Combined Voltage and Calcium Epifluorescence Imaging In Vitro and In Vivo Reveals Subthreshold and Suprathreshold Dynamics of Mouse Barrel Cortex

Thomas Berger,1,3 Aren Borgdorff,1 Sylvain Crochet,1 Florian B. Neubauer,3 Sandrine Lefort,1 Bruno Fauvet,1 Isabelle Ferezou,1 Alan Carleton,2 Hans-Rudolf Lüscher,3 and Carl C. H. Petersen1

1Laboratory of Sensory Processing and 2Flavour Perception Group, Brain Mind Institute, Ecole Polytechnique Federale de Lausanne, Lausanne; and 3Institute of Physiology, University of Bern, Bern, Switzerland

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Berger T, Borgdorff A, Crochet S, Neubauer FB, Lefort S, Fauvet B, Ferezou I, Carleton A, Lüscher H-R, Petersen CC. Combined voltage and calcium epifluorescence imaging in vitro and in vivo reveals subthreshold and suprathreshold dynamics of mouse barrel cortex. J Neurophysiol 97: 3751–3762, 2007. First published March 14, 2007; doi:10.1152/jn.01178.2006. Cortical dynamics can be imaged at high spatiotemporal resolution with voltage-sensitive dyes (VSDs) and calcium-sensitive dyes (CaSDs). We combined these two imaging techniques using epifluorescence optics together with whole cell recordings to measure the spatiotemporal dynamics of activity in the mouse somatosensory barrel cortex in vitro and in the supragranular layers in vivo. The two optical signals reported distinct aspects of cortical function. VSD fluorescence varied linearly with membrane potential and was dominated by subthreshold postsynaptic potentials, whereas the CaSD signal predominantly reflected local action potential firing. Combining VSDs and CaSDs allowed us to monitor the synaptic drive and the spiking activity of a given area at the same time in the same preparation. The spatial extent of the two dye signals was different, with VSD signals spreading further than CaSD signals, reflecting broad subthreshold and narrow suprathreshold receptive fields. Importantly, the signals from the dyes were differentially affected by pharmacological manipulations, stimulation strength, and depth of isoflurane anesthesia. Combined VSD and CaSD measurements can therefore be used to specify the temporal and spatial relationships between subthreshold and suprathreshold activity of the neocortex.

INTRODUCTION

Many computations in the neocortex are thought to occur on the millisecond timescale within maps composed of cortical columns. Most current techniques to investigate cortical processing offer limited spatial resolution because of the low density of recording electrodes or theoretical difficulties of defining the signal sources. Optical methods, however, offer both sufficient temporal and spatial resolution for real-time analysis of cortical processing.

Two of the most dynamic parameters in the active brain are membrane potential ($V_{m}$) and intracellular calcium concentration ($[Ca^{2+}]_{i}$). Both of these can be imaged optically by the introduction of fluorescent probes into the brain. Voltage-sensitive dyes (VSDs) insert into the plasma membrane and change their fluorescence intensity dependent on the potential across the lipid bilayer. Previous studies recorded VSD signals in invertebrate preparations (Antic and Zecevic 1995; Salzberg et al. 1973), from cultured cells (Bullen and Saggau 1998), brain slices (Antic et al. 1999; Contreras and Linas 2001; Laaris and Keller 2002; Petersen and Sakmann 2001), and in vivo (Borgdorff et al. 2007; Civlillico and Contreras 2006; Derdikman et al. 2003; Ferezou et al. 2006; Grinvald et al. 1984; Kleinfeld and Delaney 1996; Petersen et al. 2003a,b; Shoham et al. 1999). The VSD JPW1114 (also known as di-2-ANEPQ) has proven useful as a dye for intracellular application in individual nerve cells, allowing the spatiotemporal analysis of electrical signaling in dendrites (Antic and Zecevic 1995; Antic et al. 1999). The VSD RH1691 is optimized for in vivo measurements (Shoham et al. 1999) and, when topically applied to the neocortex, it can resolve cortical activity with millisecond and subcoluminar resolution (Grinvald and Hildesheim 2004). Fluorescent calcium-sensitive dyes (CaSDs) such as Fluo-3 and Oregon Green BAPTA-1 (OGB-1) have been developed that respond rapidly and selectively to changes in the cytosolic free calcium ion concentration (Tsien 1980). CaSDs can be applied extracellularly in a membrane-permeable ester form, which is subsequently cleaved intracellularly by esterases releasing the functional fluorescent CaSD (Tsien 1981). Recently, network activity was imaged with CaSD both in brain slices and in the intact brain (Borgdorff et al. 2007; Kerr et al. 2005; Nimmerjahn et al. 2004; Ohki et al. 2005; Peterlin et al. 2000; Stosiek et al. 2003; Wachowiak and Cohen 2001; Yaksi and Friedrich 2006).

In this study, we combined epifluorescence VSD and CaSD imaging together with whole cell (WC) $V_{m}$ recordings to allow a quantitative comparison of what is measured using these techniques, focusing on their application to the study of the mouse barrel cortex (Petersen 2003; Woolsey and Van der Loos 1970). We recorded VSD and CaSD signals in three different experimental conditions: in single cells, in brain slices, and in vivo. In single cells, we studied the dynamic range of VSDs and CaSDs during controlled membrane potential changes. In the barrel cortex in vitro, we characterized the link of the VSD and CaSD signals to sub- and suprathreshold electrophysiological network activity and thereafter studied the spatial spread of synaptic activity and action potentials. We next transferred these methods to make in vivo optical measurements of activity in layer 2/3 barrel cortex of anesthetized mice, analyzing both the evoked and the spontaneous spatiotemporal dynamics of the VSD and CaSD signals. The data are...
consistent with VSD signals reporting predominantly subthreshold activity and CaSD reporting suprathreshold activity.

**METHODS**

All experiments were carried out in accordance with the Swiss Federal Veterinary Office.

