Fiberoptic System for Recording Dendritic Calcium Signals in Layer 5 Neocortical Pyramidal Cells in Freely Moving Rats

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Murayama M, Pérez-Garci E, Löscher H-R, Larkum ME. Fiberoptic system for recording dendritic calcium signals in layer 5 neocortical pyramidal cells in freely moving rats. J Neurophysiol 98: 1791–1805, 2007. First published July 18, 2007; doi:10.1152/jn.00082.2007. Calcium influx into the dendritic tufts of layer 5 neocortical pyramidal neurons modifies a number of important cellular mechanisms. It can trigger local synaptic plasticity and switch the firing properties from regular to burst firing. Due to methodological limitations, our knowledge about Ca\(^{2+}\) spikes in the dendritic tuft stems mostly from in vitro experiments. However, it has been speculated that regenerative Ca\(^{2+}\) events in the distal dendrites correlate with distinct behavioral states. Therefore it would be most desirable to be able to record these Ca\(^{2+}\) events in vivo, preferably in the behaving animal. Here, we present a novel approach for recording Ca\(^{2+}\) signals in the dendrites of populations of layer 5 pyramidal neurons in vivo, which ensures that all recorded fluorescence changes are due to intracellular Ca\(^{2+}\) signals in the apical dendrites. The method has two main features: 1) bolus loading of layer 5 with a membrane-permeant Ca\(^{2+}\) dye resulting in specific loading of pyramidal cell dendrites in the upper layers and 2) a fiberoptic cable attached to a gradient-index lens and a prism reflecting light horizontally at 90\(^\circ\) to the angle of the apical dendrites. We demonstrate that the in vivo signal-to-noise ratio recorded with this relatively inexpensive and easy-to-implement fiberoptic-based device is comparable to conventional camera-based imaging systems used in vitro. In addition, the device is flexible and lightweight and can be used for recording Ca\(^{2+}\) signals in the distal dendritic tuft of freely behaving animals.

INTRODUCTION

In vitro studies have shown that the apical tuft dendrite of layer 5 (L5) neocortical pyramidal neurons can generate Ca\(^{2+}\) spikes that control the firing properties of the neuron (Kim and Connors 1993; Schiller et al. 1997; Williams and Stuart 1999). In addition, Ca\(^{2+}\) spikes are strongly suppressed by inhibitory neurons targeting the distal dendrites (Pérez-Garci et al. 2006). It has been suggested that this cellular property works as a form of coincidence detection, allowing the cortex to associate bottom-up and top-down information (Larkum et al. 1999b, 2004). Thus it is possible that the generation and suppression of Ca\(^{2+}\) spikes in the dendritic tuft are causally related to distinct behavioral states (Cauller 1991; Lamme et al. 1998; Llinás et al. 2002; Olson et al. 2001). So far, this conjecture could not be explored because of the difficulty in recording from the dendrites in awake behaving animals.

One-photon Ca\(^{2+}\) imaging remains an important low-cost tool. Its usage is relatively straightforward and it can be applied to record Ca\(^{2+}\) changes in very thin dendritic structures (Augustine et al. 2003), providing the opportunity for multisite recordings (Baker et al. 2005) and wide-field imaging from populations of neurons (Froemke et al. 2002). However, one-photon epifluorescence imaging techniques are not usually ideal for in vivo imaging because brain tissue scatters light (Cuche et al. 1999), resulting in smaller signal-to-noise ratios for optical measurements. In addition, the perspective of one-photon imaging in vivo is normally from above the brain looking vertically down into the tissue (as opposed to side-on as in the slice), making all but the upper portion of the cortex inaccessible. This problem has been overcome by the use of endoscopes to probe deeper into the tissue, as shown in some studies with anesthetized animals (Duff and Schmidt 2000; Göbel et al. 2004; Hirano et al. 1996; Ikeda and Matsushita 1980; Jung et al. 2004; Kudo et al. 1992) and freely moving animals (Adelsberger et al. 2005; Ferezou et al. 2006; Poe et al. 1994; Rector and Harper 1991). However, the vertical orientation of many neocortical dendrites is still unfavorable to such approaches. In addition, these studies often involve recordings from unidentified neuronal structures (e.g., soma, dendrites, and axon) of unspecified neuronal populations (e.g., various excitatory and inhibitory neurons and glial cells). The two-photon imaging approach is very attractive because it can in principle resolve structures as small as dendrites in vivo (Garschuk et al. 2006; Helmchen et al. 1999; Svoboda et al. 1997), although so far it has been possible to image structures only the size of blood vessels in the freely moving preparation (Helmchen et al. 2001). Two-photon imaging is also much more expensive and technically difficult to implement.

Our goal in this study was to develop a fiberoptic-based one-photon recording system (Flusberg et al. 2005) for specifically measuring dendritic Ca\(^{2+}\) transients in the apical dendrites of L5 pyramidal neurons in vivo. The design emphasizes ease of implementation and robustness under demanding conditions (e.g., freely moving), which would open new possibilities for studying dendritic physiology from a more functional perspective. We present a novel method based on two key features: 1) a procedure for specific loading of a Ca\(^{2+}\)-sensitive dye to the dendrites of L5 pyramidal neurons (Kerr et al. 2005) and 2) an optical fiber fitted with a gradient-index (GRIN) lens and a micropipram angled at 90\(^\circ\). This optical assembly acted as a fiberoptic “periscope,” enabling us to locally illuminate and image distal dendrites of L5 pyramidal neurons. Combining these techniques allowed us to record intracellular Ca\(^{2+}\) tran-
sents from populations of L5 pyramidal cell dendrites in freely moving rats.

**METHODS**

**Animals and surgery**

Female Wistar rats (P30–P50) were used in these experiments. For in vivo anesthetized experiments, animals were deeply anesthetized with urethane (intraperitoneal, 1.5 g/kg). The head was fixed horizontally in a stereotaxic instrument (Model SR-5R, Narishige, Tokyo, Japan) and body temperature maintained at 36 to 37°C. An outline of the craniotomy above the primary somatosensory cortex was marked with a dental drill. The craniotomy (diameter: 1–2 mm), centered at 1.5 mm posterior to bregma and 2.0 mm from midline in the right hemisphere, was performed and the dura mater was surgically removed immediately before Ca²⁺ recording (see following text). This region was chosen so that the dendrites of pyramidal cells taking up the dye lay parallel to the slice surface in the slice preparation subsequently described. Care was taken to avoid any damage to pial vessels or the cortex.

For freely moving experiments, the scalp was removed under general anesthesia (isoflurane; Baxter, Volketswil, Switzerland) and a local anesthetic (lidocaine; Sigma–Aldrich, Buchs, Switzerland) was applied to the wound. Afterwards the animal was allowed to regain consciousness and the animal was administered with an analgesic (buprenorphine, twice per day; Essex Chemie, Lucerne, Switzerland) and body temperature maintained at 36 to 37°C.

