Integration of Evoked Responses in Supragranular Cortex Studied With Optical Recordings In Vivo

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Civillico, Eugene F, and Diego Contreras. Integration of evoked responses in supragranular cortex studied with optical recordings in vivo. J Neurophysiol 96: 336–351, 2006. First published March 29, 2006; doi:10.1152/jn.00128.2006. Complex representations in sensory cortices rely on the integration of inputs that overlap temporally and spatially, particularly in supragranular layers, yet the spatiotemporal dynamics of this synaptic integration are largely unknown. The rodent somatosensory system offers an excellent opportunity to study these dynamics because of the overlapping functional representations of single-whisker inputs. We recorded responses in mouse primary somatosensory (barrel) cortex to single and paired whisker deflections using high-speed voltage-sensitive dye imaging. Responses to paired deflections at intervals of 0 and 10 ms summed sublinearly, producing a single transient smaller in amplitude than the sum of the component responses. At longer intervals of 50 and 100 ms, the response to the second deflection was reduced in amplitude and limited spatially relative to control. Between 100 and 200 ms, the response to the second deflection recovered and often showed areas of facilitation. With increasing interstimulus interval from 50 to 200 ms, recovery of the second response occurred from the second stimulated whisker’s barrel column outward. In contrast to results with paired-whisker stimulation, when a whisker deflection was preceded by a weak electrical stimulus applied to the neighboring cortex, the summation of evoked responses was predominantly linear at all intervals tested. Thus under our conditions, the linearity of response summation in cortex was not predicted by the amplitudes of the component responses on a column-by-column basis, but rather by the timing and nature of the inputs.

INTRODUCTION

Topological maps in primary sensory cortices delineate the anatomical extent of neural representations; however, the principles governing spatiotemporal interactions between physiological responses to simple stimuli are not yet understood, even for a map as well-characterized as the rodent barrel cortex. The attenuation of responses over cortical space, the degree of linearity of response interactions, and the spatiotemporal envelope within which such interactions may occur must be characterized to determine the mechanisms by which complex representations are formed and behaviors directed. Here we approach these basic questions at the large network scale by examining the interactions between responses to whisker deflections in the supragranular layer of mouse barrel cortex in vivo using optical recordings with voltage-sensitive dyes (VSDs) and electrophysiological recordings of local field potentials (LFPs) and multiunit activity (MUA).

The posteromedial barrel subfield (PMBSF) of the rodent primary somatosensory cortex contains an array of discrete multicellular elements in layer 4, termed barrels, which map in a one-to-one fashion to the whiskers on the contralateral face (Woolsey and Van der Loos 1970) and which may be visualized by staining for cytochrome oxidase (CO). Although single neurons throughout the barrel-associated cortical column respond most strongly to one principal whisker (Simons 1978, 1983; Simons and Woolsey 1979), they are also responsive to many neighboring whiskers (Brecht and Sakmann 2002a,b; Higley and Contreras 2003, 2005; Moore and Nelson 1998). Multiwhisker response integration has been addressed by numerous electrophysiological studies in vivo, both extracellularly (Brumberg et al. 1996; Ego-Stengel et al. 2005; Shimgei et al. 1999, 2000; Simons 1983; Webber and Stanley 2004) and intracellularly (Higley and Contreras 2003, 2005; Moore and Nelson 1998). Evidence points to a primarily suppressive interaction between whisker responses, with the possibility of some facilitation for coincident or near-coincident (<5 ms) whisker inputs. Integration of inputs separated in time is of particular interest in the whisker–barrel system because whiskers are likely to contact an object multiple times during the rhythmic whisking behavior (for review on frequency-dependent processing see Moore et al. 1999).