**Voltage-sensitive dye biophysics**

Defolliculated *Xenopus* oocytes were incubated for about 30 min in 1.6 mM RH1691 (Optical Imaging, Revohot, Israel) dissolved in a solution containing (in mM): 135 NaCl, 5 KCl, 5 HEPES, 1.8 CaCl₂, and 1 MgCl₂ (pH 7.3 with NaOH). The oocyte was transferred to the recording bath filled with a standard ND96 oocyte medium containing (in mM): 96 NaCl, 2 KCl, 5 HEPES, 1.8 CaCl₂, and 2 MgCl₂ (pH 7.4 with NaOH). Two microelectrodes filled with 2 M KCl were used to voltage-clamp the oocyte using an Axoclamp 2B amplifier (Axon Instruments, Union City, CA). A computer running Igor Pro (WaveMetrics, Lake Oswego, OR), interfacing with an ITC-18 board (Instrutech, Port Washington, NY), not only controlled the command potential but also sampled the achieved membrane potential at 20 kHz. The oocyte membrane was imaged using an Olympus BX51WI microscope equipped with an Olympus ×20 0.95 numerical aperture (NA) objective. Excitation light from a 100 W halogen lamp with a shutter (Uniblitz, Vincent Associates, Rochester, NY) was band-pass filtered (630/30 nm) and reflected toward the sample by a 650 nm dichroic mirror. Emitted fluorescence was long-pass filtered (665 nm) and imaged using a MiCam Ultima camera (SciMedia, Irvine, CA). Fluorescence measurements were synchronized to electrophysiology through TTL pulses. In one set of experiments, the membrane potential was stepped from a holding potential of −60 mV to test membrane potentials (−100 to +40 mV in 20 mV steps) for 200 ms periods and fluorescence changes were recorded at 10 ms frame rates (Fig. 1A). For each oocyte fluorescence signals from 10 to 20 trials were averaged. Fluorescence signals were integrated across the field of view (FOV) containing 100 × 100 pixels covering roughly 300 × 300 μm. This spatial averaging and averaging across different trials was carried out using the MiCam Ultima analysis software. The fluorescence time-course traces were subsequently exported to Igor Pro (WaveMetrics) for further analysis, where the data from six oocytes were combined and a polynomial bleaching curve was subtracted. In a separate set of experiments fluorescence was sampled at 1 ms time resolution while membrane potential was repeatedly alternated from −60 to 40 mV every 10 ms for 1 s (Fig. 1B). For each oocyte fluorescence signals from 10 trials were averaged and then the response to each of the 100 voltage steps was further averaged. Data from six oocytes were combined. The change in fluorescence, given throughout the paper, is normalized to resting fluorescence (ΔF/ΔF<sub>0</sub>), i.e., as the change divided by the fluorescence before stimulation (Figs. 1–5, 6A, and 7) or without spontaneous ongoing activity (Fig. 6, B and C).

**Brain slice preparation**

Parasagittal or thalamocortical slices (300 or 400 μm thick, respectively) (Agmon and Connors 1991) of the somatosensory cortex were prepared using a vibratome from 14- to 29-day-old C57BL6J mice in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 25 NaHCO₃, 25 glucose, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, and 1 MgCl₂ bubbled with 95% O₂-5% CO₂ (pH 7.4). Slices were incubated at 35°C for 30 min and then left at room temperature until recording.

**In vitro cellular imaging**

Current-clamp recordings in the whole cell mode of the patch-clamp technique were obtained from the somata of neurons in acute slices of the barrel cortex with Multiclamp 700A (Axon Instruments) or BVC-700A (Dagan, Minneapolis, MN) amplifiers. Patch pipettes were made from borosilicate glass with resistances of 3–7 MΩ filled with the following solution (in mM): 135 K-gluconate, 5 KCl, 5 HEPES, 1.8 CaCl₂, and 1 MgCl₂; and 7) or without

**In vitro network imaging**

Stock solutions (10 mM) of the acetoxymethyl (AM) ester forms of OGB-1 (OGB-1 AM; Molecular Probes) or Fluo-3 (Biotium, Hayward, CA) were made in 80% fresh 1:30 M N,N-dimethylformamide and 20% ACSF-filled pipette. The solution was vortexed, sonicated, aliquoted, and stored in the freezer. The AM ester solution (10 μl) was sucked into an oil-filled application pipette (tip diameter 15–25 μm). To obtain even staining of the approximately 900 × 900-μm cortical region of interest, the AM ester was injected into the slice at six to ten locations in the barrel cortex in parasagittal slices under visual control. In thalamocortical slices, the AM ester solution was applied with the same technique to label the entire layer 4 barrel cortex. OGB-1 AM was used throughout this study except in a few experiments involving the thalamocortical slice preparation, where both OGB-1 AM and Fluo-3 AM were used. The changes resulting from pharmacological manipulations were similar between OGB-1 AM and Fluo-3 AM were used. The changes in fluorescence in the FOV covering the soma and proximal dendrites (Fig. 1, D and F). Alternatively, synaptic potentials around action potential threshold were evoked with extracellular stimulation using an ACSF-filled pipette. Corresponding changes in fluorescence were imaged with a Zeiss Axioskop 2FS+ microscope [×40 0.8 NA objective; 75-W xenon short-arc lamp; Deltaram V monochromator (PTL, Lawrenceville, NJ)]. A low-resolution (80 × 80 pixel), fast (1–2 kHz) CCD camera (Redshirt NeuroCCD, RedshirtImaging, Fairfield, CT) running under computer control (Neuroplex software) was used to image changes in fluorescence in the FOV covering the soma and proximal dendrites (Fig. 1, D and F). All experiments were done at 35°C. The following filter sets were used: for OGB-1, excitation filter BP470/20 nm, dichroic mirror 510 nm, emission filter BP540/25 nm; for OGB-1114, excitation filter BP520/20 nm, dichroic mirror 580 nm, emission filter LP590 nm; for RH1691, excitation filter BP630/30 nm, dichroic mirror 650 nm, emission filter LP665 nm.