**In vivo loading of Ca²⁺-sensitive dye**

Oregon Green 488 BAPTA-1 (OGB-1) AM (O-6807, 50 μg; Molecular Probes, Eugene, OR) was mixed with 5 μL of pluronic acid (Pluronic F-127, 20% solution in DMSO, Molecular Probes) for 15 min. The solution was then diluted in 28 μL of HEPES-buffered solution (125 mM NaCl, 2.5 mM KCl, 10 mM HEPES) and mixed for a further 15 min. The OGB-1 AM solution was loaded into a glass pipette (tip diameter: 5–15 μm) and pressure-injected into L5 (see earlier text), and a metal post was fixed to the skull with dental cement. This surgery and the subsequent experiment lasted about 3 h before the animal was killed. After 1–2 h of anesthesia, the animals could move within a 50 × 50-cm environment completely freely and their behavior was not obviously affected by the presence of the attached fiber.

**In vivo Ca²⁺ recording**

A custom-made fiberoptic-based one-photon microscope was constructed for in vivo recordings (Fig. 1). A 300-W xenon lamp (Model 66902; Oriel Instruments, Stratford, CT) was used as a light source. We also tested an approximately 200-mW blue light-emitting diode (470 nm, Luxeon V Star; Philips Lumileds Lighting, San Jose, CA), which gave a smaller signal than the xenon lamp. However, we could record Ca²⁺ changes in the dendrites due to evoked signals with this light source. A cold mirror (FM 204; Thorlabs, Karlsfeld, Germany) was used for splitting the incident light into visible and near infrared light. An excitation filter, a dichroic mirror, and an emission filter (as a filter set 31001; Chroma Technology, Rockingham, VT) were used for epifluorescence Ca²⁺ recordings. Two objectives were used depending on the configuration (see following text): a ×6.3 objective (Model 04-OAS-008, 0.20 numerical aperture (NA); Melles Griot, Hessen, Germany) and a ×10 objective (Model E58-372, 0.45 NA; Edmund Optics, Karlsruhe, Germany). A tube lens (InfiniTube standard; Infinity Photo-Optical, Göttingen, Germany) was used to focus the image onto a chip of a cooled charge-coupled device (CCD) camera (MicroMax; Roper Scientific, Trenton, NJ).

Fluorescence intensities were sampled at 10 or 100 Hz. Data were acquired on a PC using WinView software (Roper Scientific). Regions of interest (ROIs) were chosen off-line for measuring fluorescence changes (see Data analysis). Bicuculline methiodide and tetrodotoxin (TTX) were bought from Tocris Cookson (Zurich, Switzerland), cadmium chloride from Fluka (Buchs, Switzerland), and kynurenic acid from Sigma–Aldrich.

**Illumination and optical fibers**

Two different optical configurations were used. 1) A one-fiber–based system (type A) in which excitation light and fluorescence light were guided through the same fiber (Fig. 1, A and C) and 2) a two-fiber–based system (type B) in which the excitation light and fluorescence light were guided through two separate fibers (Fig. 1B). We used two variations on the two-fiber system: a micropipram attached to the end of an illuminating fiber (type B-1; Fig. 1D) and a fiber without the micropipram to illuminate neurons obliquely at an angle of 20° from above the cortical surface (type B-2; Fig. 1E, see following text).

**ONE-FIBER–BASED SYSTEM.** A 2-m-long fiber bundle (IGN-06/17; Sumitomo Electric Industries, Tokyo, Japan), consisting of 17,000 fiber elements, was used for a combined illuminating–recording fiber (Fig. 1, A and C). The individual elements had a diameter of 2.5 μm and NA of 0.35. The image area of the bundle had a diameter of 0.54 mm. The total outer diameter of the bundle was 0.68 mm. Minimum bend radius of the fiber was 30 mm. The end face of the bundle was fitted with a prism-lens assembly that consisted of a right-angle prism (dimensions: 0.5 × 0.5 × 0.5 mm; GrinTech, Jena, Germany; Fig. 1E) attached to a GRIN lens (diameter: 0.5 mm; NA: 0.5; GrinTech). These optical elements were glued to each other with UV curing adhesive (Norland Optical Adhesive 63; Norland Products, Cranbury, NJ). The working distance was nominally 100 μm (a slight difference may have occurred due to the adhesive) and the magnification was ×0.73, resulting in a 685-μm-diameter field of view (FOV).

**TWO-FIBER–BASED SYSTEM.** A 2-m-long fiber bundle (IGN-08/30; Sumitomo Electric Industries), consisting of 30,000 fiber elements was used for recording with a top-down view above the surface of the brain (Fig. 1, B and D). The image area had a diameter of 0.72 mm. The total outer diameter of the bundle was 0.96 mm. Minimum bend radius of the fiber was 40 mm. The end face of the bundle was connected to a ×4.4 microscope objective that had a working distance of 300 μm and consisted of two types of GRIN lenses (GrinTech): one with NA of 0.5, geometrical length of 2 mm, and diameter of 1 mm, another with NA of 0.11, geometrical length of 11.175 mm, and diameter of 1 mm, resulting in a 155-μm FOV. The end face of the fiber was glued to a right-angle prism (dimensions: 0.5 × 0.5 × 0.5 mm; Edmund Optics). The same fiber was used for the noninvasive recording system without a prism (Fig. 1, E and G, and Supplemental Fig. 2). 1

1 The online version of this article contains supplemental data.
FIG. 1. Fiberoptic-based one-photon microscope. Two basic configurations were tested with one or two fibers. A: schematic diagram of the single fiberoptic-based recording system using a ×10 objective illuminating and recording from a 680-μm-diameter bundle of 17,000 fiberoptic elements. B: two-fiber recording system. Illumination was achieved with a ×6.3 objective and a single-core fiber. Recordings were made with the ×10 objective and a 0.96-mm-diameter fiberoptic bundle with 30,000 elements. C: optical diagram of light rays for the single-fiber system shown in A. "Periscope" system was used for illumination, consisting of a 90° prism combined with a gradient-index (GRIN) lens. Sketch (gray) of L5 pyramidal neuron drawn to scale shows the size of the periscope system relative to the apical dendrite. D: optical diagram of light rays in the periscope and the microobjective used for the two-fiber system shown in B. Sketch in gray indicates the relative size of a pyramidal neuron. E: optical diagram for a noninvasive variation on the two-fiber approach with an optical fiber oriented obliquely at 20°. F: picture of the 488-nm light passing through the periscope in a 2% agar block. Sketch of L5 pyramidal neuron (green) shows the portion of the dendritic tree illuminated by the periscope. Inset: dimensions of the microprism. G: picture of the 488-nm light from the oblique fiber. White lines indicate edges of the agar block.
less than we measured from the conventional epifluorescence microscope used for in vitro experiments (~8 mW). We never saw any evidence of photodamage in vitro or in vivo, even though the total illumination in vivo lasted 18–27 min. We assessed this by comparing the signals recorded at the beginning of the experiment to the end in terms of baseline, amplitude, and duration. We expect photodamage to be reduced in this preparation by the fact that there are relatively few dye molecules in the dendrites compared with the somata, the illumination is relatively weak, and the dendritic dye milieu is constantly being replenished by diffusion of dye from the soma.

