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Ca^{2+} imaging in the mammalian brain in vivo

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Abstract

Changes in intracellular free calcium ion concentration ($[Ca^{2+}]_i$) have been visualized over more than two decades using fluorescent dyes and optical microscopy. So far, however, most imaging studies have been performed on isolated cells or brain tissue. Here, we review approaches to measure cellular $[Ca^{2+}]_i$ changes in vivo, i.e. within the intact brain of a living animal. In particular we describe the application of two-photon microscopy to the mammalian central nervous system, which has recently enabled studies of Ca^{2+} dynamics in individual dendrites in anaesthetized rats. New developments in microscopy and labeling techniques are creating further opportunities to study Ca^{2+} dynamics in vivo and are likely to make measurements of spatio-temporal $[Ca^{2+}]_i$ distributions feasible even in awake, behaving mammals.

Keywords: Ca^{2+}; Ca^{2+} indicator; Fluorescence; Neocortex; Dendrite; Two-photon; In vivo; Olfactory bulb

1. Introduction

Ca^{2+} acts as intracellular second messenger controlling a variety of cellular phenomena including secretion, contraction, excitability, and neuronal plasticity. Astonishingly, Ca^{2+} is able to control several different processes in an individual cell simultaneously. This is possible due to compartmentation of Ca^{2+} signals (Helmchen, 1999; Wang and Augustine, 1999). An important step in understanding the role of Ca^{2+} in a particular phenomenon is therefore to determine the size, spatial spread, and time course of $[Ca^{2+}]_i$ changes in the relevant region of the cell. In excitable cells, Ca^{2+} fluxes are closely linked to the cells' electrical activity. Action potentials and/or activation of synaptic receptor channels can cause Ca^{2+} influx, which is electrogenic and may itself contribute to regenerative membrane potential changes (such as Ca^{2+} action potentials). Thus measurements of $[Ca^{2+}]_i$ changes can subserve a second function: the monitoring of electrical activity of individual neurones or of a whole region of the brain, the latter following bulk-labelling with indicator.

Measurements of spatio-temporal $[Ca^{2+}]_i$ distributions became possible with the development of fluorescent Ca^{2+} indicators (Tsien, 1989). Using these indicators and light microscopy techniques, a wealth of information about Ca^{2+} signaling mechanisms has been collected over the last two decades in many different cell types. In the mammalian central nervous system (CNS) Ca^{2+} influx into dendrites and its role in synaptic integration and plasticity have been studied extensively (for reviews see Regehr and Tank, 1994; Denk et al., 1996; Eilers and Konnerth, 1997; Mainen et al., 1999; Sabatini et al., 2001; Yuste et al., 2000). Most experiments, however, were carried out in vitro, on either isolated cells or extracted tissue such as brain slices. One of the reasons is that light scattering typically restricts the use of conventional (wide-field) and confocal microscopy to the study of cells near the surface of brain tissue. Deeper within the tissue resolution is compromised and signal-to-noise deteriorates.

From in vitro models, one can draw only limited conclusions about neuronal function in the intact brain. One cannot observe $[Ca^{2+}]_i$ changes as they occur during natural brain activity or during interactions with the outside world, either in response to sensory inputs or preceding an appropriate behavioural response. Furthermore, the properties and/or expression of proteins involved in Ca^{2+} handling (Ca^{2+}-permeable channels, Ca^{2+} buffers or pumps) may be modulated in vivo according to the behavioural state of the animal. Hence their properties could be different in vitro. It is therefore necessary to study Ca^{2+} signaling in vivo, both to establish the presence and characteristics of signaling

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mechanisms found in vitro and to establish their relevance for animal behaviour.

Until recently in vivo Ca^{2+} imaging of individual cells was feasible only in a few favorable preparations, mostly insects and lower vertebrates (for reviews, see Borst and Egelhaaf, 1994; Fetcho and O'Malley, 1997; Galizia and Menzel, 2000). These pioneering studies characterized Ca^{2+} signals that result from sensory input or precede activity of the animal. The eventual goal is to measure activity under conditions that mimic the animal's experiences as closely as possible (e.g. Kern et al., 2001). These studies constitute a benchmark for what we should like to achieve in the mammalian CNS.

With the development of two-photon laser scanning microscopy (Denk et al., 1990), we now have a tool that permits visualization of cellular compartments up to 600 μm deep within biological tissue (Denk and Svoboda, 1997). Due to its depth penetration, two-photon microscopy has contributed substantially to the current trend to study cells within their native context (Lichtman and Fraser, 2001). It has been applied in a number of in vivo studies, including work on skin (Masters et al., 1997), cortical blood flow (Kleinfeld et al., 1998), embryos (Squirrell et al., 1999), dendritic spine morphology (Lendvai et al., 2000), Alzheimer plaques (Christie et al., 2001), and tumors (Brown et al., 2001). Two-photon microscopy for the first time enabled measurements of dendritic Ca^{2+} dynamics in neocortical pyramidal neurones of anaesthetized rats (Svoboda et al., 1997), opening a new field for the investigation of the intact mammalian brain on the cellular and sub-cellular scale.

In this review, we focus on the use of two-photon microscopy for imaging neuronal $[Ca^{2+}]_i$ changes in anaesthetized rodents. Following a brief summary of previous in vivo approaches in non-mammalian preparations, we discuss some technical aspects, in particular the combination of two-photon microscopy with in vivo electrophysiology. We then give examples of $[Ca^{2+}]_i$ measurements in rat somatosensory cortex and olfactory bulb and finally present an outlook on new developments that we anticipate will further extend the opportunities to study Ca^{2+} signaling in living animals.

