiple brain areas in awake behaving animals (Buzsaki, 2004; Nicolelis *et al.*, 2003). Such measurements are, however, limited to an analysis of suprathreshold spiking activity of neurons and give no information relating to the underlying mechanisms leading to the action potentials. More detailed measurements of neuronal function have been made using intracellular recordings either using sharp microelectrodes or by the patch-clamp technique (Hamill *et al.*, 1981; Neher, 1992; Sakmann, 1992). These intracellular electrophysiological techniques can record the neuronal membrane potential capturing both the subthreshold and the suprathreshold responses of neurons. However, rather few cells (usually only one or two cells) can be recorded simultaneously due to technical difficulties. Electrophysiological measurements offer excellent temporal resolution, but are limited in their ability to map the spatial extent of brain activity (even with over a hundred extracellular electrodes).

Imaging methods have been developed to give spatial information regarding brain activity. Spectacular images of brain function have come from functional magnetic resonance imaging; however, the temporal resolution of this technique is limited. Most current functional magnetic resonance imaging techniques focus on the blood oxygenation level-dependent signal and thus temporal resolution is limited not only by the measuring apparatus but also by the delayed coupling of neural activity to hemodynamic changes.

An obvious wish would be to directly image the electrical activity of neurons in the brain. Recent advances in both imaging technology and novel voltage-sensitive dyes now offer the opportunity for high temporal

and spatial resolution recording of neocortical activity. Below, I describe a combination of a voltage-sensitive dye imaging technique with whole-cell recordings and postrecording anatomical analysis of synaptic circuits. The application of this combination to the rat somatosensory barrel cortex has advanced our understanding of cortical function.

II. VOLTAGE-SENSITIVE DYE IMAGING

Dye molecules that insert into the plasma membrane and change their optical absorption and/or emission properties, dependent upon the electrical field across the membrane, can be considered as voltage-sensitive dyes. The first optical measurements of action potentials were made on invertebrate preparations such as the squid giant axon and neurons of the leech (Salzberg *et al.*, 1973; Tasaki *et al.*, 1968). The first vertebrate in vivo voltage-sensitive dyes measurements visualized the spatiotemporal dynamics of sensory processing (Grinvald *et al.*, 1984). Since these pioneering steps, many further compounds have been successfully tested as voltage-sensitive dyes. Of equal importance, camera technology has advanced dramatically.

Voltage-sensitive dyes typically show a linear, approximately 10% change in fluorescence for 100 mV change in membrane potential. Despite the small amplitude of the signals, the spatiotemporal dynamics of the membrane potential of individual neurons can be imaged in vitro in individual mammalian neurons by loading voltage-sensitive dyes intracellularly with the whole-cell patch-clamp technique (Antic *et al.*, 1999; Djurisic *et al.*, 2004; Zecevic, 1996). Significant signal-to-noise improvements may be realized from excitation at the red spectral edge (Kuhn *et al.*, 2004).

In vivo imaging is hindered both by the small signal amplitude and by the heart-beat-pulsation-related artifacts, resulting primarily from changes in the blood oxygenation level. Shoham *et al.* (1999) made dramatic advances with in vivo imaging by using novel blue dyes, which are excited at long wavelengths where hemoglobin has little absorption. One of these dyes RH1691 (Fig. 14.1A) can be excited with 630-nm red light (Fig. 14.1B) at which wavelength it shows an increase in fluorescence (>665 nm) upon depolarization (Fig. 14.1C). RH1691 has proven extremely useful for high-resolution in vivo imaging of cortical function (Grinvald and Hildesheim, 2004) with low toxicity to the imaged neurons (Petersen *et al.*, 2003a).