In vitro experiments

Animals were anesthetized with a mixture of 95% CO₂-5% O₂ before decapitation according to the guidelines of the veterinary office of the canton of Bern. In vitro optical imaging and electrophysiological recording were performed on L⁵ pyramidal neurons of the primary somatosensory cortex in parasagittal slices of rats. After decapitation, the brain was rapidly removed into ice-cold, oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 25 NaHCO₃, 25 KCl, 1.25 NaH₂PO₄, 1 MgCl₂, 25 glucose, and 2 CaCl₂ (pH 7.4). Slices (300 μm thick) were cut with a vibrating microslicer on a block angled at 15° to horizontal and maintained at 37°C in the preceding solution for 15–120 min before use. Slices were first examined to ensure that they had dendrites running parallel to the surface of the slice (usually one to three slices per hemisphere).

Bolus loading of populations of L⁵ neurons was performed with different membrane-permeant Ca²⁺-sensitive dyes: OGB-1 AM, Fura-2 AM, Fluo-3 AM, and Calcium Orange AM (Molecular Probes). The labeling solutions were prepared as described earlier for in vivo injection. Briefly, each dye of 50 μg was mixed with 5 μL of pluronic acid in 20% DMSO and 28 μL of HEPES-buffered solution. A glass pipette (tip diameter: 5–15 μm) was used for the labeling solution and positioned in L⁵ in the slice. A small amount (<0.5 μL) of the labeling solution was pressure-injected continuously with a pressure of 8–22 kPa for 1.5–2 h into the slice perfused with the ACSF. The region of cells taking up the dye was the same in vitro as with the method of injection described earlier in vivo (see Figs. 2 and 3).

Ca²⁺ imaging and somatic whole cell patch recordings were obtained with Nikon Eclipse E600FN or Olympus BX51WI microscopes. Ca²⁺ imaging was performed using a cooled CCD camera (CoolSNAP; Roper Scientific). The fluorescence was observed using standard epifluorescence filter sets for FITC (excitation at 480 nm; used for OGB-1 AM and Fluo-3 AM), Texas Red (excitation at 560 nm; used for Calcium Orange AM and Alexa 594; Molecular Probes), and Fura-2 (excitation of 380 nm) fluorescence (Chroma Technology). Fluorescence intensities were sampled at 20–100 Hz. Pipettes (4–6 MΩ) for the whole cell patch-clamp recordings were filled with an intracellular solution containing (in mM) 135 K-gluconate, 7 KCl, 10 HEPES, 10 Na₃-phosphocreatine, 4 Mg-ATP, 0.3 GTP, 10 Alexa 594, and 0.2% biocytin (pH 7.2 with KOH). No correction was made for the junction potential between the bath and pipette solutions. The recordings were made with Axoclamp-2B (Axon Instruments, Union City, CA) or Dagan BVC-700A amplifiers (Dagan, Minneapolis, MN), digitized at 10 kHz with an A/D converter (ITC-18 or ITC-16; Instrutech, Port Washington, NY) and acquired on a PC using Igor software (WaveMetrics). Slices were perfused continuously with the ACSF at 33–35°C throughout the experiments.

Data analysis

The fluorescence signal was quantified by measuring the mean pixel value of a manually selected (off-line) region of interest (ROI) for each frame of the image stack using Igor software. For in vivo experiments, Ca²⁺ changes were expressed as ΔFI/Fo = Ft/Fo, where Fo is the fluorescence intensity of the ROI at time t during the imaging experiment and Fo is the mean value of fluorescence intensity before
stimulation. For in vitro experiments, \( \frac{\Delta F}{\Delta F_0} \) changes were expressed as \( \frac{\Delta F}{\Delta F_0} = \frac{F - F_0}{F_0} \), where \( F_0 \) is the background fluorescence measured from a region away from the recorded area. For results obtained with Fura-2, \( \Delta F/F \) was multiplied by \(-1\). Statistical analysis was performed with paired t-test, unless otherwise noted. All numbers are indicated as mean ± SD.

**RESULTS**

Our aim was to develop a method for recording changes in intracellular \( \text{Ca}^{2+} \) (\( \Delta[\text{Ca}^{2+}]_i \)) , specifically in the dendrites of L5 pyramidal neurons in vivo. We chose to take advantage of the fact that L5 pyramidal neurons project their apical dendrites through five layers of the cortex right up to the pia. This meant that by restricting the filling of neurons with a membrane-permeant dye to L5, only those structures that project out of L5 are visible in the upper layers. This approach has already been shown to be feasible using two-photon imaging (Kerr et al. 2005) and we sought to show that the same approach could be used with one-photon \( \text{Ca}^{2+} \) recordings.

This section is structured in the following order. 1) We demonstrate the feasibility of bulk loading L5 pyramidal neurons in vivo with various membrane-permeant \( \text{Ca}^{2+} \)-sensitive dyes. 2) We examine the contribution of different intracellular activities to the \( \text{Ca}^{2+} \) signal observed from a single dendrite and measure its contribution to the population response in vitro. 3) We introduce the fiberoptic-based recording device for recording \( \text{Ca}^{2+} \) signals in vivo. 4) We show that this new device is capable of recording dendritic \( \text{Ca}^{2+} \) signals in the apical tuft of L5 pyramidal neurons in vivo with a large signal-to-noise ratio and show that the signal is predominantly postsynaptic. 5) We show dendritic \( \text{Ca}^{2+} \) recordings in freely moving rats.

**In vivo bolus loading of L5 pyramidal neurons**

After bolus injection of the membrane-permeant \( \text{Ca}^{2+} \)-sensitive dye in vivo (Fig. 2A), we prepared 300-μm-thick parasagittal brain slices around the stained region that could then be viewed in profile in vitro using normal epifluorescence techniques (Fig. 2, B–D; see METHODS). The bolus loading resulted in a region (diameter: ~300 μm) in L5 with particularly high fluorescence (Fig. 2B). In this region most cell bodies and the processes of different cell types were filled with the \( \text{Ca}^{2+} \)-sensitive dye. However, closer inspection of the upper layers above this region revealed that predominantly the apical dendrites of L5 pyramidal neurons were loaded with the dye (Fig. 2, C and D). Between 5 and 20 pyramidal cell tuft dendrites were visibly filled with this method. We detected very little overlap with neighboring slices, but nonetheless we expect the maximum number will be slightly larger in vivo in some cases.