2. Imaging in non-mammalian species

While in vivo Ca^{2+} imaging in the mammalian brain was established only recently, measurements in intact insects and lower vertebrates have a longer history. Table 1 summarizes experiments in which Ca^{2+} signals have been

Table 1
Examples of in vivo calcium imaging studies

Species	Preparation	Stimulation	Resolution	Dye	Imaging method	Reference
Non-mammals						
Honeybee	Antennal lobe	Odorants	Bulk	CG-1 AM CG-2 AM	CCD camera	Joerges et al., 1997; Galizia et al., 1999
Zebrafish	Olfactory bulb	Odorants	Bulk	CG-dextran	CCD camera	Friedrich and Korsching, 1997
Zebrafish	Mid-/hindbrain of larvae	Vibration, touch	Bulk	CG-dextran	CCD camera	Nicolson et al., 1998
Drosophila	Mushroom body	Odorants	Bulk	CG-1 AM	CCD camera	Wang et al., 2001
Turtle	Olfactory bulb	Odorants	Bulk	CG-dextran	Fast CCD camera	Wachowiak et al., 2002
Cricket	Omega neurone	Cricket songs, Sound stimuli	Cellular	Fura-2	CCD camera	Sobel and Tank, 1994
Blowfly	Interneurones in visual system	Visual, Motion fields	Cellular	Fura-2	CCD camera	Borst and Egelhaaf, 1992; Single and Borst, 1998; Kurtz et al., 2001; Dürr et al., 2001
Zebrafish	Spinal cord in larvae	Head or tail tap	Cellular	CG-dextran	confocal	Fetcho and O'Malley, 1995; O'Malley et al., 1996; Ritter et al., 2001
Xenopus	Growth cones in spinal cord	Spontaneous	Cellular	Fluo 3-AM	confocal	Gomez and Spitzer, 1999
Tadpole	Retinotectal projecting axons	Visual	Cellular	CG-dextran	confocal	Edwards and Cline, 1999
C. elegans	Nerve ring neurones	Electrical	Cellular	Cameleon	CCD camera	Kerr et al., 2000
Cricket	Giant interneurone	Wind	Cellular	CG-1	confocal	Ogawa et al., 1999, 2001
Frog	Nose–brain	Electrical Odorants	Cellular	CG-dextran	2-photon wide-field	Delaney et al., 2001
Mammals						
Mouse	Olfactory bulb	Odorants	Bulk	CG-dextran	Fast CCD camera	Wachowiak and Cohen, 2001
Rat	Somatosensory cortex layer 2/3 neurones	Whisker	Cellular	CG-1	2-photon	Svoboda et al., 1997, 1999a
Rat	Somatosensory cortex layer 5 neurones	Whisker	Cellular	CG-1	2-photon	Helmchen et al., 1999
Rat	Olfactory bulb, mitral and tufted cells	Odorants	Cellular	CG-1	2-photon	Charpak et al., 2001

CG, calcium green.

measured during sensory input or motor output, including several semi-intact in vitro preparations in which the sensory organ and its associated region of the brain were both intact.

Ca^{2+} indicators have been employed for two distinct purposes in vivo: to measure bulk activity in a whole area and to measure single-cell or sub-cellular $[Ca^{2+}]_i$ changes. In the first case many neurones and/or axon fibre tracts are stained simultaneously using either the membrane-permeable acetomethoxy(AM)-ester form or the dextran-conjugated form of a Ca^{2+} indicator (Tsien, 1989; O'Donovan et al., 1993). Fluorescence changes are imaged at low resolution (without cellular resolution) representing overall functional maps of excitation, similar to intrinsic signal or voltage-sensitive dye imaging experiments. For example, odor-evoked activity patterns have been revealed in the olfactory system of honeybee, zebrafish, and Drosophila (for references, see Table 1). More recently this approach has also been applied to the olfactory bulb of mouse and turtle (Wachowiak and Cohen, 2001; Wachowiak et al., 2002).

Single-cell and dendritic Ca^{2+} dynamics can be studied following labeling of either individual neurones via an intracellular electrode or a small group of neurones using dextran-conjugated dyes. The system which has been studied in greatest detail is the visual system of the blowfly (for reviews, see Borst and Egelhaaf, 1994; Egelhaaf et al., 2002). Its large motion-sensitive interneurones are readily accessible and filled via an intracellular recording electrode with a Ca^{2+} indicator. The spatio-temporal pattern of dendritic Ca^{2+} influx during visual stimulation was monitored using a CCD camera. Remarkably, $[Ca^{2+}]_i$ elevations were localized to dendritic branches in a retinotopic fashion (Borst and Egelhaaf, 1992). Upon presentation of a moving grating, branches were activated sequentially and displayed temporal fluctuations with phase shifts that depended on local luminance changes. These branch-signals, however, were then spatially integrated, leading to a smooth $[Ca^{2+}]_i$ change in the axon (Single and Borst, 1998). More recently, the same method was applied to study synaptic transmission and its dependence on presynaptic $[Ca^{2+}]_i$ using sensory stimulation (Kurtz et al., 2001) and differences in dendritic processing between different cell classes (Dürr et al., 2001).

In another seminal in vivo study, Ca^{2+} signals were investigated in a large cricket auditory interneurone (Sobel and Tank, 1994). The time course of sound-evoked $[Ca^{2+}]_i$ changes was found to match the time course of modulation of the auditory responsiveness to sound pulses (a phenomenon called 'forward masking'). This effect can be explained by activation of a Ca^{2+}-dependent hyperpolarizing current and demonstrates the importance of cellular Ca^{2+} dynamics for behaviour.