**Figure 1A.** Distribution of threshold activity and CaSD reporting suprathreshold activity.
In vivo imaging

C57BL/6J mice aged postnatal day (P) 21 to P25 were anesthetized with urethane (1.5 mg/g body weight; Figs. 4 – 6, Supplementary Fig. S1) or isoflurane (0.5–2%; Fig. 7). Paw withdrawal, whisker movement, and eye blink reflexes were largely suppressed. A heating blanket maintained the rectally measured body temperature at 37°C. The skin overlying the somatosensory cortex was removed. The head of the mouse was glued to a metal head plate and fixed. In a first step, the location of C2 whisker representation was mapped by intrinsic optical imaging. The cortical surface was visualized through the intact skull covered with a Ringer solution sealed with a glass coverslip. The surface blood vessels were visualized using light at 530 nm to enhance contrast. The illumination was switched to 630 nm for functional imaging. The reflected light was imaged using a Quicam CCD camera (Q-imaging; 10-Hz frame rate, 800 × 800 pixels covering approximately 2.5 × 2.5-mm area). Image acquisition by a Firewire and stimulus control of the piezo by an ITC18 board were governed by custom routines running in Igor Pro. To avoid interference with VSD signals in the intrinsic imaging experiments described in Supplementary Fig. 1, we illuminated at 700 nm and to obtain the same FOV as the VSD imaging, we also imaged the intrinsic signals using a MiCam Ultima camera (20-Hz frame rate, 100 × 100 pixels covering a 2 × 2-mm area). Alternating sweeps were imaged with or without stimuli delivered to the C2 whisker. Stimuli were applied at 10 Hz for 4 s and repeated 7 to 15 times with a 120 s interval. The intrinsic signal was subtracted as the difference in the reflected light on stimulus compared with a 4 s time interval immediately before. The local signal was mapped onto the blood vessel pattern to guide surgery for the craniontomy (ranging in size from 1 × 1 to 3 × 3 mm), which was performed with extreme care so as not to damage the cortex, especially during removal of the dura. RH1691 was topically applied to the exposed cortex and allowed to diffuse into the cortex for about 40 min. Subsequently, unbound dye was washed away. OGB-1 AM was injected into layer 2/3 at multiple locations by a glass pipette connected to a hydraulic micromanipulator, avoiding blood vessels (about 50 nl of a 500 μM or 1 mM solution per injection site). Fluorescence from the esterase-cleaved OGB-1 AM increased gradually over time and stabilized 1 h after injection as previously reported (Stosiek et al. 2003). Fluorescence at an injection site covered a circular area with half-maximal values at 330 ± 70 μm diameter.

The cortex was covered with 1% agarose and a coverslip placed on top to stabilize the cortex. The excitation light was focused onto the cortical surface with a 25 mm Navitar video lens (or 50 mm Nikon lens). Fluorescence was collected by the same optical pathway but without reflection of the dichroic mirror, long-pass or band-pass filtered, and focused onto the camera by another 25 mm Navitar lens (or 135 mm Nikon lens). The voltage-sensitive dye was excited with 630 nm light emitted by light-emitting diodes (LEDs, L630, Epitex, Kyoto, Japan), reflected using a 655 nm dichroic mirror and long-pass filtered (>665 nm). Alternatively the RH1691 was illuminated with an electronically shuttered 100 W halogen lamp and directed onto the sample using fiber-optic light guides. The OGB-1 was excited either with 490/15 nm LEDs (L490-06U, Epitex) or by an electronically shuttered halogen lamp, reflected with a 500 nm dichroic mirror, and emitted fluorescence collected after band-pass filter 535/15 nm. Brief backward deflections of the C2 whisker were delivered using a computer-controlled piezoelectric bimorph. The time course and amplitude of stimuli were recorded approximately 1 mm away from the skin with an optical displacement sensor (Phittec D64-QT4, Phittec, Annopolis, MD). Images were recorded at frame rates ranging from 200 to 1,000 Hz using either a CCD camera (RedShirtImaging; Figs. 4, 5, and 7) or a dual camera MiCAM Ultima (BrainVision, Tokyo, Japan; Fig. 6 and Supplementary Fig. S1) for simultaneous measurements. Images were analyzed off-line using custom-written routines in Igor Pro.

Autofluorescence of the unstained brain was negligible (accounting for <1% of the resting fluorescence) at the wavelengths used to excite RH1691 fluorescence. However, there was considerable autofluorescence (accounting in some experiments for >80% of the resting fluorescence) of the brain at the wavelengths at which the CaSD was imaged. This autofluorescence was subtracted from the collected CaSD images to specifically measure the CaSD fluorescence signal. VSD and CaSD signals were quantified as ΔF/F₀ to correct for differences in the spatial distribution of the fluorescent dye. In some experiments, imaging sweeps were triggered at a fixed point of the electrocardiogram. The C2 whisker was deflected on alternate sweeps. Unstimulated trials were subtracted from trials with stimuli and many trials (15–60) were averaged. This analysis procedure aims to reduce both heart beat-related imaging artifacts, bleaching artifacts, and the contribution of spontaneous cortical activity. A range of stimulation strengths differing in deflection amplitude, velocity, and acceleration were studied.

No evoked signals (at the wavelengths used for intrinsic, VSD, or CaSD measurements) were detected in control experiments where the cortex was not stained with dye, but identical brief single C2 whisker stimuli were applied. The brief single-whisker stimuli that we applied thus did not produce measurable intrinsic signals originating from the optical properties of the neocortex (Grinvald et al. 1986; Shibuki et al. 2003).

Statistical tests

Data are expressed as means ± SE and were tested for statistical significance using Student’s t-test or ANOVA.