We compared the apparent transport of the dye to the apical dendrites after bolus loading of L5 cells using several different types of commonly used membrane-permeant \( \text{Ca}^{2+} \)-sensitive dyes. For these experiments, the bolus was injected into L5 in vitro (see METHODS), which gave very similar filling of both cell bodies in L5 and the dendrites to the in vivo bolus-loading method. In vitro loading was advantageous for this experiment because optimal slices with apical dendrites parallel to the plane of the slice could be chosen before loading. However, we repeated the experiments shown in Fig. 3 with in vivo bolus loading for one dye (OGB-1 AM), which gave identical results.
(n = 3 slices; data not shown). Of the four dyes tested (OGB-1 AM, Fura-2 AM, Fluo-3 AM, and Calcium Orange AM), all were taken up similarly in the cell bodies located inside the spread of the bolus that we determined by comparing the ratio of fluorescence in the somatic region to background fluorescence in L5 for each dye (12.3 ± 3.5 for OGB-1 AM, 10.7 ± 4.4 for Fura-2 AM, 7.9 ± 3.1 for Fluo-3 AM, and 11.2 ± 5.2 for Calcium Orange AM; n = 3 slices for each dye; Fig. 3B). We found large differences in apparent dendritic transport using a similar procedure comparing the ratio of fluorescence in the tuft region to background fluorescence in another region of L1. The average ratio in the tuft region for each dye was OGB-1 AM, 2.55 ± 0.88; Fura-2 AM, 1.34 ± 0.14; Fluo-3 AM, 1.05 ± 0.04; and Calcium Orange AM, 1.07 ± 0.02 (n = 3 slices for each dye). Here, a ratio of 1 would indicate no visible transport of the dye to the dendrites. Individual tuft dendrites could be seen clearly with OGB-1 AM (Fig. 1D). We never obtained comparable images for the other dyes, although we make no claims about the underlying mechanisms involved.

We also compared the relative ΔF/F after extracellular stimulus of L1 (Fig. 3D). As shown in Fig. 3, E and F, OGB-1 AM gave by far the largest ΔF/F (0.205 ± 0.07 vs. 0.013 ± 0.004 for Fura-2 AM, 0.0034 ± 0.0047 for Fluo-3 AM, and 0.0014 ± 0.0021 for Calcium Orange AM; n = 3 slices for each dye). These extracellularly evoked ΔF/F signals were consistent with the observed level of dye transport to the dendrites. On this basis, we used OGB-1 AM for all further experiments, although we make no claims about the underlying mechanisms involved.

**Examining the source of Ca\(^{2+}\) fluorescence changes in vitro**

In principle, we wanted to compare dendritic fluorescence signals to electrical activity in vivo; however, it was extremely difficult to make in vivo targeted electrical recordings from a bolus-loaded L5 pyramidal neuron. We chose an alternative but similar approach in vitro using a somatic whole cell recording from an identified pyramidal neuron in a slice that had previously been loaded using the in vitro bolus-loading technique. The patch pipette contained a nonindicator dye of a different wavelength (Alexa 594) to identify the apical dendrite (Fig. 4A), but no extra OGB-1 in addition to the OGB-1 AM already taken up by the cell. There was very little leak of the dendritic OGB-1 into the patch pipette, which was confirmed by the fact that there was no significant difference in the reduction of OGB-1 fluorescence in the double Alexa/OGB-1–stained dendrite compared with a nearby dendrite over a 30-min period (88 ± 11% vs. 89 ± 12%, respectively; P = 0.27, n = 3 cells). Most of this reduction was presumably due to bleaching of the dye during the imaging.

By using the “critical frequency” (CF) test (Larkum et al. 1999a; Pérez-Garcí et al. 2006), we could measure Δ[Ca\(^{2+}\)]\(_i\) in the tuft region of the dendrite that resulted from backpropagating action potentials (BPAPs) at a subcritical frequency (Sub-CF, typically <60 Hz) and with regenerative Ca\(^{2+}\) channel activation at a supracritical frequency (Supra-CF, typically >70 Hz) (Fig. 4, B and C). At Supra-CFs, Ca\(^{2+}\) influx into the apical dendrite is equivalent to a Ca\(^{2+}\) spike and the signature of this event can be recorded as an afterdepolarizing potential.

**FIG. 4.** Dendritic Ca\(^{2+}\) spikes detected in L5 neurons loaded with OGB-1 AM in vitro. A: schematic diagram of experiment. Left column: OGB-1 AM was injected extracellularly in L5 in vitro and incorporated by a population of pyramidal neurons. A whole cell recording with a pipette filled with Alexa 594 was made from the soma of a single OGB-1–loaded neuron. ROIs were positioned on the apical tuft of the Alexa-labeled dendrite (ROI 1) and on an adjacent tuft containing only OGB-1 AM (ROI 2). Middle column: sequence of pictures showing the same group of neurons using a FITC filter set (OGB-1 AM, top), a rhodamine filter set (Alexa, middle), and a superimposed picture of the 2 images above (bottom). Right column: magnified views of the areas marked by squares in their respective pictures in the middle column. ROIs for Ca\(^{2+}\) imaging are shown in the bottom picture. B and C: dendritic changes in intracellular Ca\(^{2+}\) (Δ[Ca\(^{2+}\)]\(_i\)) from ROI 1 (solid line) and ROI 2 (dashed line) were measured during trains of action potentials (APs) by the somatic pipette. Two firing frequencies were tested by injecting trains of depolarizing current pulses (bottom traces): one at a subcritical frequency (Sub-CF) of 60 Hz (B) and the another at a supracritical frequency (Supra-CF) of 110 Hz (C), which evoked a Ca\(^{2+}\) spike in one dendrite. Each trace represents the average of 5 trials. D: whole cell somatic recording showing the falling phase of the last AP in the train at the Sub-CF superimposed on the same time period for the Supra-CF (boxes in B and C). Arrowhead indicates the afterdepolarizing potential indicative of a dendritic Ca\(^{2+}\) spike at the Supra-CF. E: summary of the results for B and C (n = 7 cells). *P < 0.001.
EPSP evoked a proportionately larger of one neuron. We refer to the Supra-CF stimulus as a “Ca\textsuperscript{2+} using a theta-glass stimulating pipette (Fig. 5C). The equivalent in vivo data.) The kynurenic acid are shown later in Fig. 7 for comparison with OGB-1–stained neuron (normalized single neuron. No signals were observed outside the Alexa/OGB-1 AM. Moreover, it shows that the Ca\textsuperscript{2+} spike dominates the dendritic response compared with BPAPs in a single neuron. No signals were observed outside the Alexa/OGB-1–stained neuron (normalized \Delta F/F: 0.1 ± 0.02%, n = 3 slices; Figs. 4, B, C, and E), indicating that the response came only from the dendrite of the stimulated neuron, not from the neuropil (e.g., other dendrites and/or axons).