III. WHOLE-CELL PATCH-CLAMP RECORDING

The patch-clamp technique developed by Bert Sakmann and Erwin Neher provided the first direct recordings of single ion channels (Neher and Sakmann, 1976). Further development of different recording configurations together with the remarkable mechanical stability and low noise

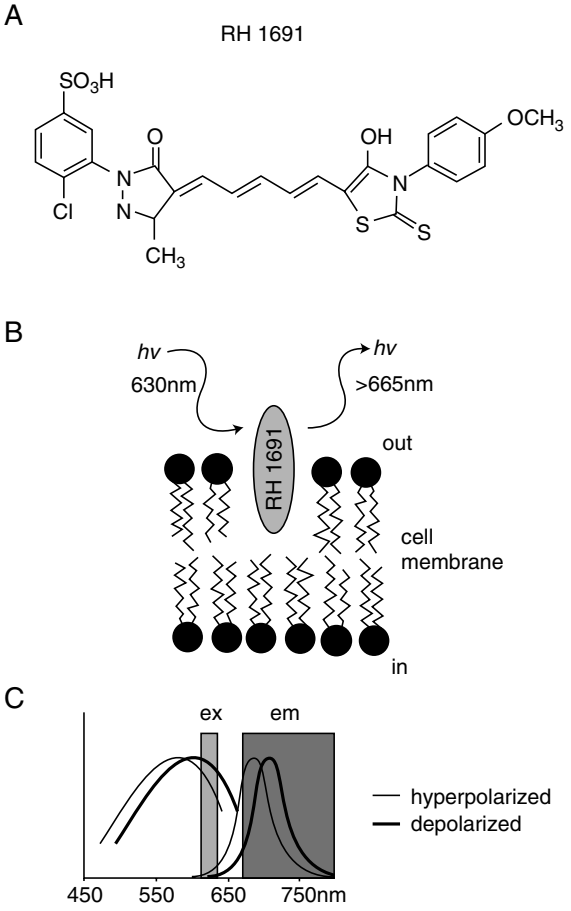

Figure 14.1. Part A shows the chemical structure of voltage-sensitive dye RH1691. After extracellular application, the dye molecules insert into the plasma membrane and upon excitation with 630-nm light they emit fluorescence that can be measured after 665-nm long-pass filters, as schematically indicated in Part B. A schematic drawing of the spectral shifts induced by changes in membrane potential is shown in Part C. During depolarization there is an increased absorption at 630 nm and an increase in fluorescence can be detected.

of the "gigaseal" (Hamill *et al.*, 1981) has extended the usefulness of this electrophysiological recording technique to virtually all biological preparations. The whole-cell patch-clamp configuration has proven particularly useful for recording both in acute brain slices and in vivo in the intact living animal (Fig. 14.2A,B). This is possible because the tip of the glass electrode patch-pipette can be kept clean by applying positive pressure to its inside with a resultant continuous outflow of intracellular solution. During the "search" for a cell to be recorded (Fig. 14.2C) this flux of liquid prevents debris from sticking to the patch-pipette and also "cleans" the cell membrane

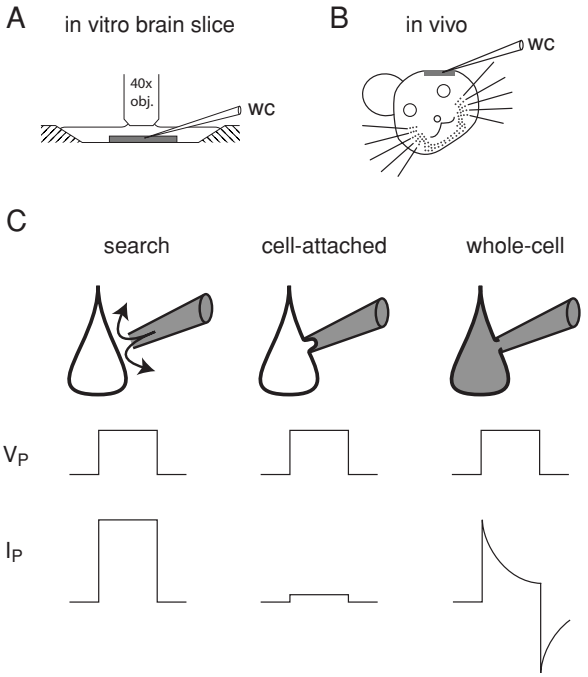

Figure 14.2. Whole-cell (WC) patch-clamp recordings can be made in vitro from neurons in brain slices (A) or in vivo from the intact living animal (B). Part C schematically shows various configurations of the patch-clamp technique. In the search mode, the pipette is under positive pressure, which keeps the tip of the electrode clean as it penetrates brain tissue. Large square shape currents (I_P) are evoked by square-shaped voltage command pulses (V_P) to the patch-clamp amplifier. When a cell is encountered current flow is decreased and gentle suction is applied. This allows formation of the gigaseal. This cell-attached configuration is characterized by very little current flow during voltage pulses, indicating a high resistance. Further gentle suction breaks down the membrane patch inside the tip of the electrode while maintaining the tight electrical seal of the membrane in contact with the glass pipette. This whole-cell configuration is characterized by large capacitative current transients in response to voltage pulses. The whole-cell configuration allows exchange of molecules from the patch-pipette with the inside of the cell.