RESULTS

VSD fluorescence correlates linearly with Vₘ

Our first goal was to characterize the relationship between membrane potential changes and fluorescence changes under controlled conditions. We applied the VSD RH1691 to voltage-clamped Xenopus oocytes and found linear submillisecond changes in fluorescence with respect to Vₘ (n = 6 oocytes; Fig. 1, A–C). However, we were unsuccessful in our attempts to obtain functional fluorescence signals from single neurons labeled with RH1691. As a positive control for our experimental procedures, we introduced the VSD JPW1114 into individual layer 2/3 neurons in brain slices of the mouse barrel cortex by the patch pipette during WC recordings. VSD fluorescence (Fig. 1, D and E) linearly followed both sub- and suprathreshold changes in neuronal Vₘ induced by alpha currents (n = 9 cells). In agreement with previous studies (Antic and Zecевич 1995; Antic et al. 1999), these data indicate that VSD fluorescence (from both RH1691 and JPW1114) correlates linearly with Vₘ at submillisecond precision.

CaSD signals reflect action potential firing

Having found a linear relationship of Vₘ and VSD fluorescence in individual cells, we next investigated the behavior of

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1 The online version of this article contains supplemental data.
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Cortical spatiotemporal dynamics of VSD and CaSD signals in vitro

After delineating the behavior of VSD and CaSD at a cellular level, we next investigated how their fluorescence signals compared when applied to the study of neuronal networks in vitro. Brain slices of the mouse somatosensory barrel cortex were labeled with VSD RH1691 by topically applying the dye to the slice surface. CaSD OGB-1 was injected into multiple locations across the cortical area of interest in its AM ester form and nearly 1 h later cells were strongly labeled (Stosiek et al. 2003). In some experiments, the imaging was combined with WC recordings of $V_m$ from neurons in layer 2/3. The imaged area covered three to four barrel columns and spanned from cortical layer 2 to layer 5. Neuronal network activity was evoked with a stimulation electrode in a layer 4 barrel and the time course quantified in layer 2/3 (Fig. 2, A–C). Compared with the VSD signal, the CaSD signal was roughly 20 times larger (peak amplitude $\Delta F/F_0$ VSD $0.34 \pm 0.07\%$, $n = 9$; CaSD $8.3 \pm 2.2\%$, $n = 13$; in four experiments only CaSD and not VSD signals were imaged) and lasted about five times longer than the VSD signal (half-width duration VSD $35.5 \pm 3.1$ ms, $n = 9$; CaSD $181 \pm 14$ ms, $n = 13$).

Pharmacological manipulations were carried out to explore the underlying nature of these signals. Bath application of the ionotropic glutamate receptor antagonists $\alpha$-2-amino-5-phosphonovaleric acid (n-APV, 50 $\mu$M) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 $\mu$M) blocked WC responses and VSD signals (1.3 $\pm 0.8\%$ of control; $n = 9$ slices) (Fig. 2, C and D). This suggests that the VSD response almost exclusively reflects postsynaptic potentials (PSPs). In contrast to the profound block of the VSD response, adding n-APV/CNQX reduced the peak of the CaSD response to only 56.3 $\pm 6.9\%$ of the control value ($n = 6$ slices; Fig. 2, C and D). This reveals that the CaSD signal evoked by local electrical stimulation is not driven primarily by synaptic potentials. Additional application of 1 $\mu$M tetrodotoxin (TTX) abolished all responses (WC recording, VSD and CaSD) (Fig. 2, C and D). Evoked $V_m$ and CaSD signals were not changed by blockade of metabotropic glutamate receptors [500 $\mu$M (S)-$\alpha$-methyl-4-carboxyphenylglycine (MCPG); peak CaSD response was 92.6 $\pm 3.2\%$ of control; $n = 7$] or the endoplasmic reticulum calcium ATPase (5 $\mu$M thapsigargin; peak CaSD response was 101.7 $\pm 6.0\%$ of control; $n = 5$). These results indicate that the entire CaSD signal is thus mediated by action potential firing. The fraction of the CaSD signal that is sensitive to ionotropic glutamate receptor antagonists likely reflects APs in postsynaptic cells.
driven by synaptic potentials. The majority of the CaSD signal (which is not blocked by d-APV/CNQX) is likely evoked by the direct stimulation of APs in neurons with axons near the stimulation electrode.

To separate the stimulation site from the measuring location and, in addition, to excite the cortical network in a more physiological manner, we prepared thalamocortical slices (Agmon and Connors 1991), with CaSD injected into the barrel cortex (Fig. 2E). Stimulation of the VPM nucleus of the thalamus evoked calcium signals in the connected layer 4 barrels (Beierlein et al. 2002; MacLean et al. 2005). These thalamically evoked responses were almost completely abolished by blockade of postsynaptic ionotropic glutamate receptors. We found that bath application of 50 μM d-APV and 10 μM CNQX reduced the peak of the thalamic evoked cortical CaSD response to 7.6 ± 1.6% of the control response (n = 10 slices; Fig. 2E). These calcium transients are therefore not mediated by signals in the thalamic axons, but instead are likely to relate primarily to cortical action potentials evoked after the release of glutamate from the thalamocortical synapses.