Next, we compared the contribution of different types of intracellular activities [Ca\textsuperscript{2+} spikes, BPAPs, and excitatory postsynaptic potentials (EPSPs)] to the \Delta F/F signal recorded from a single neuron and a population of neurons in vitro (Fig. 5). Here, we again used trains of three to four APs generated in a single identified pyramidal neuron (as shown in Fig. 4A) at a Supra-CF and a Sub-CF and we also evoked synaptic input to the tuft region by stimulating L1 with a weak pulse (~50 \mu A) using a theta-glass stimulating pipette (Fig. 5B). The stimulus strength was chosen by turning up the stimulus until a small electrical response (~1–5 mV) could be measured at the soma of one neuron. We refer to the Supra-CF stimulus as a “Ca\textsuperscript{2+} spike” (see Discussion), the Sub-CF stimulus as “BPAPs,” and the L1 stimulus as an EPSP. We showed that the L1 stimulus evoked a synaptically activated potential because bath application of kynurenic acid abolished the response (Fig. 5B, bottom traces). (Note: Ca\textsuperscript{2+} imaging data for this result with kynurenic acid are shown later in Fig. 7 for comparison with the equivalent in vivo data.) The \Delta F/F from a Ca\textsuperscript{2+} spike recorded in the same neuron was much larger than both the \Delta F/F from BPAPs (Fig. 4) and a L1 stimulus-evoked EPSP (Fig. 5C). Furthermore, all three types of stimuli failed to evoke detectable \Delta[Ca\textsuperscript{2+}]\textsubscript{i} in a nearby region (ROI 2) away from the patched cell where other L5 neurons were loaded with OGB-1 AM. However, when the signal over the whole FOV (108 × 145 \mu m) was averaged (ROI 3, “Wide Field”), the EPSP evoked a proportionately larger \Delta F/F compared with the ROI confined to a single dendrite (Fig. 5, D and E). This is probably explained by the fact that the L1 extracellular stimulus evoked an EPSP response in many different neuronal dendrites in the FOV because this response was blocked by addition of kynurenic acid (Fig. 7, B and C). Nonetheless, the \Delta F/F measured from the whole FOV (ROI 3) during a Ca\textsuperscript{2+} spike in a single pyramidal neuron was still significantly larger than the response during BPAPs (normalized \Delta F/F: 21.0 ± 15.5%, P = 0.01) or the population EPSP response from many neurons (normalized \Delta F/F: 16.3 ± 4.4%, P < 0.01, n = 3 slices). This result indicates that even one Ca\textsuperscript{2+} spike from a population of dendrites can be easily detected.

FIG. 5. Ca\textsuperscript{2+} spikes dominate the fluorescence signal recorded in vitro. A: experimental setup. Left: schematic diagram of experiment (same as in Fig. 4A) showing a population of OGB-1 AM–filled neurons with one L5 pyramidal neuron filled with Alexa 594 by a patch pipette. In addition, a theta glass extracellular stimulating electrode was placed in L1 about 450 \mu m away from the recorded dendrite. Right: sequence of pictures showing a bundle of apical dendrites labeled with OGB-1 AM (top), the tuft of a single Alexa 594/OGB-1–filled neuron (middle), and a superimposed picture indicating the 3 ROIs (bottom). ROI 1 and ROI 2 were set on the Alexa/OGB-1–labeled tuft branch and on an OGB-1 AM–labeled adjacent branch, respectively, and ROI 3 represented the whole FOV (108 × 145 \mu m). B: somatic whole cell recordings from the single Alexa-filled neuron. Top: train of APs evoked by depolarizing current pulses at a Supra-CF (100 Hz). Middle: train of APs evoked at a Sub-CF (30 Hz). Bottom: excitatory postsynaptic potential (EPSP) evoked by the L1 stimulation before (green) and after (black) the application of kynurenic acid (1 mM). Arrowhead indicates the time of the stimulation. C: simultaneously recorded \Delta F/F in ROI 1 (solid lines) and ROI 2 (dashed lines) with the corresponding whole cell recordings in B. Each trace represents the average of 10 trials. D: \Delta F/F from the whole FOV (ROI 3, “Wide Field”) acquired from the same experiment shown in B and C. Each trace represents the average of 10 trials. \Delta F/F was calculated similarly to the in vivo experiment (see Methods). E: summary of the results for D (n = 3 slices). *P = 0.01, **P < 0.01.
Fiberoptic-based epifluorescence recording system

Having established that fluorescence signals can be recorded specifically from the tuft dendrites of populations of L5 pyramidal neurons in vitro using bolus loading in L5, we sought to develop an equivalent method for recording population fluorescence responses in vivo using a fiberoptic one-photon recording system (Fig. 1; see METHODS). We used two basic approaches: 1) a single-fiber-bundle epifluorescence system (Fig. 1, A and C), where illumination and detection were achieved through the same fiber bundle, and 2) two two-fiber systems, where a single optical fiber was used for illumination and a fiber bundle used for detecting fluorescence (Fig. 1, B, D, and E). To confine illumination to the upper layers of the cortex we used a “periscope” fiber using a prism with a 90° angle (Fig. 1, C, D, and F) or an obliquely oriented (20° angle) fiber for the noninvasive version (Fig. 1, E and G).

Using the single-fiber-bundle periscope approach (Fig. 1, A and C, type A; see METHODS), we achieved a side-on view of the upper 685 μm of the cortex equivalent to the in vitro slice perspective (Fig. 6A). We could choose the ROI that exclusively covered the tuft dendrites (Fig. 6A, ROI 1) or predominantly covered the apical dendritic shafts (Fig. 6A, ROI 2).

Ca²⁺ signals recorded from the dendritic tuft region with the fiberoptic-based recording system in anesthetized rats

Under urethane anesthesia, as shown in Fig. 6B, a strong L1 stimulus (~1 mA) with a tungsten electrode similar to that used in vitro (Fig. 3) evoked a similar-sized ΔF/ΔF response in...
vivo (peak amplitude of \( \Delta F/F \) in vitro, 0.11 ± 0.01, n = 3 slices vs. in vivo, 0.083 ± 0.02, n = 8 rats, P = 0.13, unpaired t-test). We used a single extracellular stimulus pulse to L1 both in vitro and in vivo. The in vitro data (taken from the same experiments shown in Fig. 3) were calculated using the whole FOV for the ROI and no background subtraction for comparison with the in vivo data. This shows that the fiberoptic-based periscope system in vivo is equivalent to the conventional epifluorescence recording system used in vitro.

In addition, a spontaneously fluctuating \( \Delta F/F \) signal (average, 1.2 ± 0.2 Hz; range, 0.78–1.6 Hz, n = 8 rats) was observed in vivo (Fig. 6B), which was absent in vitro. The spontaneously fluctuating signal was much smaller than the L1 stimulus-evoked \( \Delta F/F \) signal. The extracellularly evoked fluorescence transient reached peak within 100 ms of the stimulus and decayed toward baseline within 1 s of the peak (Fig. 6C). Surprisingly, the addition of kynurenic acid and bicuculline [to block glutamate and \( \gamma \)-aminobutyric acid type A (GABA\(_A\)) receptors] to the cortical surface led to a significant increase (normalized \( \Delta F/F \) = 142.7 ± 36.2%, P < 0.02, n = 8 rats) in the evoked signal (Fig. 6, B–D). This would be consistent with direct activation of the dendrite by the strong stimulus and reduced dendritic inhibition (Pérez-García et al. 2006). All responses were blocked in the upper ROI (ROI 1) when \( \text{Cd}^{2+} \) was added to the cortical surface above the craniotomy (normalized \( \Delta F/F \) = 2.55 ± 3.0%, P < 0.001, n = 4 rats; Fig. 6D, right) but initially there was no effect in the lower region (ROI 2; Fig. 6, B and C). With extended application of \( \text{Cd}^{2+} \) for >1 h, the ROI 2 signal was also eventually abolished (n = 4; data not shown). Thus the spontaneously fluctuating \( \Delta F/F \) signal was due to voltage-sensitive \( \text{Ca}^{2+} \) channel activity—not, for instance, due to tissue movement caused by cardiovascular pulsations.