A vertebrate system in which cellular Ca^{2+} signals can be linked directly to behaviour is the hindbrain and spinal cord of the intact larval zebrafish (for reviews, see Fetcho and O'Malley, 1997; Fetcho et al., 1998). Neurones are labeled retrogradely (or by injection of Ca^{2+} indicator into blastomeres; Cox and Fetcho, 1996) and can be imaged with high resolution in the live fish due to its transparency. In a set of hindbrain neurones, different activation patterns have been observed during escape behaviour in response to two different stimuli, a tap to either the head or the tail of the fish (O'Malley et al., 1996). Furthermore, activation patterns were different for different behaviours (Ritter et al., 2001). These studies are beautiful examples of how physiologically relevant activity patterns can be resolved in vivo in a neural network.

In summary, these studies of non-mammalian CNS demonstrate how Ca^{2+} signals that were measured in vivo in neuronal processes can be related directly to sensory processing, the generation of behaviour and developmental processes (Table 1). Unfortunately, in trying to perform similar experiments in mammals we are confronted with multiple problems. One is that cells of interest are typically located deeper in the tissue and that depth penetration is limited due to relatively strong light scattering. Only recently has the application of two-photon laser scanning microscopy enabled deep imaging in the intact mammalian brain.

3. Two-photon imaging of the mammalian CNS

3.1. In vivo two-photon microscopy

Two-photon laser scanning microscopy (2PLSM) is based on two-photon excitation of fluorophores using near-infrared pulsed laser light (Denk et al., 1990). The key advantages of 2PLSM, relative to wide-field and confocal microscopies, are (1) reduced light scattering due to the longer excitation wavelength and (2) confinement of excitation to the focal volume due to the nonlinear dependence of two-photon excitation rate on illumination intensity (Denk et al., 1995). As a consequence, 2PLSM offers high-resolution optical *z*-sectioning deep within brain tissue (Denk and Svoboda, 1997).

Fig. 1A shows schematically a two-photon microscope adapted for in vivo imaging in rodent brain. The animal is deeply anaesthetized and a cranial window is opened. A metal plate is cemented to the animal's skull and the head is held immobilized under the microscope objective. These procedures have been described elsewhere (Svoboda et al., 1999b; Kleinfeld and Denk, 1999), but several points warrant emphasis: (1) A 10-W laser is necessary as pump for the infrared laser to reach imaging depths of 500–600 μm (Kleinfeld et al., 1998; Oheim et al., 2001). (2) Brief laser pulses are essential to maximize penetration depth, because high peak power is necessary and average laser output is limited. Hence femtosecond pulses are essential for in vivo imaging, whereas picosecond pulses are adequate in brain slices (Koester et al., 1999). (3) Depth penetration largely depends on tissue properties and may therefore differ

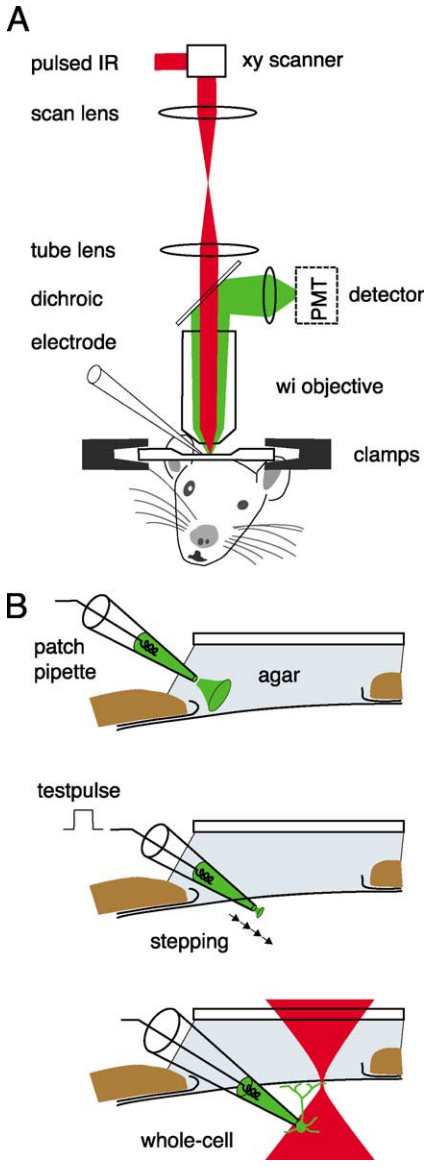

Fig. 1. Combined in vivo imaging and electrophysiology. (A) Schematic of a two-photon microscopy setup. A plate is cemented to the skull of an anaesthetized rat and the skull and head-plate are immobilized using two clamps. A microelectrode or patch pipette is inserted at a shallow angle and used to fill individual neurones with a Ca^{2+} indicator via an intracellular recording. Fluorescence light can be collected efficiently with a detector positioned close to the objective. (B) Protocol for obtaining an in vivo whole-cell recording in an agar-filled cranial window. The patch pipette is lowered through the agar and inserted into the brain while applying high positive pressure (30 kPa). Then the pressure is lowered (0.3 kPa) and the current response to a voltage test pulse is monitored while stepping the pipette forward (2 μm steps). Upon a sharp increase in resistance a GΩ-seal is formed with the help of slight suction. The whole-cell configuration is established and the cell fills with the indicator by diffusion.

between different brain regions. From our experience, the surface distribution of blood vessels has a particularly strong effect on imaging depth. This is probably due to both absorption of laser light and distortion of the illuminating wavefront, with each vessel acting as a small lens. (4) Underfilling the back aperture of the objective is recommended as it minimizes loss of excitation intensity at the back aperture with only a slight decrease in resolution. (5) Optimization of the fluorescence light collection is particularly important. Due to the confinement of excitation all fluorescence photons contribute useful signal. Therefore no detection pinhole is needed and descanning is unnecessary. The detection optics should be designed to collect as much light as possible, for example by placing the detector close to the objective (Fig. 1A). For further technical details on 2PLSM, the reader is referred to a number of references (Denk et al., 1995; Denk, 1996; Mainen et al., 1999; Majewska et al., 2000).