priming it for the gigaseal. The cell to be recorded can be either visualized by various microscopy techniques (Margrie *et al.*, 2003; Stuart *et al.*, 1993) or electrically sensed by the increased resistance encountered as the pipette hits a cell (Blanton *et al.*, 1989). Upon encountering a target cell, the positive pressure is reversed by gentle sucking and an electrically tight gigaseal is formed. The gigaseal has resistance of >1 GΩ and has remarkable mechanical stability. This recording configuration is termed cell attached and can be used to record single channels in the patch membrane. The whole-cell recording configuration can be entered by further suction and can be monitored by the large increase in capacitance associated with the whole-cell membrane area.

The whole-cell configuration can remain stable for hours even in vivo despite the small movements of the brain (caused, for example, by heart-beat-pulsation), allowing high-quality measurements of membrane potential. The ease and stability with which whole-cell recordings can be obtained allow this experimental technique to be combined with other techniques such as voltage-sensitive dye imaging (Fig. 14.3).

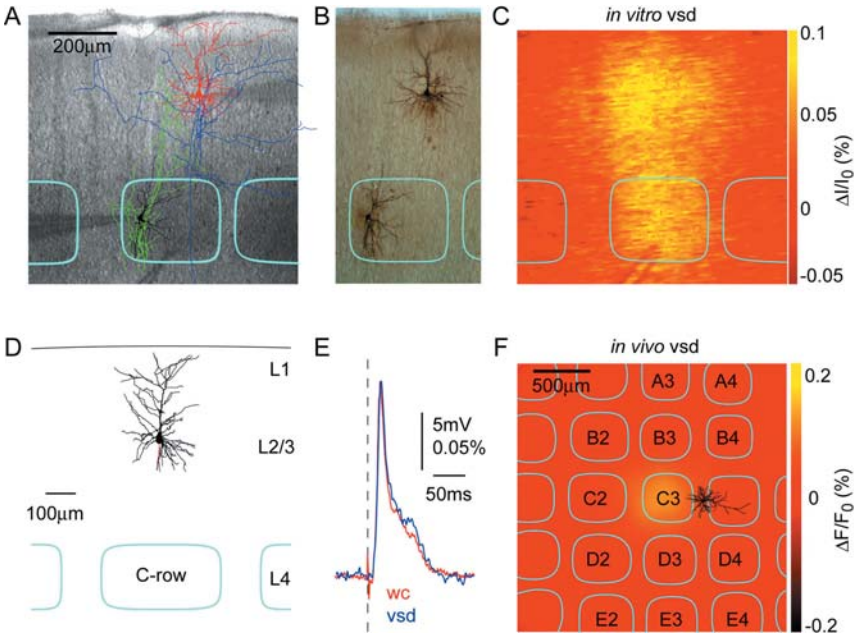

Figure 14.3. Part A shows a bright field photograph of a brain slice of the rat somatosensory cortex. The layer 4 barrels are outlined in cyan. One layer 4 neuron was recorded and later reconstructed with dendrites shown in black and axon in green. One layer 2/3 neuron was recorded and drawn with dendrites in red and axon in blue. A photograph of the stained neurons is shown in Part B. While the two neurons were recorded by the whole-cell patch-clamp technique, large-scale network activity was evoked by an extracellular stimulus delivered by a third electrode. This stimulation electrode was placed in layer 4 and the ensemble response was visualized with millisecond resolution with voltage-sensitive dye. Part C shows the voltage-sensitive dye image captured 12 ms after stimulation. The image shows columnar excitation. Part D shows a reconstruction of the somatodendritic compartment a layer 2/3 neuron recorded in vivo (viewed in a plane normal to the pial surface and along the row). During the whole-cell recording the cortical dynamics were imaged with voltage-sensitive dye. Part E shows the quantification of the C3 whisker-evoked voltage-sensitive dye response quantified over a 200 × 200 µm region centered on the soma of the recorded neuron. The time course of the optical response closely matches the time course of the changes in membrane potential of the recorded neuron. Part F indicated the lateral locations of the recorded neuron relative to the layer 4 barrels (cyan) and the evoked voltage-sensitive dye response recorded 15 ms after stimulation. (Reprinted in modified form with permission from Petersen and Sakmann, 2001 (© 2001 Society for Neuroscience), and Petersen *et al.*, 2003a (© 2003 Society for Neuroscience)).