Single-neuron recordings are inherently limited in scope because electrodes must be targeted to particular columnar locations. Multielectrode arrays overcome this limitation, but are constrained to a predetermined shape and spatial resolution that may not be in register with the anatomical and physiological features of highly variable in vivo preparations. VSDs, by contrast, allow the measurement of activity, at fine temporal and spatial scale, of selected functionally significant regions of arbitrary size and shape. Recent VSD studies have described the spatiotemporal properties of the supragranular response to individual whisker stimuli in vivo (Derdikman et al. 2003; Kleinfeld and Delaney 1996; Orbach et al. 1985; Petersen et al. 2003a,b) and to electrical stimulation in vitro (Laaris and Keller 2002; Laaris et al. 2000), and the technique is well suited to describe the interaction of responses to multiple stimuli as well.

Previous imaging studies that addressed the question of multiwhisker integration used blocks of stimuli trains and images averaged over hundreds of milliseconds. Here we report high-speed optical recordings of response interactions at 0- to 200-ms interstimulus intervals between paired whisker deflections over the entire barrel cortex. A frame rate of 250 Hz...
allowed visualization of response development at the timescale of synaptic events. Responses to single stimuli and, accordingly, interactions between responses occurred over wide distances (hundreds of micrometers) and long intervals (>100 ms). At intervals of 0 and 10 ms, summation occurred that was linear or sublinear. At intervals of 50 and 100 ms, response interaction was consistently sublinear but, more importantly, the second response was strongly suppressed. For intervals >100 ms the second response recovered from suppression to control values, producing responses identical to the linear sum. The second whisker’s main barrel column consistently participated in the earliest recovery. In contrast to sensory responses, electrically evoked responses in cortex showed predominantly linear interactions with the whisker responses.

METHODS

Results are based on 30 adult C57 mice (7 wk old, 20 g). From these, 25 experiments were selected for analysis based on the following criteria: 1) homogeneous staining of the preparation as judged by visual inspection of the baseline image (14-bit) obtained at the beginning of each recording, 2) stability of the optical responses throughout the experimental session, 3) stability of the EEG pattern recorded from the same electrodes used for electrical stimulation, 4) stability of the evoked LFP responses recorded by electrodes adjacent to the stimulating one, and 5) large signal-to-noise ratio (10:1) of VSD responses to deflections of at least two different vibrissae.

Surgery and preparation

Mice were deeply anesthetized with ketamine–xylazine (100 and 20 mg/kg, respectively, administered intraperitoneally) and mounted in a stereotaxic apparatus. Supplemental anesthesia (25 and 5 mg/kg, respectively) was administered as necessary to maintain cortical slow oscillations and weak or absent foot withdrawal reflex. A craniotomy was made that extended 2 mm in the anterior–posterior direction starting from bregma, and 2–4 mm in the mediolateral direction starting from the midline. In most animals this was sufficient to expose most of the PMBSF. The dura was resected over the entire craniotomy.

Once electrodes were inserted, hand stimulation of the whiskers with audio feedback from the cortical LFPs was used to determine the approximate location of the electrodes within the PMBSF. This information was used to determine the whiskers most suitable for VSD imaging.

Staining

Following Kleinfeld and Delaney (1996), a 1-mm³ piece of gelfoam (Upjohn Pharmacia) was soaked in a warm solution of the voltage-sensitive dye RH795 (1 mg/mL; Molecular Probes, Eugene, OR) or RH1691 (1 mg/mL; Optical Imaging, Mountainside, NJ) in 0.9% saline and placed on the exposed cortex. Additional dye was added to keep the gelfoam soaked for 1.5 h. After staining and before recording the exposed surface of the brain was generously washed with saline to remove unbound dye. Throughout the experiment the brain surface was rinsed with saline to prevent desiccation. RH795 (Grinvald et al. 1994; Obaid et al. 2004) and RH1691 (Shoham et al. 1999) are potentiometric styryl dyes that attach to cell membranes and show a decrease (RH795) or increase (RH1691) in fluorescence on a microsecond timescale in response to membrane depolarization. For consistency with convention all VSD responses shown here are oriented so that positive-going deflections indicate depolarization. When applied topically in vivo, the dyes stain the supragranular cortical layers most intensely (Kleinfeld and Delaney 1996; Petersen et al. 2003a). Potentiometric dyes are linear indicators of $V_m$ over physiological ranges (Cohen and Salzberg 1978; Cohen et al. 1978; Salzberg et al. 1983). The dye is taken up preferentially by dendrites and cell bodies. Because layer 2/3 is primarily neuropil, the signal source in vivo is considered to be mostly from dendrites (Contreras and Llinas 2001; Grinvald et al. 1994; Yuste et al. 1997), although a recent detailed stereological analysis revealed that far more axonal than dendritic membrane per unit of volume is present in the neuropil of layers 2 and 3 (C. Avendaño, personal communication). Some contribution also comes from glial cells (Konnert et al. 1987; Salzberg 1989).