3.2. Combination of 2PLSM with electrophysiology

To date, in vivo $[Ca^{2+}]_i$ measurements from individual neurones in the anaesthetized rat have been obtained with a combination of 2PLSM and intracellular recordings. In these experiments, intracellular access was essential in order to load the neurone with a water-soluble Ca^{2+} indicator (e.g. Calcium Green-1 or Oregon Green BAPTA-1).

These experiments present several technical challenges to the experimenter. Firstly, a large cranial window must be opened over the region to be examined (at least 1 × 1 mm). With such a large cranial window, pulsatile motion of the brain poses a severe problem. This motion is caused by the heartbeat, and to a lesser extent, the breathing cycle. Furthermore, removal of the dura mater across much of the cranial window is necessary, both to allow the insertion of electrodes and to optimize optical access. (Although it is possible to obtain images through the dura, image quality and depth penetration are both reduced). Unfortunately, removal of the dura substantially exacerbates pulsatile motion of the brain, often resulting in oscillatory movements of 5–10 μm at around 8 Hz, both laterally and/or perpendicular to the focal plane. In addition, motion lowers the probability of obtaining stable intracellular recordings, which are necessary to load cells with sufficient amounts of dye. Under these conditions, it is almost impossible to obtain acceptable Ca^{2+} signals from a small structure such as a dendrite. One key requirement for high-resolution imaging is therefore the reduction of this pulsation. Sufficient stabilization can generally be obtained by closing the cranial window with 1–2% (w/v) agar, which is then capped with an immobilized glass coverslip (Fig. 1B and Svoboda et al., 1999b). Further reduction of brain motion may be desirable when imaging particularly small structures (such as dendritic spines; see Section 4.1) or when imaging repeatedly (such as when acquiring a time-lapse series). For these situations, triggering image acquisition from the animal's heartbeat (as measured with an electrocardiogram) proves beneficial.

In initial studies intracellular recordings were obtained with flexible sharp microelectrodes, which tended to bend as they were advanced through the agar. This made it hard to position the tip of the electrode on the brain surface. More

recently, we have performed whole-cell patch clamp recordings in vivo (for technical details, see Margrie et al., in press). Most importantly we have found that patch pipettes can be inserted through the agar while applying positive pressure to prevent the pipette tip from clogging (Fig. 1B). It is even possible to navigate the patch pipettes within the agar in three dimensions, which is useful to avoid hitting surface blood vessels. With reasonable access resistance (<40 MΩ) and a pipette dye concentration of 200 μM cells and proximal dendrites can be imaged within about 10–15 min.

Several cell types have been visualized in the intact rat brain with these techniques. Some examples are shown in Fig. 2.

3.3. Dendrites in the mammalian CNS

In 1994, Denk et al. explored the application of 2PLSM in vivo for the first time, studying the neocortex of anaesthetized rats (Denk et al., 1994). The cortical surface was soaked with a fluorescent dye, staining the extracellular space, and two-photon images were obtained at different focal depths. Neuronal somata and proximal dendrites could be resolved as unstained structures. These early experiments demonstrated that 2PLSM provides a penetration depth large enough to image neurones in supra-granular cortical layers (layers 1–3).

The combination of intracellular recording and in vivo Ca^{2+} imaging was first applied to layer 2/3 neocortical pyramidal neurones in rat barrel cortex (Svoboda et al., 1997). The apical dendrite of these cells extends toward the pial surface of the cortex (i.e. toward the microscope objective; see Fig. 1), typically branching near the border of layers 1 and 2 and giving rise to a distal apical tuft with many branches in layer 1. The entire apical dendritic tree is therefore accessible to 2PLSM such that $[Ca^{2+}]_i$ transients can be measured sequentially at different dendritic locations (Fig. 3; see also Svoboda et al., 1999b).

Ca^{2+} influx into dendrites may occur via different pathways, including voltage-dependent Ca^{2+} channels, NMDA-type glutamate receptors or by release from intracellular stores. One of the main questions in the initial study was under what conditions and through which pathway dendritic Ca^{2+} influx may occur during sensory stimulation. Whisker deflections evoked dendritic Ca^{2+} signals, which required action potentials and scaled with action potential number (Svoboda et al., 1997), suggesting that the observed Ca^{2+} influx was due to opening of voltage-dependent Ca^{2+} channels. Ca^{2+} influx through synaptic receptor channels