In addition to measuring the electrophysiology of neurons, the whole-cell technique allows the introduction of small molecules into the cell cytoplasm. By including biocytin (biotinyl lysine) in the solution in the patch pipette, this label diffuses into intracellular milieu entering both axonal and dendritic compartments. After recording of the neuron, the brain can be fixed and sectioned. The location of the biocytin molecules can be revealed by the specific binding of the biotin motif (part of the biocytin molecule) to avidin conjugated to peroxidase, which in a reaction with diaminobenzidine forms a dark deposit. This allows visualization of the neuronal structure under light microscopy, and computer-aided tracing of dendritic and axonal compartments in three dimensions (Fig. 14.3).

IV. AN APPLICATION: ANALYZING THE SENSORY RESPONSE IN RODENT BARREL CORTEX

The rodent primary somatosensory cortex has aroused much interest in neurobiology because of its high degree of anatomical organization (Petersen, 2003). The mystacial vibrissae representation in this cortical area is segregated into discrete units termed barrels present in layer 4, which can be visualized in living brain slices (Petersen and Sakmann, 2000) as well as by numerous staining techniques. The layout of the barrels across the cortical map is identical to the layout of the whiskers on the snout of the rodent. This suggests that each of these barrels is intimately involved in processing the information from its corresponding whisker (Woolsey and Van der Loos, 1970).

The first level of neocortical processing begins with the layer 4 barrel neurons, which are directly connected to thalamic VPM neurons through glutamatergic synapses (Agmon and Connors, 1991). Both glutamatergic excitatory (spiny stellate and star pyramidal neurons) and diverse classes of GABAergic neurons in layer 4 receive direct VPM input (Bruno and Simons, 2002; Porter *et al.*, 2001). Excitatory layer 4 neurons within the same barrel are strongly connected with approximately every third pair of neurons being synaptically connected, but there is very little synaptic connectivity between neighboring barrels (Feldmeyer *et al.*, 1999; Petersen and Sakmann, 2000, 2001; Schubert *et al.*, 2003; Shepherd *et al.*, 2003). As seen in Fig. 14.4A,B, this pattern of physiologically measured synaptic connectivity likely results from the highly polarized dendritic and axonal arbors of these neurons, which rarely enter neighboring layer 4 barrels (Lübke *et al.*, 2000; Petersen and Sakmann, 2000). Each layer 4 barrel, therefore, is an independent and irreducible unit consisting of a few thousand neurons which process information relating primarily to its isomorphic whisker. The ability to define the neuronal network in terms of both the number of participating neurons and the normal physiological input (since the location of the barrel in the sensory map can be established) makes the barrel cortex an ideal starting point for quantitative modeling of neocortical networks (Petersen, 2002).