Optical recordings

Recordings were made with a modified upright microscope (BX50WI; Olympus, Tokyo, Japan). Epi-illumination was provided by a 12-V halogen lamp. Excitation light was band-pass filtered around 540 ± 20 nm; light emitted from the preparation was long-pass filtered <600 nm. The optical signal was collected with a CCD camera (MiCam01; BrainVision, Tokyo, Japan) with a detector array of 96 × 64 pixels (87 × 60 imageable) at a frame rate of 250 Hz (4 ms/frame). Frame times given in figures and text refer to the end of acquisition of a frame; for example, a frame labeled 24 ms is a measurement of light emitted from 20 to 24 ms. The microscopic objective was ×4 (N.A. = 0.28; Olympus), resulting in an imageable area of 1 × 2 mm and a pixel size of 22 × 22 μm (484 μm²). Optical recording was controlled by the MiCam software. All data were collected as single trials, with no on-line blank subtraction or on-line averaging.

The fractional fluorescence change received little contribution from intrinsic metabolic signals related to oxygen delivery. The longest time after a stimulus for which we analyzed data was 350 ms, i.e., the time between the first whisker deflection and the peak response to the second whisker deflection at the maximum interdeflection interval of 200 ms. In contrast, hemoglobin-associated absorbance changes have been shown to begin several hundred milliseconds poststimulus. For example, in Devor et al. (2003), blood-flow–related signals were recorded from 1.5 to 2.5 s after the stimulus, whereas LFP and MUA were integrated from 0 to 300 ms after the stimulus. The “early signal” or “initial dip” corresponding to the increase in deoxyhemoglobin by oxygen delivery to neurons takes almost one full second to develop (Frostig et al. 1990; Kim et al. 2000). Additionally, in four experiments with RH1691, which is not sensitive to hemoglobin changes as a result of its shifted absorbance spectrum, the kinetics of the responses and the time course of suppression were identical to those seen with RH795.

Electrophysiological recordings

To record LFP and deliver electrical stimulation, we manufactured arrays of three or four pairs of tungsten electrodes (FHC, Bowdoinham, ME), with vertical tip separation of 0.5 mm and horizontal separation between pairs in the array of 0.75 mm. For each experiment one array was advanced into the cortex at the lateral edge of the craniotomy, normal to the cortical surface, with the upper electrode resting on the pial surface. Recording and stimulation were in bipolar configuration. The signal from the electrodes was band-pass filtered between 0.1 and 300 Hz to obtain LFP recordings and between 300 and 10,000 Hz to obtain multiunit recordings (MUA).

Our measurements of MUA and LFP were made at the periphery of the stimulated area, to allow for imaging over the greatest possible cortical area. Therefore our electrophysiological recordings are not likely to reproduce the findings of supralinear summation in the principal barrel as measured by others (Shimegi et al. 1999). The dual purpose of recording electrophysiological signals was to confirm the basic temporal properties of the VSD responses and to increase our confidence that the VSD responses represented primarily neuronal signals.
**Electrical stimulation**

Electrical stimuli consisted of single 100-μs pulses of 0.1- to 0.3-mA intensity delivered through the recording electrodes in a bipolar configuration. In a healthy stained cortex, the response to an electrical stimulus spreads across all visible barrel cortex (Civillico and Contreras 2005). We exploited this property to assess the health of the preparation during an experiment. If the spatial spread of the response to electrical stimulation decreased significantly during the course of an experiment, it was terminated and the data were not used for analysis.