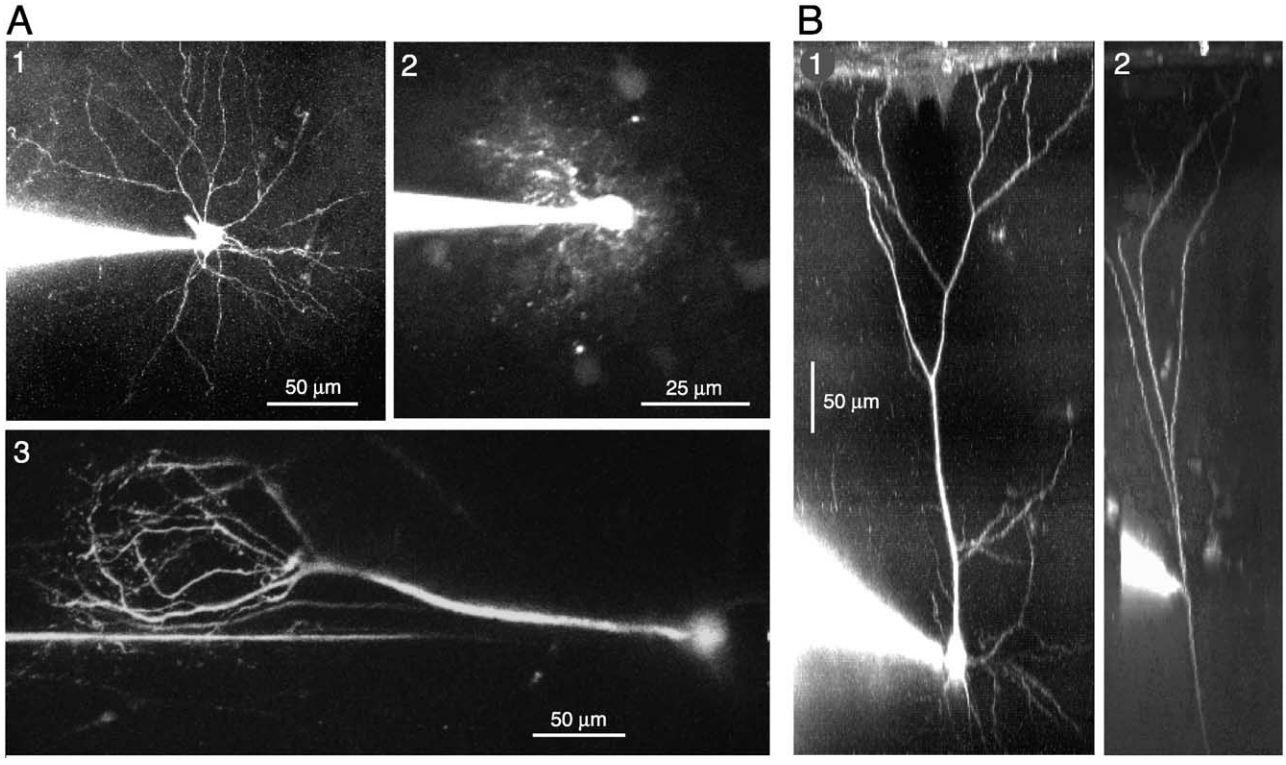

Fig. 2. In vivo two-photon images of different cell types. Stacks of fluorescence images were collected following labelling of individual cells. (A) Maximum-intensity projections from the surface down into the brain; (1) Basal dendritic tree of a layer 2/3 neurone filled via a patch pipette with 500 μM Oregon Green BAPTA-1; (2) Glial cell (astrocyte) filled with 500 μM Oregon Green BAPTA-1; (3) Mitral cell in the olfactory bulb filled via a sharp electrode with Calcium Green-1 (with permission from Charpak et al., 2001). (B) Side projections calculated from 3D stacks; (1) Same layer 2/3 pyramidal neuron as shown in (A). Note the shadowing effect of the large blood vessel near the surface; (2) Apical dendritic tuft of a presumed layer 5 neurone filled via a dendritic patch clamp recording (500 μM Oregon Green BAPTA-1).

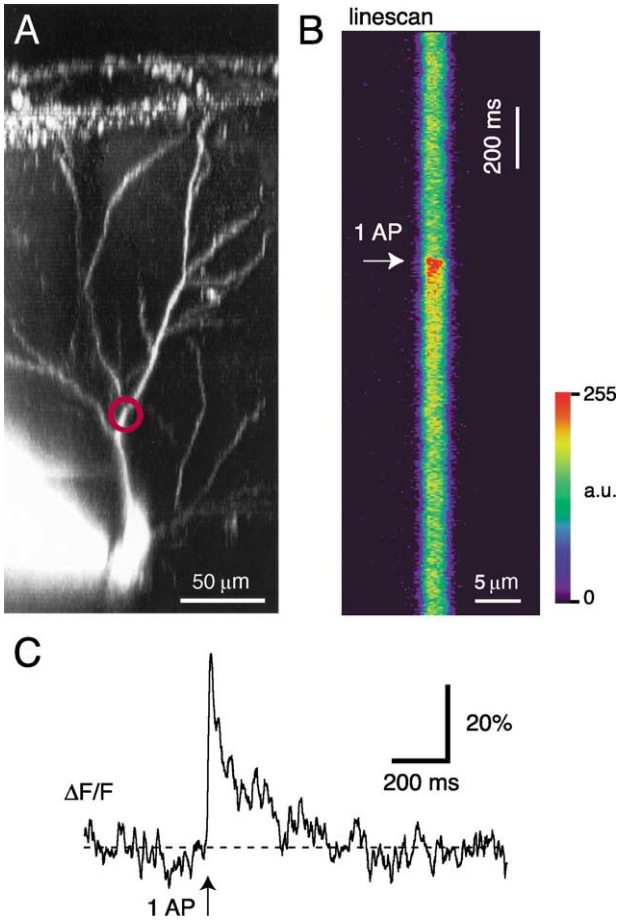

Fig. 3. Action potential-evoked dendritic $[Ca^{2+}]_i$ transient. (A) Side projection of a layer 2/3 pyramidal neurone in rat somatosensory cortex. The soma was about 300 μm below the pial surface. (B) Line scan image showing the fluorescence increase (in arbitrary units, a.u.) evoked by a single action potential in the proximal apical dendrite (red circle in A). (C) Time course of the relative fluorescence change ($\Delta F/F$) obtained from the linescan image shown in (B).

is presumably highly localized and therefore difficult to resolve in vivo (see Section 4.1, below).