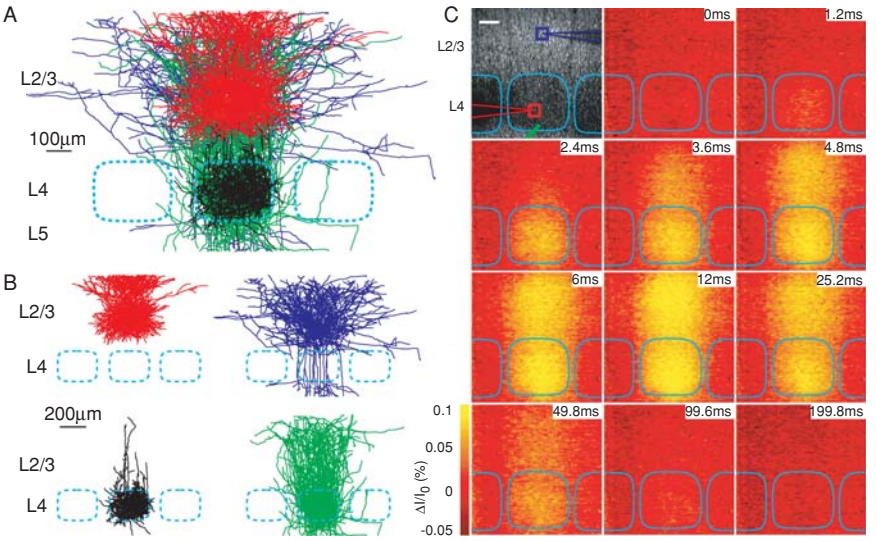

Figure 14.4. Parts A and B show the superposition of many excitatory neurons reconstructed from in vitro brain slice recordings and normalized according to the barrel width. Dendrites and cell bodies of layer 4 neurons shown in black are largely confined to the layer 4 barrel. The axons of the layer 4 neurons shown in green are laterally confined to the width of the layer 4 barrel but project heavily to both layers 2/3 and 4. The dendrites and cell bodies of the layer 2/3 pyramidal neurons are indicated in red and their axons in blue. The layer 2/3 axon spreads far laterally. Part C shows the functional activation of a barrel column evoked by extracellular stimulation of the layer 4 barrel and measured by voltage-sensitive dye imaging. In addition to the large stimulation electrode (green) in the layer 4 barrel, there is also a whole-cell recording pipette in layer 4 (red) and another in layer 2/3 (blue). The images demonstrate remarkably tight columnar activation throughout the duration of the response. (Reprinted in modified form with permission from Petersen and Sakmann, 2001 (© 2001 Society for Neuroscience)).

The excitatory layer 4 neurons project most densely into layer 2/3 with the horizontal axonal field spreading little wider than the underlying layer 4 barrel, thus defining anatomically a neocortical column (Fig. 14.4A, B). Excitatory synaptic connections from layer 4 to layer 2/3 pyramidal neurons occur frequently but have smaller efficacies and smaller NMDA receptor components than the layer 4 to layer 4 synapses (Feldmeyer *et al.*, 2002). The flow of excitation is strictly feed forward since there are no reciprocal excitatory connections from layer 2/3 to layer 4. Layer 2/3 pyramidal neurons synapse with their neighboring layer 2/3 pyramidal neurons, layer 5/6 pyramidal neurons (Reyes and Sakmann, 1999) and project to other cortical areas including contralateral somatosensory cortex, motor cortex, and secondary somatosensory cortex. Within the local circuits the axonal fields of layer 2/3 pyramidal neurons do not respect barrel column boundaries (Fig. 14.4A,B) extending far into the neighboring barrel columns.

To probe how this neuronal network operates when many neurons are excited, we imaged the membrane potential with voltage-sensitive dye (Fig. 14.4C). Stimuli were delivered to a single layer 4 barrel causing local excitation and spread of activity to the supragranular layer in a columnar fashion (Petersen and Sakmann, 2001). This was the first demonstration of a functional neocortical column at the subthreshold synaptic level, which matches the anatomically defined extent of the layer 4 axons.

The activity of the excitatory neuronal network is likely to be strongly regulated by the many diverse types of cortical GABAergic interneurons (Gupta *et al.*, 2000). When GABAergic inhibition is blocked in vitro, synaptic excitation can spread horizontally in layer 2/3 presumably through local excitatory synapses (Petersen and Sakmann, 2001).

During behavior, the whiskers usually operate in concert as a sensory organ. Therefore, the exchange of information related to the individual whiskers is likely to play a prominent part in cortical processing. One role for the barrel cortex is then to distribute the information related to the movement of a single whisker and compare this with information relating to movements of other whiskers. Such a process may occur in a defined spatial and temporal integrative process in the cortex.