**Response interaction experiments**

For each interaction experiment, a reliable response was first obtained from a control whisker in response to a 100-ms ramp-and-hold deflection (8-ms rise time, 1,300°/s, calibrated as for Wilent and Contreras 2004) in the ventral direction with a piezolectric device (Simons 1983). An air puff (10 ms, 2–5 PSI; Picospritzer, Intracel, Herts, UK) was then directed to deflect another single whisker or group of whiskers in the ventral direction using a 1-mm-diameter capillary tube. Great care was taken to ensure that no whiskers were unintentionally stimulated; when necessary, nearby whiskers were trimmed away. Once clear responses had been evoked from two whiskers, pairs of deflections were presented, with the interstimulus interval (ISI) cycling through a set of five to seven interdeflection intervals plus both controls. The intervals used were 0, 10, 50, 100, and 200 ms. In some experiments 5- and 150-ms intervals were also used. To avoid confounds caused by changes in cortical responsiveness over the duration of an interaction experiment, whisker deflections were presented in interleaved order, one every 10 s, with a complete cycle of intervals and controls being presented before any interval was repeated. For some experiments, the protocol was repeated with the first whisker deflection replaced with an electrical stimulus to the cortex, delivered through one of the tungsten bipolar electrodes of the array. In all experiments, the timing of the second deflection within the trial remained constant, whereas stimulus 1 was moved to different times to produce the desired interval.

**Cytochrome oxidase histology**

At the conclusion of an imaging experiment, two fiducial marks were made by advancing a single electrode into the cortex. Reference images in register with the VSD recordings were taken with these new marks. Animals were perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS). Brains were postfixed overnight in a mixture of 0.1 M PBS with 10% methanol (BP1105-1, Fisher Scientific) and 30 mg of cytochrome oxidase per 100 ml of phosphate buffer (PB). The following day, tissue was washed in PBS, mounted in subbed glass slides, dehydrated, and coverslipped.

**Barrel binning**

The tracks left by the field potential electrodes, in combination with the additional fiducial marks made at the end of the experiment, were used to align the barrel outlines from histology with the fractional fluorescence images (Fig. 1). This allowed binning of pixels into signals corresponding to the average activity within barrel columns while also increasing the signal-to-noise ratio. The margin of error for the alignment of barrels was half the width of a single electrode, which is close to the width of septa. Thus we made no attempt to describe responses in septa as has been done for single cells (Brecht and Sakmann 2002a; Brecht et al. 2003). For simplicity, we did not discriminate barrel substructure.

**Data analysis**

Optical data were collected as differential fluorescence and divided by a reference image acquired automatically at the start of each trial to produce fractional fluorescence \((ΔF/F)\) data used for all analysis and figures. Postprocessing consisted of averaging of single trials after screening (see following text) followed by spatial averaging using a flat 5 × 5 window, except in Fig. 1A where unfiltered data are shown. All analysis was done with custom routines written in Igor Pro (WaveMetrics, Lake Oswego, OR).

**Quantification of response interaction**

Interactions between whisker responses were quantified with two measures: the linearity index \((LI)\) [analogous to the facilitation index \((FI)\) used in Shimegi et al. (1999, 2000)] measures the linearity of the response interaction, whereas the response ratio \((RR)\) measures the effect of response 1 on response 2. The \(LI\) was computed as follows: for intervals of 0 and 10 ms, we constructed a linear prediction by adding together the single control responses with the appropriate temporal offset. \(LI\) was then computed as the observed response peak amplitude divided by the predicted peak amplitude. For intervals of \(≥50\) ms, variability in response decay kinetics made the interpretation of \(LI\) measured in this way difficult. Therefore for ISIs of \(≥50\) ms, \(LI\) was computed as the response 2 measured from its point of departure from response 1, divided by response 2 alone. This retains the measure's meaning as an index of linearity \((\text{linearity } = 1)\). The \(RR\) was computed as the ratio of the signal level at the expected time of the second response peak to the level of the second response peak when evoked alone. This value was measured irrespective of whether a second response transient in the combined response was discernible. As with intracellular recordings, \(RR\) could assume negative values.