Consistent with our cellular measurements (Fig. 1), these data from neuronal networks suggest that VSD signals measure predominantly PSPs and that CaSD signals measure local AP activity. We therefore applied these two optical techniques to image the spatiotemporal dynamics of subthreshold and suprathreshold activity evoked by electrical stimulation of a layer 4 barrel in the mouse somatosensory cortex (Fig. 3; Supplementary Movie 1). Excitation began in the stimulated layer 4 barrel, then spread in a columnar fashion into layer 2/3, and finally into neighboring columns. In layer 2/3 of the neighboring barrel column, the VSD response was reduced to 71.7 ± 9.3% (n = 9 slices) and the CaSD response was reduced to 25.5 ± 4.0% (n = 13 slices) of the peak response in the stimulated layer 4 barrel (P = 0.0001; Fig. 3C). These data likely reflect a signaling sequence that begins with the stimulation of APs in layer 4 evoking PSPs in the columnar layer 2/3 through the strong axonal projection from layer 4 to layer 2/3 (Laaris and Keller 2002; Petersen and Sakmann 2001; Wirth and Lüscher 2004). If in turn the layer 2/3 neurons fire APs, then these will evoke PSPs across a large area of cortex through the extensive horizontal axonal arborizations of the layer 2/3 pyramidal neurons (Petersen et al. 2003a). The VSD signal therefore spreads further than the CaSD signal, which remains more tightly localized to the stimulus location. These results are consistent with the hypothesis that VSD images PSPs, which originate from neurons firing APs localized close to the stimulating electrode and are imaged with CaSD.

In vivo VSD and CaSD imaging of neocortical sensory processing

To investigate the spatiotemporal dynamics of sensory processing evoked by whisker deflection, we recorded VSD RH1691 and CaSD OGB-1 signals in vivo from layer 2/3 of the barrel cortex of anesthetized mice. The staining and imaging...
procedures did not alter cortical sensory processing as assessed by intrinsic optical imaging (Supplementary Fig. 1; Slovin et al. 2002). The in vivo VSD and CaSD fluorescence measurements were combined with WC recordings targeted to layer 2/3 neurons in the C2 barrel column (n = 7 cells, each in a different mouse). The optical signals were quantified over a barrel-sized area, immediately surrounding the recorded neuron. The C2 whisker was deflected in a highly controlled manner and each whisker deflection measured with an optical displacement sensor. Layer 2/3 neurons typically responded by subthreshold \( V_m \) depolarizations accompanied by APs on some trials (Fig. 4, A–E; peak whisker deflection averaged across the seven experiments was 33 ± 1 µm). The time course of the VSD signal closely followed \( V_m \) changes both in individual experiments (Fig. 4, A–C) and in the traces computed by averaging across the seven experiments (Fig. 4, D and E). Our in vivo VSD measurements thus reflect the mean \( V_m \) changes of layer 2/3 neurons. These \( V_m \) changes in response to whisker stimulation were almost entirely dominated by subthreshold activity. Comparing averages of raw \( V_m \) with averages of median-filtered \( V_m \) (the median filter used was adjusted in a way that APs were specifically removed) showed that APs made very little impact on the averaged evoked \( V_m \) response (Fig. 4D).

The CaSD signal evoked by whisker deflection also showed a rapid onset, but it was somewhat delayed relative to the VSD signal (difference 2.9 ± 1.2 ms; n = 11 animals, \( P = 0.019 \)). The CaSD signal lasted longer than the VSD signal (222 ± 26 vs. 97 ± 11 ms half-width). The delayed calcium response can relate to the fact that the neurons first need to depolarize through summation of PSPs before reaching AP threshold. In our WC recordings the delay between PSP onset and the first occurring AP was 7.7 ± 2.6 ms (n = 7 cells, range 0.9–21.7 ms). The correlation of single-cell \( V_m \) and the VSD signal suggests that, on the scale of a single barrel, many neurons behave in a similar subthreshold manner.

Similar to our observations in vitro (Fig. 2, C–E), we found that topical application of 500 µM d-APV and 125 µM CNQX to the cortical surface blocked the sensory response measured with both CaSD and VSD after a 10 min wash-in period (Fig. 4, F and G). Both VSD and CaSD therefore predominantly measure the local cortical processing of sensory information rather than axonal signals from long-range (e.g., thalamocortical or corticocortical) inputs.

### CaSD and VSD spatiotemporal dynamics

Different stimulation strengths evoked different temporal and spatial patterns of activity and an example experiment is shown in Fig. 5, A–C. At low stimulation strength (peak whisker displacement amplitude 14 µm) localized VSD responses could be observed without any CaSD signal (Fig. 5A). This likely reflects subthreshold potentials in layer 2/3 neurons without evoking APs. In contrast, at an intermediate stimulation strength (peak whisker displacement amplitude 18 µm), the VSD signal spreads and a localized CaSD signal was recorded (Fig. 5B). The spreading VSD signal likely results from AP firing of layer 2/3 neurons in the C2 barrel column evoking PSPs on target cells distributed across the extensive lateral axonal arborizations of the L2/3 pyramidal neurons. A stronger stimulus (peak whisker displacement amplitude 26 µm) evoked spreading VSD and CaSD signals (Fig. 5C). The horizontal spread of VSD and CaSD signals evoked by C2 whisker stimulation was quantified in seven experiments by comparing the peak amplitude of evoked responses in the C2 barrel column relative to a location about 500 µm away. The VSD signal evoked by an intermediate whisker stimulus (19 ± 3 µm) was reduced to 34 ± 3% and the CaSD response was reduced to 14 ± 2% (\( n = 7, P < 0.01 \)). Increasing stimulation strength increased both the amplitude and spread of the evoked responses. Doubling whisker deflection amplitude (38 ± 4 µm) increased peak responses in the C2 barrel column to 161 ± 8% for VSD and to 259 ± 52% for CaSD. In a region about 500 µm away from the C2 barrel column, the VSD response amplitude was reduced to 59 ± 4% and the CaSD signal was reduced to 28 ± 5% (\( n = 7, P < 0.001 \)). In vivo CaSD signals evoked by sensory stimuli thus remain significantly more localized compared with the VSD signals, which can spread over large cortical areas. These results are consistent with small suprathreshold receptive fields reflected in localized CaSD signals and larger subthreshold receptive fields (Brecht et al. 2003; Moore and Nelson 1998; Petersen and...