The distributed nature of sensory signals originating from single brief sensory stimuli has been highlighted by combined in vivo voltage-sensitive dye imaging and whole-cell recordings (Fig. 14.5). This direct measurement of how cortical activity evoked by a single whisker is spatiotemporally distributed across the barrel cortex (Petersen *et al.*, 2003a) correlates well with measurements of receptive field properties of individual neurons analyzed by sequentially deflecting many whiskers (Armstrong-James *et al.*, 1992; Brecht *et al.*, 2003; Brecht and Sakmann, 2002; Moore and Nelson, 1998; Simons, 1978; Zhu and Connors, 1999). The earliest sensory response occurs ~8 ms following whisker deflection and is localized to the direct targets of the VPM input, the layer 4 barrel neurons and a fraction of neurons in mid-layer 5/6. In the next milliseconds, excitation propagates into layer 2/3 in a columnar fashion. Thus a functional neocortical column, bounded laterally by the layer 4 barrel structure, is depolarized 10–12 ms after whisker deflection (Fig. 14.5C). In the following milliseconds both infragranular neurons and neurons in neighboring barrel columns become excited, apparently mainly through local cortical synaptic circuits. Excitation spreads preferentially along the row orientation of the barrel cortex, for example deflection of the D2 whisker evokes first a response in the D2 barrel column and over the next milliseconds the largest responses are found in D1 and D3 neighboring barrel columns with smaller responses in the C2 or E2 columns. This oriented spread of excitation may serve a useful physiological function. The whisking behavior involves rapid whisker movements oriented largely in a plane along the rows. Thus during the forward motion of the whiskers, the D3 whisker will pass through a point in space a few milliseconds before the D2 whisker, which in turn will be followed by the D1 whisker moving through the identical spatial location. Thus whiskers lying in the same row will often

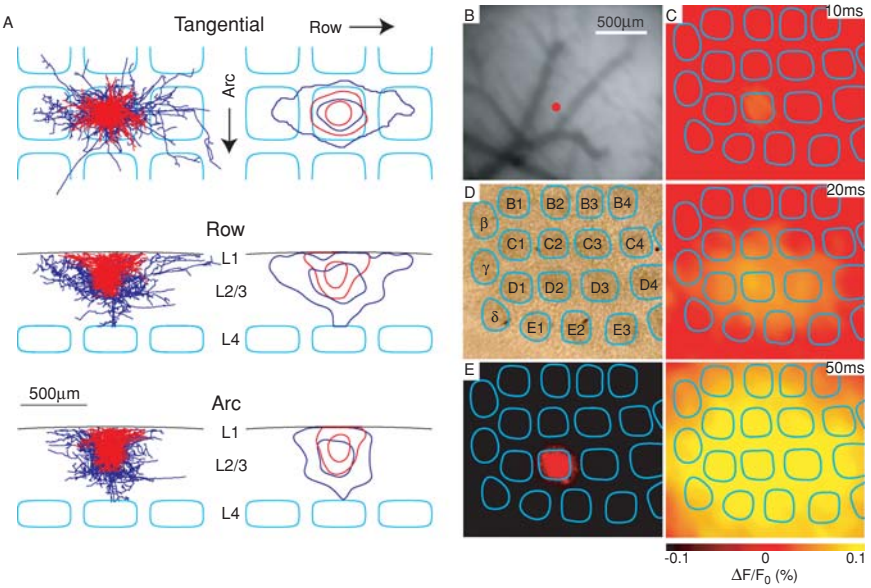

Figure 14.5. Part A shows the projection in three orthogonal directions of three dimensionally reconstructed axons (in blue) and dendrites (in red) of layer 2/3 pyramidal neurons recorded in vivo. The right-hand column shows the 10 and 50% contours of the length density of axon (blue) and dendrite (red) computed from the superimposed, gaussian smoothed, normalized computer-aided three-dimensional reconstructions. The axons extend preferentially in the row direction of the barrel cortex organization. Part B shows the blood vessels at the surface of the somatosensory cortex. Part C shows the voltage-sensitive dye signals recorded in response to deflection of the D2 whisker. The earliest signals occur ~10 ms following whisker deflection and are localized to the homologous barrel column. In the next tens of milliseconds the signal propagates over a large cortical area preferentially in the row direction. After the functional imaging, DiI was injected into the location of the epicenter of the response. The DiI was allowed to diffuse and later the brain was sectioned tangentially to locate the layer 4 barrels (viewed under transillumination without staining in Part D). DiI fluorescence in layer 4 was found in the D2 barrel (E) in agreement with the location of the response to the D2 whisker deflection. (Reprinted in modified form with permission from Petersen *et al.*, 2003a (© 2003 Society for Neuroscience)).