**Thresholding and visualization**

For each recording, the baseline noise was quantified by obtaining the SD at each pixel during 100 ms before the first stimulus, and then plotting the distribution of these values, which was always unimodal. The peak, which represents the most common SD over the image, was used to set the range of each color for pseudocolor images. In all pseudocolor images only statistically significant activity >2 SD is shown. Pseudocolor images in \(ΔF/F\) are shown superimposed on the baseline fluorescence in grayscale. For contour plots, thresholds were chosen to emphasize the localization of response peaks within the PMBSF.

**Trial screening**

The interaction of sensory- or electrically evoked responses with anesthesia-dependent slow oscillations is well documented (Rosanova and Timofeev 2005; Sachdev et al. 2004; Timofeev et al. 1996). In this study, we did not address the effect of the slow oscillation on response interactions; however, we did remove from the averages experimental trials in which the first stimulus caused a transition to the depolarizing phase of the slow oscillation (also called an “up-state”). Up-state trials were identified by examination of the VSD signal as
responses with a normal onset latency and initial slope, but amplitude up to twice that of other evoked responses, with no return to baseline observed during the several hundred milliseconds of an optical recording trial. Trials identified in this way consistently corresponded to stimulus-evoked transitions in the simultaneously recorded LFPs. Their removal addresses an important potential confound by eliminating trials in which stimuli fell in two different states. Such trials would be likely to produce an overestimation of suppression resulting from reduction of the driving force and a change in $V_m$ of almost all cortical cells between the first and second stimuli. The number of trials removed was between 10 and 20% of the total in each experiment.

RESULTS

Our goal was to characterize, using optical recordings from the cortical surface, the spatiotemporal properties of the interaction between responses to sensory stimulation and between sensory and electrical stimulation. We used the responses to paired whisker deflections with different interdeflection intervals and brief low-intensity electrical shocks applied directly to the cortical surface. The data presented here are based on 25 experiments and a total of 374 imaged barrel columns. We will briefly describe the response to single-whisker deflections and
then characterize the response to paired deflections. Finally, we will characterize the interaction between sensory- and electrically evoked responses.

**Responses to single-whisker deflections**

Responses were visible before filtering in the averaged optical recordings (Fig. 1A, n = 20 trials each). Each frame in Fig. 1A represents fractional fluorescence (%ΔF/ΔF in grayscale) integrated from $t = 20$ to $t = 24$ ms after the onset of a 5-ms ramp-and-hold deflection of each of four different whiskers (C1, D1, D3, and E1, indicated above the optical frames). Superimposed on the raw data images are yellow isofluorescence contours (thresholded at 0.07% ΔF/ΔF, 6 SDs above baseline) constructed after convolving the images with a $5 \times 5$ (about $100 \times 100 \mu m$) flat kernel. The effect of the kernel is to enhance the signal-to-noise ratio by scaling the signal from each pixel proportionally to its correlation with its neighbors. The isofluorescence contours from the filtered data agree with the responses visible in the raw images. Data in all subsequent figures were filtered using this procedure and data analysis was based on the filtered responses; however, the main features of the responses illustrated in the results can be seen in the raw data.

To understand the spatiotemporal distribution of cortical activation with respect to the location of layer 4 barrels, we aligned the optical data with the barrel outlines obtained from CO staining (Fig. 1B). Because the barrels in layer 4 typically varied in size and shape, forming an irregular grid of rows and arcs, it was necessary to align the histological and fluorescence images for each experiment. We used the marks left by the three bipolar tungsten electrodes (e1, e2, e3), which form a straight line parallel to the midline from anterior (A) to posterior (P), and two medial fiducial marks (f1 and f2), made after completion of recording. The alignment procedure is illustrated in Fig. 1C, in which isofluorescence contours at 24 and 32 ms of the responses to each of four whiskers deflected individually are superimposed on the CO barrels, and the landmarks are aligned between the two images (arrowheads). Presumably because of the small size of the mouse brain and its relative lack of convolutions and curvature over the region of barrel cortex, only minimal corrections along the two dimensions were necessary to align the images. No skewing, warping, or other nonlinear image transformations were needed.