The evoked signal shown in Fig. 6 resulted from strong stimulation of L1 that was used to produce a large dendritic signal suitable for comparison of the different fiber recording methods. For a more precise estimation of the presynaptic component of the fluorescence signal, we used a weak stimulus of L1 to avoid direct stimulation of the dendrite before and after the addition of kynurenic acid in vivo (Fig. 7A) and in vitro (Fig. 7B). Here we found a significant reduction of the fluorescence response (normalized \( \Delta F/F \) = 14.7 ± 5.2%, P = 0.001, n = 3 rats) after the addition of kynurenic acid for in vivo experiments (Fig. 7, A and C). Because the evoked signal after blocking glutamatergic transmission was smaller than the spontaneously fluctuating \( \Delta F/F \), we assume that presynaptic \( \text{Ca}^{2+} \) influx was a very small component (<10%) of the fluorescence signal. We also found the same result in vitro using a similar stimulus to L1. Here, the stimulus evoked an EPSP that was 1–5 mV when measured at the soma (see Fig. 5). As shown in Fig. 7, B and C, the \( \Delta F/F \) signal averaged over the whole FOV after L1 stimulus was significantly reduced (normalized \( \Delta F/F \) = 16.3 ± 4.4%, P < 0.001, n = 3 slices; the values were estimated from the same data set in Fig. 5, D and E) by the application of kynurenic acid. Taken together, we conclude that the method detects predominantly postsynaptic \( \text{Ca}^{2+} \) changes.

The spontaneously fluctuating \( \Delta F/F \) signal might have been due to synaptic input to the tuft or to other influences such as BPAPs. Two lines of evidence already suggested that this activity was not synaptic: 1) it was not altered by the addition of blockers of synaptic transmission (Fig. 6) and 2) it was greatest in the lower ROI where there would be less dendritic surface area and therefore fewer synaptic inputs (Fig. 6). We measured the spontaneously fluctuating \( \Delta F/F \) signal by creating histograms of the deviation from the median value in a 5-s window. The average fluctuation was taken as the width of a Gaussian fit to the histogram using the equation: \( y_n = A \exp\left[-(x - x_0)/\text{width}^2\right] \), where \( y_n \) is baseline, \( A \) is amplitude, \( x_0 \) is peak position, and width is 2\( \sigma \). With glutamate and GABA\(_A\) receptors blocked, we applied TTX first to L1 by the cortical surface and then puffed into L5 (Fig. 8A). Whereas the addition of TTX to L1 almost completely abolished the evoked dendritic \( \Delta F/F \) signal (normalized \( \Delta F/F \) = 9.3 ± 7.9%, P = 0.002, n = 3 rats; Fig. 8, B and C), it did not significantly affect the fluctuating \( \Delta F/F \) signal before and after the stimulus (normalized change in fluctuation width: 78.4 ± 9.8%, \( P = 0.063 \), n = 3 rats; Fig. 8B). On the other hand, TTX puffed into L5 significantly reduced the fluctuating \( \Delta F/F \) signal (normalized change in fluctuation width: 32.3 ± 6.0%, \( P < 0.05 \), n = 3 rats; Fig. 8, B–E), consistent with the hypothesis that this activity was mostly generated by BPAPs. \( \text{Cd}^{2+} \) applied to the...
cortical surface blocked the fluctuating ΔF/F signal (Fig. 6) and we assume that cortically applied TTX affects a similar region. Because TTX only partially reduced the ΔF/F signal in this region we assume BPAPs were only partially blocked. This might happen if there was still some passive spread of BPAPs from the edge of the TTX-affected region, which itself was quite superficial. On the other hand, TTX to L5 would be expected to abolish AP initiation in most L5 pyramidal neurons. The effect of application of TTX to L1 on the evoked response is most likely due to a block of the initial Na⁺ component of dendritic Ca²⁺ spikes (Larkum et al. 2001) because synaptic transmission was already blocked. Given that

FIG. 8. Contribution of Na⁺ channels to the dendritic Ca²⁺ signal in vivo. A: schematic diagram of the experiment. Tetrodotoxin (TTX) was applied to L1 via the cortical surface (left) and to L5 via a patch pipette (right). B: typical effect of TTX on ΔF/F with application of TTX to L1 and L5. Arrowhead indicates the time of the stimulus to L1. Top: kynurenic acid (1 mM) and bicuculline (20 μM) were applied to L1 (control). Middle: TTX (1 μM) to L1. Bottom: TTX to L5. C, top: averaged ΔF/F in kynurenic acid and bicuculline (black) and TTX (gray) to L1. Bottom: summary of the effect of TTX to L1 on peak amplitude of ΔF/F signal (n = 3 rats). D: Gaussian distribution of fluorescent fluctuations made from the fluorescence signals in B (see text). E: normalized peak width was determined from the Gaussian distribution (D) for 3 rats to compare the spontaneously fluctuating ΔF/F signal in control conditions, after application of TTX to L1 and to L5. *P < 0.05, **P = 0.002.
our main aim was to establish that the method can be used for recording dendritic Ca\textsuperscript{2+} signals, we did not pursue this question any further.

Next, we used a second method (type B-1; see METHODS) with an optic-fiber bundle for recording from neurons with a single fiber and a 90°-angled prism for illumination (Fig. 9A) as described in Fig. 1. This system achieved a better signal-to-noise ratio (Fig. 9B) and could be used to search for ΔF/ΔF signals in a circular area of the cortical surface with a diameter 155 μm. In these experiments, with the fiber bundle placed directly over the bolus-loading site, signals were approximately the same in amplitude in all regions of the FOV (Fig. 9B). As in the experiments with the periscope single-fiber bundle (Fig. 6), addition of kynurenic acid and bicuculline led to larger Ca\textsuperscript{2+} fluorescence responses with L1 stimulus (normalized ΔF/ΔF: 160.3 ± 6.4%, P < 0.005, n = 3 rats), which were blocked by addition of Cd\textsuperscript{2+} to the surface of the brain (normalized ΔF/ΔF: 2.5 ± 2.2%, P < 0.001, n = 3 rats; Fig. 9, C and D). Similar to the single-fiber-bundle periscope approach (Fig. 7A), the L1 stimulus-evoked ΔF/ΔF signal was significantly reduced by application of kynurenic acid (Supplemental Fig. 1).

Finally, we used a third noninvasive recording method (type B-2) with an oblique optical fiber for illuminating neurons as described in Fig. 1E. This provided both an alternative method involving no damage to the cortex and a means to assess the importance of damage by comparison with the periscope data. We successfully recorded stimulus-evoked ΔF/ΔF signals that were blocked by application of Cd\textsuperscript{2+} (n = 3 rats; Supplemental Fig. 2). Stimulus-evoked ΔF/ΔF signals could even be recorded through the dura mater (n = 3 rats; data not shown), which would be advantageous for chronic recordings.