Cortical responses evoked by identical whisker stimuli are highly variable from trial to trial (Arabzadeh et al. 2005; Crochet and Petersen 2006). An interaction of ongoing spontaneous activity with the evoked response can largely account for the response variability, with small responses, both in terms of PSPs and APs, being evoked during spontaneous depolarizations (Petersen et al. 2003b; Sachdev et al. 2004). To investigate the single-trial variability of the evoked responses, we made simultaneous fluorescence measurements of VSD and CaSD signals using two cameras. Single-sweep simultaneous VSD and CaSD recordings revealed strong variability in both optical signals (Fig. 6A, n = 7 mice). The sensory-evoked CaSD and VSD response amplitudes covaried trial by trial, with small CaSD signals accompanying small VSD signals and large CaSD signals accompanying large VSD signals. These results are consistent with both evoked APs (reflected in the CaSD signal) and evoked PSPs (reflected in the VSD signal) competing with the spontaneous activity (Petersen et al. 2003b; Sachdev et al. 2004). The spontaneous activity itself, occurs as propagating waves, which are nearly simultaneous in CaSD and VSD signals (Fig. 6B; Supplemental Movie 2). Topical application of 500 μM d-APV and 125 μM CNQX to the cortical surface after 10 min blocks this spontaneous activity as measured by both VSD and CaSD (Fig. 6, C and D). These spontaneous fluorescence changes similar to the evoked sensory responses are thus also dominated by local cortical activity rather than long-range axonal signals.

Deep isoflurane anesthesia suppresses CaSD signals more than VSD signals

We were interested to see whether further experimental conditions could be found that would affect the CaSD and the VSD responses differently, in addition to the effect of varying stimulus strength described earlier. Striking differences were found between the two optical signals measured under different levels of anesthesia. We imaged both CaSD and VSD responses under different concentrations of isoflurane and found that VSD responses were relatively little affected by depth of anesthesia (Ferezou et al. 2006), whereas CaSD responses were strongly suppressed by anesthesia (Fig. 7A). Deep (1.5–2%) isoflurane anesthesia reduced the whisker-evoked response amplitude to 85.7 ± 13.5% for VSD and to 33.4 ± 6.8% for...
CaSD relative to the responses recorded at low (0.5–1%) concentrations of isoflurane (Fig. 7B). Because our results suggest that the VSD signals reflect subthreshold changes and CaSD reflects suprathreshold signaling (Figs. 1–5), these data then suggest that increasing isoflurane concentration strongly reduces AP firing with a much smaller effect on subthreshold PSPs. To test whether these observations hold true at the level of individual neurons, we made whole cell V_m recordings to study evoked responses at different concentrations of isoflurane. Some cells responded with only subthreshold PSPs (Fig. 7C) and others also fired APs (Fig. 7D). Deep isoflurane anesthesia reduced PSP amplitude to 73.8 ± 9.5% (n = 8) and reduced evoked AP activity to 2.0 ± 2.2% (n = 5) of the evoked response recorded under light isoflurane anesthesia (Fig. 7E). Increased isoflurane anesthesia therefore slightly reduces evoked synaptic depolarization of cortical neurons and strongly reduces AP firing. The nonlinear threshold for AP initiation likely allows a small reduction in VSD signal and PSPs to be translated into a large reduction in CaSD signal and suprathreshold activity.

**DISCUSSION**

We have demonstrated that VSD and CaSD epifluorescence measurements can be combined to image the spatiotemporal dynamics of cortical activity both in vitro and in vivo. The VSD signal was found to correlate with subthreshold membrane potential changes and the CaSD signal reflected predominantly action potential firing. We therefore imaged in vivo VSD and CaSD to define the sub- and suprathreshold spatiotemporal dynamics in layer 2/3 of spontaneous activity and sensory processing evoked with different stimulus strengths and under different conditions of isoflurane anesthesia.

**Imaging membrane potential with VSD and action potentials with CaSD**

In vivo, the time course of the VSD RH1691 fluorescence followed V_m changes recorded in individual layer 2/3 neurons of mouse somatosensory barrel cortex (Fig. 4, A–C), similar to...
this single AP on surrounding postsynaptic neurons in the
2003a,b) and mouse barrel cortex (Ferezou et al. 2006). Com-
results described previously in rat barrel cortex (Petersen et al.
VSD and CaSD signals (gray circles represent individual
anesthesia, whereas the CaSD response was strongly suppressed.
increasing the isoflurane concentration in this example
10% of the AP-evoked calcium signal in the
network is orders of magnitude larger, causing a network
depolarization of approximately 1,000 cells \( \times 1 \text{ mV} \times 25 \text{ ms} \)
(\(=25,000 \text{ mV-ms} \)). The \( V_m \) changes observed in the averaged
traces of the WC recording and the accompanying VSD signals
are therefore dominated by subthreshold \( V_m \) changes. This
situation applies to networks where neurons are on average far
from threshold and require substantial depolarization to evoke
an action potential. This is the case for both the in vitro and the
in vivo measurements. The correlation of single-cell \( V_m \) and
the VSD signal suggests that, on the scale of a single barrel,
many neurons behave in a similar subthreshold manner and it
is likely that specificity in encoding information arises from the
subset of neurons that reach threshold to fire action potentials.

Because the VSD stains the brain tissue nonspecifically,
changes in \( V_m \) of glial cells will also contribute to the fluores-
cence signals. However, this is likely to be a small effect
because glial responses to direct neuronal transmitter release
have small amplitudes as a result of the relatively high glial
membrane conductance (Lin and Bergles 2004) and because
glial VSD responses resulting from increased extracellular
potassium concentrations have much slower kinetics (Konnerth
et al. 1987).