At 24 ms after deflection onset (Fig. 1C), each response was localized to a discrete region loosely associated with the barrel corresponding to the deflected whisker (main barrel). In some cases (barrels E1 and C1; see labeling in Fig. 1B), this initial contour and the main barrel outline were concentric, whereas in others (D1, D3) they were not. The initial contour of the response to the D3 deflection (black) clearly extended into the D4 barrel column. This variability in response onset location within the main barrel was a feature of all single-whisker recordings. By 32 ms, all single-whisker responses encroached extensively on neighboring barrels and overlapped considerably with one another. By 40 ms postdeflection (not shown), each response had activated the majority of the visible cortical area.

The amplitude of the optical response decreased and the latency to peak increased with increasing distance between the deflected whisker and the column being recorded. Figure 1D (left) shows the optical response in the histologically identified C1 column to the deflections of the C1, D1, D3, and E1 whiskers (same recordings as in Fig. 1C). Simultaneously recorded LFP and MUA responses from electrode 2 (e2 in Fig. 1B) are shown to the right. (For additional simultaneously recorded LFP, MUA, and VSD responses see Supplementary Fig. 1.)

The histology showed that electrode 2 was located in the C2 and C3 barrels. The amplitude of the LFP and the number of spikes per stimulus recorded on the electrode decreased with increasing distance of the deflected whisker from this location. The largest response was seen to the C1 deflection, intermediate responses to D1 and D3, and no response to the deflection of the E1 whisker. The VSD and electrophysiological measurements clearly reflected the overlap of single-whisker representations in supragranular barrel cortex, which has been well characterized in the literature (Kleinfield and Delaney 1996; Petersen et al. 2003a; Simons 1978). To estimate the temporal window in which two responses might interact in the cortex, we quantified the onset and peak latencies of the individual responses to each of the two deflections used in the paired protocols over the whole population of barrel-column recordings ($n = 374$, Fig. 1E). The half-height ranges for both responses, to the nearest 4 ms, were 16–32 and 40–60 ms for the onset and peak times, respectively (average onset time: response 1 = 18 ± 8 ms, response 2 = 22 ± 8 ms; average peak time: response 1 = 44 ± 12 ms, response 2 = 49 ± 10 ms). The distributions of the two responses (light and dark gray traces) showed complete overlap, indicating very similar time course. The large variance of the distributions is expected, given that the optical signal incorporates responses from many thousands of cells, and that these distributions include data from barrels at a wide range of distances from the deflected whiskers' barrels. The broad envelope defined by the range of onsets and peaks predicted that responses would overlap at interdeflection intervals as high as 50 ms.

**Responses to paired whisker deflections**

Responses to simultaneous whisker deflections were monophasic and of larger amplitude than that of either response alone. Over most of the imaged area in each experiment, these responses showed varying degrees of sublinearity, with <15% of the imaged area displaying supralinearity. In the example of Fig. 2, the top two rows show the responses to deflection of whiskers D1 and D3 (statistical pseudocolor as described in METHODS). For clarity, every other frame is shown. D1 was deflected with an air puff (10 ms, 4 PSI), whereas D3 was deflected with a piezoelectric ramp (1,300°/s, 8-ms rise time). Statistically significant activity first appeared within the main barrel at $t = 32$ ms for D1 and $t = 28$ ms for D3 (poststimulus times are indicated above top row) and propagated to nearly all visible barrels within 12 ms. Both responses showed a clear tendency to propagate along the row axis into higher-numbered arcs. To show the distribution of activity during the response irrespective of latency, we plotted the maximum amplitude attained at each pixel (peak maps, at right). The highest amplitudes distributed over a wide area and were not well localized to a single barrel.