Insertion of the prism into the cortex involves some damage that may change important aspects of the signals. We examined the extent of damage from the periscope method in three ways. First, slices were made from brains that had been previously damaged with insertion of the periscope. We recorded from cells (n = 4) whose apical dendrites were close enough to the

![Diagram](http://www.jn.org/content/vol98/sep/1801/Figure9.large.jpg)

**FIG. 9.** In vivo Ca\textsuperscript{2+} recordings with a two-fiber system. A: schematic diagram of the experiment with a two-fiber system. Fiber with a ×4.4 microobjective and single-core fiber with a prism were used for recording and illuminating, respectively. Inset: image of a grid slide for calibration taken with the fiber. One box has a dimension of 25 × 25 μm. B: typical ΔF/ΔF signals taken with the system. Numbers (1–5) of ROIs in the FOV (left) correspond to those in the traces (right). Bottom trace: superposition of all ROIs. Arrowhead indicates time of stimulation to L1. C: effect of kynurenic acid with bicuculline and Cd\textsuperscript{2+} on the Δ[Ca\textsuperscript{2+}], Top: typical ΔF/ΔF in ROI 2, evoked by L1 stimulation. Middle: after application of kynurenic acid (1 mM) and bicuculline (20 μM) to L1. Bottom: after application of Cd\textsuperscript{2+} (1 mM) to L1. D: summary of the results for C (n = 3 rats). *P = 0.005, **P < 0.001.

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prism to be affected by the periscope damage and from cells (n = 5) too far away (>300 μm) to be significantly affected (Supplemental Fig. 3, A and B). Neurons in regions near to the prism face and far away had normal resting membrane potentials (near: −70.8 ± 5 mV, n = 5 cells; far: −73.1 ± 2.2 mV, n = 4 cells; Supplemental Fig. 3D). Moreover, the distal dendrites showed typical suprathreshold influx of Ca\(^{2+}\) in response to Supra-CF trains of APs (Supplemental Fig. 3C), indicating that the mechanisms for regenerative Ca\(^{2+}\) entry into the tuft were still intact. [Note: we also recorded from three cells that were directly under the damaged area (“underneath”.)] The tuft dendritic arborizations in these neurons appeared to have more or less returned to their original position in the area previously occupied by the prism, their morphology appeared partially restored and the resting membrane potentials were normal (−71.6 ± 3.3 mV, n = 3 cells; Supplemental Fig. 3C). We did not measure the response to Supra-CF trains for this group because their dendrites would not contribute to the signal recorded by the periscope. This highlights the relative robustness of dendrites in the cortex. Second, we recorded stimulus-evoked ΔF/F signals with the periscope from anesthetized rats in vivo using specific local illumination with a 125-μm fiber (100-μm core diameter) inserted next to and 200 and 400 μm from the face of the microprism. As expected, illumination near the prism face gave larger signals but we still found a substantial contribution from regions further away (Supplemental Fig. 4). There were no apparent differences in the time course of the responses and nothing that would indicate a pathological response from the area close to the periscope face.

Finally, we recorded L1 stimulus-evoked ΔF/F signals in vivo with the periscope system after previous recordings using the noninvasive recording system in the same animals. The amplitude and time course of ΔF/F signals acquired with both systems were similar to each other (normalized ΔF/F with periscope system, 107 ± 30% of noninvasive system, n = 3 rats; data not shown). In all the tests used to assess the effect of damage using the periscope, both in vivo and in vitro, we could not detect any significant differences to undamaged tissue. Nonetheless it may be advisable to check this with the noninvasive approach where possible.

**Dendritic Ca\(^{2+}\) signals in freely moving rats**

To demonstrate that the recording technique introduced here is feasible and easy to implement for experiments with awake, freely moving animals, we developed a system for rigidly attaching the periscope to the brain using a periscope holder that consisted of a base and post components (Fig. 10A). Surgery was performed on the rats 2 days in advance under isoflurane anesthesia to prepare the region of the skull intended for the periscope holder. Local anesthesia (lidocaine) and an analgesic (buprenorphine) were given over this period. On the day of the experiments a small craniotomy was made and the AM dye injected. Immediately after the injection, the base was fixed on the skull with dental cement and three anchoring screws. The post was then fixed on the base with dental cement. Last, the periscope was inserted into the brain through the post and then fixed rigidly with a screw.

While the rats were still deeply anesthetized with isoflurane they were transferred to the arena for behavioral observation (37 × 27 cm) and the effects of the anesthetic allowed to wear off. In all freely moving rats (n = 3), we obtained stable dendritic Ca\(^{2+}\) signal recordings with the periscope recording system (type A) with normal (10 Hz; data not shown) and high-speed (100 Hz) sampling rates while the rat was moving in the arena (Fig. 10, B and C). Moreover, whisker-stimulation-evoked ΔF/F signals could be recorded after an air puff (100-ms duration) was applied to whiskers, even without averaging signals (Fig. 10, D and E). The whisker-stimulus-
evoked signal and the fluctuating background signals were blocked by application of Cd²⁺ to the brain surface (normalized ΔF/F: 7.4 ± 5.2%, P < 0.002 for evoked signals; normalized ΔF/F: 43.2 ± 0.7%, P < 0.001 for fluctuating signals, n = 3 rats for each experiment; Fig. 11, A–D), demonstrating that these signals are due to neuronal activities and that under normal circumstances movement artifacts were not important. This last configuration also demonstrated the feasibility of performing pharmacological manipulations in the freely moving preparation.

To measure the effect of movement on the signals recorded with the freely moving system we mechanically gyrated the whole head and body of the rat under isoflurane anesthesia with a frequency of about 2.5–230 Hz using a benchtop stirrer (for 2.5–40 Hz; IKA-VIBRAX VXR; IKA Werke, Staufen, Germany) to gyrate the whole rat and an electric fan (for 230 Hz, Arctic Fan 8; Scythe, Tokyo, Japan) coupled to a plastic tube to vibrate the head about 1 mm. Here, there were no significant changes (P = 0.89 for 2.5 Hz, P = 0.48 for 20 Hz, P = 0.67 for 40 Hz, P = 0.60 for 230 Hz, n = 3 rats for each frequency), indicating that there was no additional signal due to movement (Supplemental Fig. 5).

Because this was primarily a study for the feasibility of freely moving recordings we chose not to try to interpret the data from the freely moving experiments in terms of correspondence to behavior other than the test for movement artifacts and the whisker-stimulation experiments.

**DISCUSSION**

We have presented a new method for recording fluorescence signals directly from the apical dendrites of L5 neocortical pyramidal neurons in freely moving animals. The main features of this method are: 1) loading of dendrites in the upper cortical layers by bolus application of a membrane-permeant dye to the cell bodies in L5 (Kerr et al. 2005) and 2) the use of a “periscope” system—a fiberoptic-based microendoscope—to record Ca²⁺ changes in the apical dendrites of populations of L5 neocortical pyramidal neurons in vivo. The major advantages of our approach are that it is easy to implement, is cost-effective, and provides signals from populations of dendrites that match in vitro recordings in terms of signal to noise. The major disadvantage of this approach is that it is difficult to resolve single dendrites.