In layer 2/3 pyramidal neurones, the action potential-evoked Ca^{2+} signals are largest in the proximal dendrite and decrease in the distal region. Typically in adult rats a single action potential does not induce Ca^{2+} influx in the terminal branches in layer 1. This decline was first attributed to strong action potential attenuation (Svoboda et al., 1999a). However, recent data indicate that action potentials attenuate only mildly and that backpropagation is active in vivo (Waters et al., 2001). It is more likely therefore that the gradual decrease in action potential size is sufficient that depolarization drops below the threshold for Ca^{2+} channel activation in the distal part of the dendritic arbor. Several observations, nevertheless, indicate the presence of functional Ca^{2+} channels in this distal region. First, direct current injection into the dendrite in some neurones evoked regenerative Ca^{2+} spikes with accompanying large $[Ca^{2+}]_i$ transients (Svoboda et al., 1999a). Second, high-frequency bursts of action potentials were able to evoke Ca^{2+} influx in distal branches (Waters et al., 2001). It remains to be determined, however, under what circumstances distal Ca^{2+} influx, which could be involved in plasticity of layer 1 synapses, might be induced by natural stimulation.

Neuromodulatory afferents are known to affect various channel types and their activation could change cellular properties and affect dendritic computations in neocortex. For example, tail pinch is known to activate subcortical areas projecting to the neocortex (Detari and Vanderwolf, 1987). The effect of a brief tail pinch on dendritic Ca^{2+} signals was investigated by Svoboda et al. (1999a). Neuronal excitability was generally increased, resulting in more action potentials upon sensory stimulation, but the Ca^{2+} influx per action potential was unchanged. Thus no specific effect on Ca^{2+} signaling mechanisms has been found so far, but more experiments are needed (perhaps focussing on different and more specific stimuli).

Another cell type that has been studied in vivo using 2PLSM is the layer 5 pyramidal neurone. Its long apical dendrite can extend more than a millimeter and form a tuft in layer 1, similar to that of the layer 2/3 neurone. Findings from in vitro studies showed that L5 cell dendrites contain voltage-dependent sodium and Ca^{2+} channels, supporting active backpropagation of somatically induced action potentials and enabling initiation of regenerative events in the distal dendrite (Amitai et al., 1993; Yuste et al., 1994; Schiller et al., 1997; Larkum et al., 1999a,b). As dendritic Ca^{2+} action potentials may enable the cell to associate proximal and distal inputs (Larkum et al., 1999b), it is important to ask whether such dendritic Ca^{2+} action potentials occur in vivo.

In adult rat brain, the somata of layer 5 neurones are located more than 800 μm below the pial surface—too deep to be visualized with 2PLSM. Hence studying their dendritic properties with a combination of electrophysiology and 2PLSM is particularly challenging. In some instances dendritic recordings were obtained in vivo and no soma could be found within the superficial 500 μm of cortex, indicating that the dendrite was that of a deep-layer (presumed layer 5) pyramidal neurone (Helmchen et al., 1999; see also Fig. 2). In these dendrites large depolarizing potentials were observed, suggesting the generation of regenerative Ca^{2+} events (Helmchen et al., 1999).

To confirm that the dendrites of somatically filled layer 5 neurones display these properties, recordings were made from deep-layer somata. Imaging the distal dendrites of somatically filled cells was possible although locating the labeled distal apical tuft proved difficult, requiring a search in three dimensions following trigonometric calculations. Each somatically impaled neurone was also filled with neurobiotin, enabling subsequent histochemical identification of the neurone. Consistent with the data from dendritic recordings, large Ca^{2+} signals occurred in distal apical dendrites in conjunction with bursts of action potentials observed in the somatic electrical recording (Fig. 4). Den-