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1 The Supplementary Material for this article (two Figures) is available online at http://jn.physiology.org/cgi/content/full/00128.2006/DC1.
The response to simultaneous deflection (observed, third row) was larger than either response alone for its entire duration. At 28 ms, a continuous region of statistically significant activity appeared spanning from D1 to D3, and partially invading the C and E rows. By 36 ms the combined response had clearly invaded the B row and most of the E row. Interestingly, the small area that exhibited the highest amplitude response (in red in the peak map at right) did not correspond to either of the two main barrels activated. The maximum amplitude of 0.15% ΔF/F occurred in the C3 column. Relative to the predicted linear sum (fourth row), the obtained response showed more activity at 20 ms and occupied a greater area at 28 ms. However, the linear prediction surpassed the observed response in the D row by 36 ms, and across most of the barrel field by 44 ms. The peak maps of the observed and obtained responses showed that the observed response was much smaller in amplitude over most of the imaged area, indicating a primarily sublinear interaction.

We quantified the relationship between the obtained and predicted responses by calculating the ratio between the obtained and predicted peak maps (bottom). A value of 1 indicates linear summation, whereas values <1 or >1 indicate sub- or supralinear summation, respectively. We refer to this measure as the linearity index (LI; see METHODS). Optical recordings allow computation of this value at every point on the image to generate an LI map. The peak of the distribution of LI values on the map in Fig. 2 was between 0.6 and 0.7, but there were also regions with values ≈ 1 (white) and >1 (blue). When trials were divided into even and odd groups and averaged separately, the locations of the supralinear regions varied, although their size did not. Because the most robust finding was the wide-ranging sublinearity, we did not further characterize the supralinear regions. The degree of sublinearity varied over space and, more importantly, was not correlated with the locations of the stimulated whiskers’ barrels or the areas of larger response amplitude as shown in the peak maps.

To illustrate the difference between regions in the LI map, examples are shown from regions in which the summation was supralinear (top left, brown and black traces are the expected and the observed sum, respectively), sublinear (middle and bottom traces at left), and linear (bottom). When the signals were averaged over the barrel-delimited regions, the spatial substructure was averaged out and the responses were mostly sublinear (e.g., in the D1 and C3 barrel columns, shown at right in Fig. 2) and, in a minority of cases, linear (e.g., in the B2 column). In summary, these results show that the degree of linearity of response summation was not related to the structure or amplitude of the individual responses. For a second example, see Supplementary Fig. 2.

The response to the combined deflection at 10-ms interval was also monophasic (showed a single peak). We quantified the response by calculating the LI at each pixel as with the responses to the simultaneous deflections. The distributions of LI values for the whole population, for the 0- and 10-ms deflection intervals, are shown in Fig. 3A. The mean LI value for 0 ms was 0.69 ± 0.39; for 10 ms it was 0.86 ± 0.54. The distribution for the 10-ms interval indicates that this interval yielded responses closer to linearity but with larger variation...
and showing a greater incidence of supralinear summation (29% of the distribution’s integral is above LI = 1, vs. 16% for 0 ms). As with the 0-ms interval, the spatial substructure of the supralinearity varied among groups of trials analyzed separately (not shown).

Sublinear summation may be the result of a ceiling effect, in which a limit is imposed on the size of the combined response by either the spike threshold or the reversal potential of the synaptic response. We reasoned that if such a ceiling effect were the source of LI values <1, the degree of sublinearity would correlate with the linear prediction on a pixel-by-pixel basis. To test this hypothesis, we plotted the LI values for 10 experiments against the predicted response amplitudes at every responding pixel (Fig. 3B, top). To correct for variations in response amplitude across experiments, we replotted the values with each predicted response map normalized to its peak value (Fig. 3B, bottom). In both the raw and normalized plots, for both 0- and 10-ms intervals, the LI values correlated poorly with the predicted response amplitudes on a pixel-by-pixel basis. In all plots, LI values <1 occurred across a wide range of predicted response sizes, indicating that small responses, not only large responses, commonly added sublinearly. The plots also show that supralinear summation was most frequently observed when the predicted responses were small (<0.1 ΔF/F% raw, or <30% of the maximum normalized). A typical cluster of supralinear pixels is shown in the ratio map in Fig. 2, with their averaged signal and expected linear sum plotted at the left.