CaSD signals require AP firing and reflect suprathreshold
neuronal activity. In addition to the AP-evoked calcium sig-
als, we found only a minor subthreshold contribution (esti-
mated to be roughly 2% of the AP-evoked calcium signal in the
cellular measurements; Fig. 1, F and G). The nonspecific
loading with AM esters labels both neurons and glia, both of
which might contribute to our calcium measurements. How-
ever, it was recently demonstrated that glial and neuronal
calcium signals do not temporally correlate (Nimmerjahn et al.
2004) and that sensory-evoked glial calcium signals are nearly
two orders of magnitude slower in onset and duration com-
pared with neuronal calcium signals (Wang et al. 2006). The
calcium signals evoked by sensory or electrical stimulation that
we recorded are fast, linked to neuronal activity, and are thus
unlikely to substantially involve glial calcium signals. We
did not observe slow CaSD signals unrelated to VSD signals,
although very slow signals are more difficult to detect and so
may have gone unnoticed. We conclude that the CaSD signals
we observed are likely to result predominantly from AP firing
in neurons (Smetters et al. 1999). In particular, local action
potential activity seems to play a dominant role for the calcium
signal we measured, whereas long-range axonal signals from
the thalamus or other cortical areas appear to make only a
minor contribution (Figs. 2E, 4, F and G, and 6, C and D). In
contrast, the VSD signal is driven by PSPs and dominated by
changes in neuronal subthreshold \( V_m \) which it follows with
millisecond precision. Although VSD and CaSD signals are
clearly related, the highly nonlinear aspect of AP generation
allows the two signals to be differentially manipulated.

Imaging sensory processing with VSD and CaSD

Whisker deflections evoke responses in the primary somato-
sensory barrel cortex, through a signaling pathway from
the trigeminal nerve to the brain stem, and then to the
thalamus, which provides input to the neocortex. Three
qualitatively different processing modes of layer 2/3 barrel
cortex were observed depending on the strength of the
whisker stimulus. The weakest stimuli evoked localized

results described previously in rat barrel cortex (Petersen et al.
2003a,b) and mouse barrel cortex (Ferezou et al. 2006). Com-
plete truncation of action potentials through median filtering
makes only a small difference to the mean \( V_m \) trace (Fig. 4, D
and E). Experimentally, it is therefore clear that APs contribute
little directly to the ensemble network membrane potential
changes. The absence of a substantial direct AP-related VSD
signal, while imaging network activity, can also be understood
in terms of the relative ensemble electrical impact of APs
versus PSPs. A single AP in a single pyramidal neuron will
directly cause a depolarization of approximately 1 cell \( \times 100 \text{ mV} \times 1 \text{ ms} \)
(\(=100 \text{ mV-ms} \)). However, the synaptic impact of
this single AP on surrounding postsynaptic neurons in the

FIG. 7. Deep isoflurane anesthesia strongly suppresses evoked CaSD sig-
als and APs with a smaller reduction in VSD signals and postsynaptic
potentials (PSPs). A: increasing the isoflurane concentration in this example
did not change the peak VSD RH1691 signals evoked by whisker stimulation,
but strongly reduced CaSD OGB-1 signals (each trace is an
average of 10 trials). B: plot of the ratio of the peak response amplitude evoked
during deep anesthesia (1.5–2% isoflurane) relative to light anesthesia (0.5–1%
isoflurane) for VSD and CaSD signals (gray circles represent individual
experiments with a line linking VSD and CaSD measurements; black circles
show the average \(+ \text{SE}, n = 7\). VSD signal was weakly reduced during deep
isoflurane anesthesia, whereas the CaSD response was strongly suppressed.
C. top: 10 superimposed individual sweeps of evoked PSPs measured with a WC
recording under 0.75% isoflurane (left) and 1.5% isoflurane (right). Increasing
the concentration of isoflurane did not affect the amplitude of PSPs evoked
by whisker stimulation (averaged sweeps below). D: in a different cell, which
responded with suprathreshold activity to whisker deflection during light
isoflurane anesthesia, the AP firing was almost abolished at deeper isoflurane
anesthesia. Ten superimposed trials are shown (above) at 0.75% isoflurane
(left) and 1.5% isoflurane (right). PSTH for this cell is plotted below, quanti-
fied as the probability of evoking an AP in a given 2-ms time bin. E: plot of
the ratio of the evoked PSP amplitude and APs during deep isoflurane
anesthesia (1.5–2%) relative to light isoflurane anesthesia (0.5–1%) (gray
circles represent individual experiments). APs were not evoked in all neurons
and a line links experiments where both PSPs and APs were recorded. Black
circles show the average \(+ \text{SE}, n = 8 \text{ PSPs}; n = 5 \text{ APs}. PSP amplitude is
much less affected than AP firing by increasing isoflurane anesthesia, mirroring
the strong reduction in CaSD signals with smaller effects on the VSD
signals described earlier.
VSD signals without any CaSD signal, implying a localized subthreshold excitation without supragranular AP activity. Such a local subthreshold signal could modulate other inputs, for example, by summatng with other sensory inputs from neighboring whiskers, other sensory modalities, or with top-down influences. Intermediate stimuli evoked propagating PSPs with localized suprathreshold signals in layer 2/3. In response to this intermediate stimulation strength, the sensory processing of the single whisker stimulus in terms of action potential activity is therefore restricted to the principal whisker barrel column, although the spreading subthreshold VSD signal would allow multiwhisker integration to occur. The strongest stimuli evoked propagating VSD and CaSD signals. Therefore even a single whisker, if deflected sufficiently strongly, can evoke a propagating wave of sensory responses involving regenerative AP firing. These data are in good agreement with receptive field analysis of individual neurons in layer 2/3 of barrel cortex, which are very broad at the subthreshold level, but show more tightly tuned suprathreshold classical receptive fields (Brecht et al. 2003; Moore and Nelson 1998; Petersen and Diamond 2000; Simons 1978; Wilent and Contreras 2005; Zhu and Connors 1999).