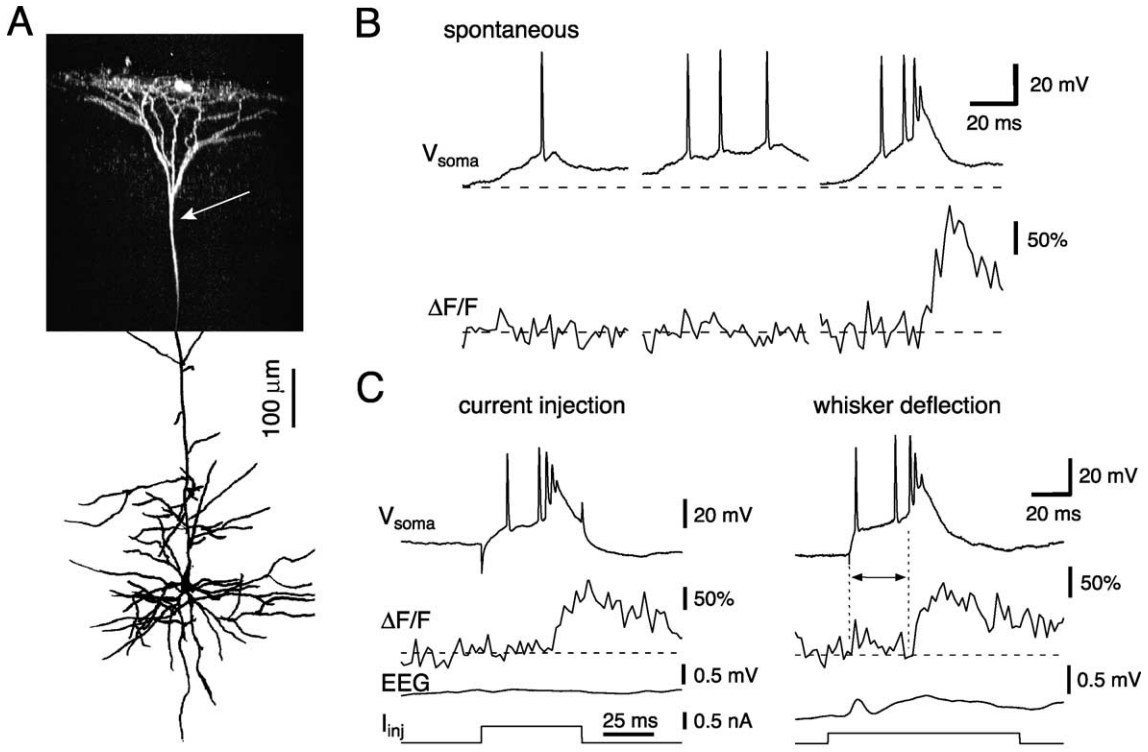

Fig. 4. Burst-associated distal dendritic Ca^{2+} signals in layer 5 pyramidal neurones. (A) Fluorescence image side-projection of the apical tuft of a layer 5 pyramidal neurone in rat barrel cortex filled via a somatic sharp electrode with Calcium Green-1. The histological (neurobiotin) reconstruction of the perisomatic region is overlayed. Arrow indicates the main bifurcation. (B) Somatic membrane potential (V_{soma}) and dendritic fluorescence changes measured near the main bifurcation (different cell from A). A single action potential or multiple action potentials at low frequency caused no measurable $[Ca^{2+}]_i$ increase although a large calcium signal was evoked by a spontaneous high-frequency burst of action potentials. (C) Distal $[Ca^{2+}]_i$ transients evoked by bursts induced by somatic current injection (left) and by a deflection of the whiskers (right). Adapted from Helmchen et al. (1999).

dritic Ca^{2+} action potentials presumably are involved in the generation of action potential bursts by inducing additional current flow at the soma following synaptic activation or a somatically induced action potential. However, induction of distal dendritic Ca^{2+} influx and action potential bursts with whisker stimulation was quite variable (Helmchen et al., 1999). More experiments will therefore be needed to reveal the rules governing burst generation in the intact neocortex.

In another two-photon study odor-evoked Ca^{2+} signals were measured in the dendrites of mitral cells and tufted cells in the rat olfactory bulb (Charpak et al., 2001; see Fig. 2). In mitral cell dendrites, action potentials induced $[Ca^{2+}]_i$ transients throughout the apical dendritic tree, consistent with in vitro findings that action potentials actively back-propagate in the apical dendrite of these cells without attenuation in size (Bischofberger and Jonas, 1997). In addition, substantial Ca^{2+} signals were observed during odor-induced subthreshold depolarizations and were attributed to the opening of low-threshold voltage-dependent Ca^{2+} channels. As mitral cell dendrites release transmitter as well as receiving synaptic inputs, such graded $[Ca^{2+}]_i$ changes could influence release from dendrodendritic synapses.

In summary, these first in vivo Ca^{2+} imaging studies of the mammalian brain have characterized basic patterns of dendritic $[Ca^{2+}]_i$ accumulations during action potential firing. The future prospect is that the in vivo approach will enable us to reveal in more detail sensory-evoked $[Ca^{2+}]_i$ distributions in dendrites and to understand their role in dendritic processing and neocortical function. On-going developments in labeling techniques and microscopy are creating even more opportunities for such studies.

4. Outlook

4.1. Ca^{2+} imaging of spines

One exciting new possibility is the resolution of synaptic activation patterns using sensory stimuli. Natural stimuli could cause clustered activation of synapses, perhaps on individual dendritic branches, they could cause activation of synapses distributed throughout the dendritic tree or perhaps the reality is somewhere in between. This is very much an open question at present. These input patterns can greatly influence information processing in dendrites (Poirazi and Mel, 2001). In the case of clustered input, it remains to be determined whether localized dendritic branch activation occurs in vivo, as reported for neocortical and hippocampal neurones in brain slices (Schiller et al., 2000; Wei et al., 2001). The barrel cortex, with its topographical organization, is an ideal system in which to address this question.

However, since excitatory synapses onto neocortical pyramidal neurones are typically at dendritic spines, this means studying $[Ca^{2+}]_i$ transients within spines in vivo.

2PLSM offers sufficient resolution for this task; in brain slice preparations two-photon microscopy has been used to reveal detailed information about Ca^{2+} signaling in individual dendritic spines (Yuste and Denk, 1995). Over the past years, several studies have addressed which sources contribute to Ca^{2+} influx into spines in vitro and how buffering and extrusion mechanisms shape the local $[Ca^{2+}]_i$ transient (for reviews, see Denk et al., 1996; Yuste et al., 2000; Sabatini et al., 2001). Fig. 5 demonstrates that dendritic spines and spine Ca^{2+} signals can be resolved in vivo using 2PLSM. Spines can be resolved on apical dendritic branches and in the case of layer 2/3 pyramidal neurones also on basal dendrites, even several hundred micrometers below the pial surface. In principle therefore similar studies of Ca^{2+} signaling at individual CNS synapses should be possible in vivo. The reason for the lack of such studies to date is probably that in vivo one is confronted with several additional experimental difficulties. First, brain pulsation in most cases still occurs on the micrometer level