sample the same point in space within milliseconds of each other. In order for the animal to process this information relating to individual whiskers distributed across the neocortical barrel field, it is likely to be important that this single-whisker-related information is rapidly exchanged along the rows of the barrel cortex. The rapid spread of the sensory response may mediate this integrative process, with propagation velocities along the row being twice as fast as along the orthogonal arc direction (Petersen *et al.*, 2003a). This spread of excitation may be mediated by local excitatory synaptic connections in layer 2/3 since their axons are preferentially oriented along the rows of the barrel cortex (Fig. 14.5A). The combined methodologies

of single-cell recording, anatomical reconstruction, and ensemble imaging are therefore beginning to describe the synaptic events underlying sensory processing.

V. SUMMARY OF ADVANTAGES AND LIMITATIONS

Recent advances in imaging technology and new voltage-sensitive dyes now allow high-resolution imaging of cortical dynamics (Petersen *et al.*, 2003b). However, the current in vivo technique does not allow signals from individual neurons to be resolved within the stained neocortical network, but only ensemble activity. To overcome this limitation will likely require future generations of much improved voltage-sensitive fluorescent proteins (Ataka and Pieribone, 2002; Cacciatore *et al.*, 1999; Sakai *et al.*, 2001; Siegel and Isacoff, 1997). However, calcium as a measure of neuronal activity can be readily imaged with single-cell resolution within networks stained with cell permeant calcium-sensitive dyes (Peterlin *et al.*, 2000; Stosiek *et al.*, 2003; also see the chapter of Goldberg *et al.*, 2005, in this volume).

Since voltage-sensitive dye imaging is technically straightforward, this imaging technique can be readily combined with whole-cell patch-clamp recordings. Thus simultaneous measurements of single neuron and spatiotemporally resolved ensemble membrane potential measurements can be made. Together with anatomical analysis we can therefore begin to reconstruct the synaptic pathways, underlying simple sensory responses in the neocortex.

APPENDIX

A. Commercial Sources of Voltage-Sensitive Dye, Camera, and Imaging Software

1. Commercial Source of Voltage-Sensitive Dye RH1691

Optical Imaging Inc., PO Box 1262, Mountainside, NJ 07092-1262 (http://www.opt-imaging.com).

2. Commercial Sources of Cameras and Imaging Software

Imager 3001: Optical Imaging Inc., PO Box 1262, Mountainside, NJ 07092-1262 (http://www.opt-imaging.com).

NeuroCCD/NeuroPDA: RedShirtImaging LLC, 2 Stoneleigh Road, Fairfield, CT 06825 (http://www.redshirtimaging.com/).

MiCAM: SciMedia Ltd, 4 Executive Circle, Suite 170, Irvine, CA 92614 (http://www.scimedia.com).

B. Staining Neocortex with Voltage-Sensitive Dye

1. It is important to carry out animal experiments in accordance with local legislation. Anesthetize animal (e.g., juvenile 200 g Wistar rat injected intraperitoneally with urethane at 1.75 mg/g). Maintain the body temperature at 37°C.
2. Remove or reflect the skin covering the skull.
3. Carefully scrape the bone clean. Further cleaning of the bone can be performed, if necessary, with 1% hydrogen peroxide.
4. Apply a thin layer of cyanoacrylate glue to the surrounding region of the bone, away from where the craniotomy will be performed (this helps dental cement adhesion). Glue a metal head-plate to the skull with dental cement. The plate must be tangential to the region of cortex to be imaged and the hole in the plate must be positioned over this region.
5. When the dental cement has hardened, immobilize the skull and minimize movement of the brain by fixing the head-plate firmly between metal posts.
6. Perform a craniotomy of desired size by drilling within the hole of the head-plate. Be careful not to damage the underlying brain tissue.
7. Finally, remove the dura, leaving the pia of the underlying cortex exposed.
8. Dissolve the voltage-sensitive dye RH1691 (Shoham *et al.*, 1999) to 0.1–1 mg/ml in Ringer's solution: 135 mM NaCl, 5 mM KCl, 5 mM HEPES, 1.8 mM $CaCl_2$, and 1 mM $MgCl_2$.
9. Apply a small quantity (~250 μl) of this dye solution to the craniotomy. Seal this chamber with a glass coverslip and very slight pressure is applied to prevent brain edema.
10. Leave the cortex to stain for 1–2 h. During this period, the dye will diffuse into the superficial layers of the neocortex. At the end of the staining period, remove unbound dye by washing the cortex extensively with Ringer's solution. The cortex should now have a pale blue color.
11. Cover the craniotomy with 1% agarose, dissolved in Ringer's solution, and place a glass coverslip on top. The glass coverslip should not be just large enough to cover the width of the craniotomy, but little more. This will allow access for whole-cell recordings to be made.