We based our method on similar fiberoptic approaches used before to record calcium changes in neurons in anesthetized (Kudo et al. 1992) and freely moving animals (Adelsberger et al. 2005; Duff and Schmidt 2000). Of particular importance was the approach taken by Adelsberger et al. (2005) who used laser illumination and a photomultiplier to record with a single
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core fiber ("optrode") from cortical neurons in resting newborn mice. We also found that the optrode approach gave better signals than the fiber bundle and appears to be a good strategy for maximizing signal to noise. Another advantage of the optrode approach is that it allows thinner and more flexible fibers to be used. However, we found that added stiffness of the fiber bundle did not noticeably restrict the movement of the rats. Ferezou et al. (2006) were able to increase the flexibility of a fiber bundle by removing the cladding.

A key element of the method described herein is the use of a microprism (Bird and Gu 2003) for right-angled illumination (Duff and Schmidt 2000) of the upper cortical layers, ensuring that virtually all fluorescence changes measured are due to Δ[Ca\(^{2+}\)]\(_i\) in the apical dendrites of L5 pyramidal cells. The method is particularly suited to record responses from populations of dendrites from a particular cortical area or column for which there is no satisfactory equivalent electrical recording method at present. It would also be relatively simple to combine with other approaches, such as whole cell recordings (Ferezou et al. 2006) or extracellular recordings (Kudo et al. 1992) to simultaneously monitor firing activity of neurons in deep layers with the Ca\(^{2+}\) changes in upper layers. Although in principle high enough to image single dendrites, the resolution of the fiber bundle is compromised to some extent by light scattering and by the fact that the underlying structures (apical tuft dendrites) are very overlapping. We found that using a fiber above the cortex resulted in a homogeneous signal from all locations, whereas the side-on method allowed us to resolve the tuft and the shaft regions, which may be important to determine the origin of dendritic signals (e.g., backpropagating vs. locally initiated). We chose to record signals with acquisition rates between 10 and 100 Hz. Large Ca\(^{2+}\) signals in the dendrites of L5 pyramidal cells are generally slow using high-affinity dyes and can therefore be adequately captured within this range of temporal resolution (Helmchen et al. 1996, 1999).

We presented two main techniques for viewing the dendrites either side-on (type A; see METHODS) or from above the cortex (type B). Signals were slightly better with the second approach from above the cortex, although the side-on approach had the advantage that signals could be recorded at different cortical depths. The use of a microprism-based illumination system requires insertion of a small prism (500 × 500 μm at the base) into the cortex. This procedure caused some damage to the cortex but L5 neurons still had normal resting membrane potentials and were apparently normal in their dendritic properties after the in vivo damage with the periscope (Supplemental Fig. 3). Nonetheless, this damage may be an important issue for long-term recordings in living animals. We showed that similar recordings could be made without tissue damage using an obliquely orientated illumination fiber. In cases where damage may be important, we suggest comparison of the signals produced using both methods.

This study combined the use of in vitro with in vivo recordings to ascertain the source of Ca\(^{2+}\) responsible for the fluorescence changes. This combined approach proved of particular importance for correlating fluorescence changes to intracellular activity. We evoked suprathreshold dendritic Ca\(^{2+}\) activity using strong extracellular stimulus of L1 and Supra-CF BPAPs. Supra-CF trains of BPAPs have been shown to activate the same voltage-gated Ca\(^{2+}\) channels that are activated during a dendritic Ca\(^{2+}\) spike (Pérez-Garci et al. 2006). For this reason, we treated the dendritic fluorescence signal recorded under these conditions as equivalent to dendritic Ca\(^{2+}\) spikes.

The data point to the following conclusions. 1) Ca\(^{2+}\) signals were nearly exclusively postsynaptic with little or no contribution from the neuropil because the dendrites of L5 neurons were the only fluorescent elements in the upper layers (Fig. 1), as already indicated by Kerr et al. (2005). 2) The Ca\(^{2+}\) influx accompanying EPSPs did not contribute much to the fluorescence signal (Figs. 5 and 6). This is consistent with studies in L2/3 neocortical pyramidal neurons (Garaschuk et al. 2006; Waters et al. 2003). 3) On the other hand, it is likely that Ca\(^{2+}\) spikes are easily detectable in the population signal even when they occur in only a single dendrite as was shown in vitro (Fig. 5). 4) The spontaneously fluctuating ΔF/F signal appeared to be periodic with a frequency of 1.2 ± 0.2 Hz (range: 0.78–1.6 Hz). The amplitude of the signal was small compared with the extracellular stimulus-evoked ΔF/F signal and was probably caused by BPAPs in L5 pyramidal neurons. We concluded this because a) the spontaneously fluctuating signal was greatest in the lower part of the FOV (lower L3 and L4) where there would be less dendritic surface area and therefore fewer synaptic inputs but bigger BPAPs (Fig. 6), b) it was suppressed by local application of TTX to L5 but not L1 (Fig. 8), and c) it was not changed significantly by addition of blockers of synaptic transmission (Figs. 6 and 8). On the other hand, the signals were blocked by Cd\(^{2+}\) applied in a similar manner (Fig. 6), indicating that the spontaneously fluctuating signal was not due to tissue movement. Last, we hypothesize that there is a tonic inhibitory suppression of dendritic Ca\(^{2+}\) activity that can be relieved by application of bicuculline, a GABA\(_{A}\)-receptor antagonist. Stimulus-evoked inhibition of dendritic Ca\(^{2+}\) influx has been observed in vitro (Kim et al. 1995; Pérez-Garci et al. 2006; Tsubokawa and Ross 1996) and in vivo (Buzsáki et al. 1996).

We showed that the in vivo fiberoptic system is easy to implement in freely moving animals (Fig. 10). We chose not to try to interpret the signals in terms of behavior in this study, although we were able to show responses to whisker stimulus demonstrating that the signals are biological in origin. Interpretation of these kinds of experiments can present difficulties in terms of signal averaging and controlling the number of free parameters, although we were able to record significant signals on single trials that were blocked with Cd\(^{2+}\), indicating that they were due to changes in [Ca\(^{2+}\)]\(_i\). In the case of whisker-stimulation–evoked dendritic Δ[Ca\(^{2+}\)]\(_i\) (Fig. 10E), the averaged data were more or less identical to those of single trials. Also encouraging was the fact that movement artifacts did not appear to be a significant problem using this method, which may present some advantage over a two-photon freely moving approach for certain behavioral experiments (Helmchen et al. 2001). Thus we are confident that it will be possible to analyze ΔF/F signals without any averaging using this fiberoptic system.

In summary, we have presented a novel in vivo method for monitoring dendritic Ca\(^{2+}\) in the distal dendrites of populations of L5 pyramidal neurons in the neocortex. This method is straightforward and easy to implement in vivo and even in the freely moving animal, and should also be easy to combine with...
other experimental methods such as electrophysiological recordings.

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