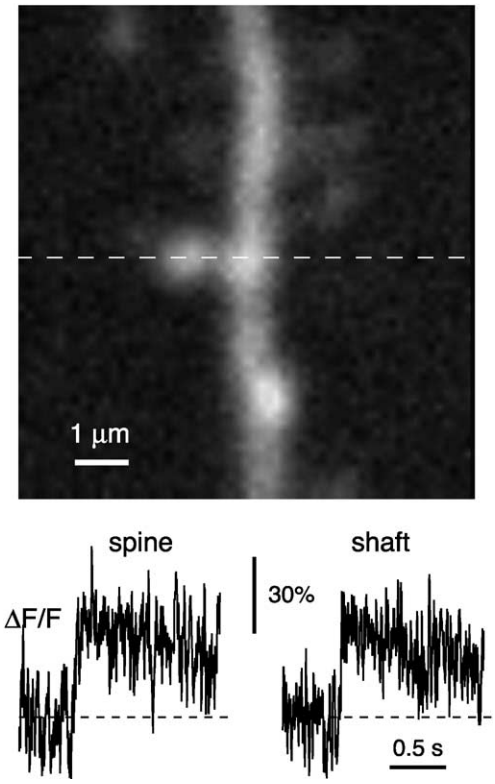

Fig. 5. In vivo $[Ca^{2+}]_i$ measurement from a dendritic spine. Two-photon excited fluorescence image of a distal apical dendrite of a neocortical pyramidal neurone filled with 500 µM Oregon Green BAPTA-1. The branch was located about 20 µm below the pia. Fluorescence in a spine and the parent dendrite was measured using a linescan (dashed line). $[Ca^{2+}]_i$ transients were elicited both in the spine (lower left) and the shaft (right) following a high-frequency burst of action potentials induced by somatic current injections.

even with agar over the cranial window (see Section 3.2, above). Perhaps an even bigger hurdle, however, is locating a synaptically activated spine. This will almost certainly prove extremely difficult since only a few of a cell's many thousands of spines are likely to be activated by a sensory stimulus. Measuring synaptically induced Ca^{2+} signals has in fact proved difficult even in brain slices, where an extracellular electrode typically has to be placed within a few micrometers of a dendritic branch to permit activated spines on that branch to be identified (Yuste and Denk, 1995). Whether it will be possible to find spines activated in vivo during sensory stimulation is therefore an intriguing question.

4.2. Other cell types

Obviously, the combination of intracellular recordings and 2PLSM can be applied to other parts of the mammalian CNS. The only restriction is that the cell (or at least the region of the cell to be studied) has to be relatively superficial and accessible with a standard water-immersion objective typically used for imaging (working distance 2–3 mm). Readily accessible regions include other neocortical areas (e.g. higher-order sensory areas and motor cortex) and the cerebellum.

In principle other cell types within the neocortex may be studied, including both interneurones and glial cells (see Fig. 2). The likelihood of obtaining a cell penetration using a sharp electrode or a GΩ-seal using a patch pipette is, however, much lower than for pyramidal neurones. From our experience using the blind patch clamp approach about 5–10% of recordings are from glial cells and less than 5% from fast-spiking presumed interneurones. In addition these recordings are typically brief.

4.3. GFP-based Ca^{2+} indicators

Green fluorescent protein (GFP) has revolutionized biological fluorescence microscopy. It is genetically encoded and can be directed to specific neuronal populations using appropriate promoters (Feng et al., 2000). The expressed GFP can be cytosolic or it can be fused to another protein, e.g. glutamate receptors (Shi et al., 1999). A particularly promising development for in vivo $[Ca^{2+}]_i$ measurements are Ca^{2+}-sensitive GFP-fusion proteins, several of which are now available (Miyawaki et al., 1997, 1999; Nakai et al., 2001; Truong et al., 2001). These indicators make it possible to generate transgenic animals, which express a Ca^{2+}-sensitive protein in one or more neuronal population(s) and thus allow the Ca^{2+} signals in the network to be monitored, as recently demonstrated in *C. elegans* (Kerr et al., 2000).

In vivo imaging studies in mammalian brains using GFP-based indicators therefore are foreseeable. The gene encoding the Ca^{2+} indicator could be delivered either by viral transfection (Lendvai et al., 2000) or by insertion in trans-

genic animals (Tucker, 2000). An alternative, non-genetic approach to label neuronal populations could be biolistic loading of organic Ca^{2+} indicators using coated particles (Kettunen et al., 2001).

4.4. Imaging in awake animals

In another exciting development an optical fibre was used to build a miniaturized two-photon microscope that permits high-resolution imaging in awake, freely moving rats (Helmchen et al., 2001). The device is small enough, at about 25 g, to be carried by an adult rat and thus can be used in a similar manner to chronic multi-electrode implants.

Using this two-photon fiberscope neurons were visualized down to 250 μm below the brain surface in anaesthetized rats and $[Ca^{2+}]_i$ transients could be resolved in dendrites. Furthermore, stained blood capillaries were imaged in awake, freely moving rats. Although technical improvements are still required to increase depth penetration and further reduce the size of the instrument, the fiberscope promises the measurement of $[Ca^{2+}]_i$ transients in single mammalian neurones during behavioural tasks. Thus, experiments similar to those performed in the zebrafish (Fetcho et al., 1998) come within reach in the mammalian brain.

5. Summary

The remarkable depth penetration of two photon microscopy now enables neuroscientists to conduct types of cellular $[Ca^{2+}]_i$ measurements in the intact mammalian brain that previously were possible only in insects and lower vertebrates. To date most experiments have examined the responses of single cells in anaesthetized rodents to artificial or simple sensory inputs. New labelling techniques should enable small groups of neurones to be imaged in real time at cellular resolution, without recourse to labelling through an intracellular pipette. The use of miniaturized microscopes should also extend these studies to awake, freely moving mammals. Aided by these technical advances, future experiments are likely to include studies of more complex sensory stimuli and of cortical activity during behaviours such as motor planning or attentional processing.

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