C. Voltage-Sensitive Dye Imaging

1. Illuminate with ~530-nm green light and record the blood vessel pattern on the cortical surface using the camera.
2. Move the focal plane 300 μm into the cortex and excite the voltage-sensitive dye with epifluorescent light at 630 nm. Emitted light is long-pass filtered (>665 nm), forming the voltage-sensitive dye signal, which

should be recorded at frame rates faster than 100 Hz. Heart-beat-related signals form the largest artifacts. The timing of these known artifacts can be recorded via an electrocardiogram. The artifacts can then be removed by computer processing to improve the resolution of the collected signals.

D. Whole-Cell Recording

1. Fill whole-cell pipettes with intrapipette solution: 135 mM potassium gluconate; 4 mM KCl; 10 mM HEPES; 10 mM phosphocreatine; 4 mM MgATP; and 0.3 mM Na_3GTP; pH 7.2 adjusted with KOH and include 3 mg/ml of biocytin (to allow staining of the recorded neurons).
2. Pipettes should have a resistance of ~5 MΩ. Monitor the tip resistance by applying brief voltage steps of 5 mV in the voltage-clamp mode while measuring the current flow on an oscilloscope. Apply a positive pressure of ~200 mbar on the pipette.
3. Slowly advance the electrode through the agarose under the coverslip and into the cortex. When the tip is close to the chosen recording site, reduce the positive pressure to ~30 mbar.
4. Advance the pipette in 2 μm steps until the tip resistance suddenly increases (indicating contact with a cell membrane). Release the pressure in the pipette and apply light suction until a gigaseal is formed.
5. Establish whole-cell recording configuration by rupturing the membrane in the pipette. This can be achieved by applying either brief suction pulses or slowly increasing the suction pressure.
6. After collecting data, slowly retract the whole-cell recording pipette while monitoring whole-cell capacitance transients with 5 mV voltage steps. During the retraction the excised patch configuration should be established. This insures that the neuron remains intact and viable for later anatomical staining.

E. Anatomical Analysis of Neuronal Structure and Position

1. Supplement anesthetic (e.g., by an additional intraperitoneal injection of 1 mg/g of urethane). Perfuse the animal transcardially with ice-cold 0.1 M phosphate buffer (pH ~7.3) and then with ~50 ml of 4% formaldehyde. Remove the brain from the skull and postfix overnight at 4°C.
2. Section the cortex tangentially with a vibratome at 100 μm and stain for biocytin using ABC kit (Vectastain Laboratories). The angle of mounting the brain for tangential sectioning is difficult to determine exactly, but is helped by the slightly blue color of the brain in the craniotomy remaining from the voltage-sensitive dye staining.

3. The blood vessels initially imaged with green illumination now provide the link between the location of the neuronal processes visualized by the anatomical stain and the functional voltage-sensitive dye images. The blood vessels, barrel patterns, and the axonal and dendritic processes can be traced in three dimensions, using an image-combining computerized microscopy system (e.g., Neurolucida, MicroBrightField, Inc. Williston, VT). For further details please refer in this volume to the chapter by Ascoli and Scorcioni and also the chapter by Bjaalie and Leergard.
4. In addition, other fluorescent labels can be introduced into the brain during the in vivo experiment for additional position information. Pipettes filled with DiI (1 mg/ml dissolved in dimethylformamide) can be inserted into the brain and DiI ejected through a brief pressure pulse. The DiI diffuses into the nearby tissue labeling axon and dendrites.

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