Deep isoflurane anesthesia strongly reduces evoked action potentials and CaSD signals

In a further test to probe the relationship of the VSD and CaSD signals, we found that the level of isoflurane anesthesia led to appreciable differences in the behavior of the two fluorescence signals. The VSD signals and PSPs were only weakly affected by increasing isoflurane concentration, whereas the CaSD signals and APs were strongly suppressed (Hentschke et al. 2005). Even small changes in subthreshold activity may strongly affect suprathreshold activity at low firing rate conditions (Brecht et al. 2003; Crochet and Petersen 2006). Thus the action of isoflurane is likely to reflect a small change in subthreshold activity that nonlinearly translates into a strong effect in action potential discharge. Equally, one can construct a membrane potential trajectory by summing either many or few excitatory and inhibitory synaptic inputs. It might therefore be possible to record a similar PSP resulting from sparse AP firing compared with higher levels of AP activity, simply by appropriately balancing excitation and inhibition. Finally, the large CaSD signal at low anesthesia might suggest that AP firing rates are considerably higher in awake mice than in mice under deep anesthesia. Independent of the underlying mechanisms, these results stress the importance of measuring with both dyes because together they give information, which imaging either dye alone could not provide. The nonlinear function linking $V_m$ to firing rate makes it difficult to relate PSPs to APs at a network level. The combined imaging approach presented here is thus likely to be a useful experimental tool for many investigators probing cortical network properties and seeking to differentiate between sub- and suprathreshold effects of a specific manipulation.

Outlook and limitations

The current technique is attractive in that it is relatively simple, requiring only epifluorescence optics and a fast camera. The data collected by this method demonstrate that CaSD and VSD signals can be recorded in the same preparation with millisecond time resolution and that they image different aspects of cortical function, with the VSD reflecting subthreshold PSPs and the CaSD being dominated by APs. Currently, these methods applied in vivo are well suited only for the study of supragranular layers because VSD RH1691 does not penetrate deeply into the cortex (Supplementary Fig. 1; Ferezou et al. 2006). Also the fluorescence excitation light is unlikely to penetrate substantially below the supragranular layers and indeed the blue light needed to excite OGB-1 will penetrate less deeply into the brain than the red excitation light for VSD RH1691. Equally, the clear correlation between the VSD signal and subthreshold activity that we observe in the neocortex may not hold true for other brain areas. In hippocampal Schaffer collaterals, a high degree of synchronized action potential activity results in the detection of the suprathreshold activity with VSDs (Grinvald et al. 1982). Comparably, highly synchronous spiking activity of thalamocortical axons traversing the striatum can be observed with RH1691 in thalamocortical slices after stimulation in the thalamus (Neubauer and Berger, unpublished observations). In the cortical gray matter, however, the part of the VSD signal related to spiking activity (compare Fig. 1D) is extremely small because only a small fraction of membranes experience an action potential, but all neuronal membranes experience subthreshold depolarization. Thus either dye alone (VSD or CaSD) does not seem to be well suited to reflect the complexity of electrical signals in the cortical network. The CaSD responses measured under our conditions with epifluorescence do not have a dynamic range that allows the detection of subthreshold activity and VSDs on the gray matter network level do not reflect AP activity. However, what initially seems to be a limitation turns into an advantage if both dyes are used together, as done in this study for the first time. A combined use of VSDs and CaSDs enables the simultaneous but separate study of synaptic and action potential activity in the barrel cortex.

Using two-photon microscopy combined with OGB-1 AM labeling, Kerr et al. (2005) previously described the detection and separation of local input and output activity in neocortex by imaging the fluorescence of a single dye. Fluorescence changes in the neuropil were suggested to relate to axonal calcium transients, which would reflect local synaptic input. Somatic fluorescence changes resulted directly from action potential discharge of the imaged neuron, which thus provided a measure of output. With epifluorescence optics, the calcium signals of incoming long-range axons do not seem to have a major impact on the overall calcium signal (Figs. 2E and 4, F and G). Thus the calcium signal in our study appears to reflect only the local action potential activity of the local cortical area imaged.

A disadvantage for the use of CaSDs in vivo (in contrast to VSDs) is the need to inject the AM ester dye. AM esters can be applied with pipettes into the brain but they stain only neurons in a small volume of about 300-$\mu$m diameter (Stosiek et al. 2003). In a spatially restricted field of three cortical columns in a parasagittal slice, a complete staining can be achieved (Fig. 3), but even with many injections we were unable to obtain uniform staining of a complete cra-
niotomy measuring several millimeters in diameter. Our analyses of the spatial extent of CaSD signals in vivo (Fig. 5) was therefore limited to three well-defined separate injection spots across the cortical map. This spatially restricted labeling is of course true for both epifluorescence and two-photon imaging. However, two-photon imaging is generally applied at high magnification and correspondingly small fields of view, which can be stained easily with one AM ester injection (Kerr et al. 2005; Stosiek et al. 2003). The recent development of transgenic mice expressing genetically encoded calcium-sensitive fluorescent proteins may provide an approach to wide-field epifluorescence measurements of calcium signaling in vivo (Díez-García et al. 2005; Hasan et al. 2004; Nagai et al. 2004). However, current data suggest that the genetically encoded calcium indicators are not sensitive to single action potentials (Pologruto et al. 2004). In the rodent somatosensory cortex, where low-frequency action potential firing was previously observed (both for spontaneous and evoked activity) (Brecht et al. 2003; Crochet and Peterson 2006), the genetically encoded calcium indicators might then miss the majority of activity.

Our approach of wide-field epifluorescence imaging of CaSDs and VSDs therefore appears to be a promising combination to study different aspects of cortical computation. Such simultaneous imaging of subthreshold and suprathreshold neuronal activity will help advance our understanding of sensory processing.

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Present address of A. Borgdorff: Pelvipharma SAS, Domaine CNRS, Bat 5, 1 Avenue de la Terasse, 91910, Gif-sur-Yvette, France.

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