To determine whether the linearity of response summation was predicted by the relative amplitude of either response alone at any given barrel column, we plotted the residuals from linearity (predicted − observed) for barrel-column–averaged signals from all visible barrel columns (see METHODS), for 0- and 10-ms intervals, against the magnitude of each response alone (r1 and r2) in the respective column. Despite the low correlation of single pixel values with the linearity of the sum (as in Fig. 3), it was possible that a better correspondence would exist with the signals averaged over the barrel column. Therefore we used barrel-column–averaged traces rather than single-pixel traces for this analysis. In Fig. 4A, five representative examples are shown, plotting the raw residual amplitude in fractional fluorescence against the normalized control response amplitudes (different colored symbols; horizontal dotted line represents linearity). In some experiments weak correlations were observed in the raw residuals, particularly at the 0-ms interval (Fig. 4A, left, pink squares and green crosses). In Fig. 4B the population is represented (n = 374 barrels, 25 mice), with the residuals normalized by the amplitude of the expected linear sum at each column. This normalization results in a measure identical to the linearity index. At the population level, no correlations were apparent between the LI measure and r1 and r2 amplitudes at either interval, demonstrating that the percentage sublinearity was not correlated with the amplitude of the individual responses on a column-by-column basis. One effect of computing the LI from column-averaged VSD traces was to decrease the number of supralinear data points relative to the pixel-based plots in Fig. 3. This indicates that regions of supralinearity did not generally dominate an entire barrel (see LI map in Fig. 2).

Because at 10 ms the combined response was monophasic, we increased the deflection interval to study the interaction between the two responses at intervals at which a linear
interaction would predict a distinct second response. To quantify the linearity of the interaction, we computed LI values at each pixel for these longer intervals as described in METHODS. A representative example is shown in Fig. 5. Stimulation of two neighboring whiskers (D1 and D2) produced largely overlapping responses, as shown by the isofluorescence contours superimposed on the barrel grid (Fig. 5, top left; D2 in blue, contours drawn at 0.066% ΔF/F, or 6 SD above baseline noise; D1 in red, contours at 0.044% ΔF/F or 4 SD). D2 was deflected with an air puff (10 ms, 4 PSI), whereas D1 was deflected with a piezoelectric ramp (1,300°/s, 8-ms rise time). Both single-whisker responses were circumscribed to their main barrel at 24 ms (thick contours), but rapidly spread over a large portion of the barrel field by 28 ms (thin contours). Consistent with other reports, the single-whisker responses exhibited a preferential spread along the row dimension of the barrel grid as shown by the elongated contours along the rows.

At the 50-ms interdeflection interval (second row), the response to the deflection of D1 was completely suppressed. This is shown by the empty LI map (left column) and by the complete absence of the expected second peak (brown traces) in the observed response (black traces) in the example barrel columns shown (D2, D1, C2, and B1 in italics at top; corresponding barrels are drawn with thicker lines in the barrel maps at left). The expected linear sum traces were generated from the control responses shown in red and blue at the top of each column. Strong, but not complete, suppression also occurred at the 100-ms interval (third row). The LI map (left column) illustrates the small region in which a response to D1 could be observed, with response ratio values between 0.1 and 0.35. The barrel traces show a small response in the D1 (arrow) and C2 barrel columns, but not in the D2 column. Consistent with the data for short intervals, suppression of the second response at ≥100 ms was not proportional to the amplitude of the first response at a particular cortical location, as exemplified in the B1 column, in which, despite a very small response to the preceding D2 deflection, the response to D1 was completely suppressed.

At the 150-ms interdeflection interval, the LI map shows that the response to deflection of D1 had recovered to control amplitude (linear summation) in some areas, including most of the D1 barrel, but large areas of sublinearity remained, particularly in the D2 and D3 barrels. In addition, the second response (black traces) was longer lasting than expected (brown traces). At the 200-ms interval, the LI map shows larger areas in which the response to D1 was larger than expected (blue, supralinearity). This is also illustrated by the larger amplitude values of the observed compared with the expected response in the selected example barrels. In the 150- and 200-ms LI maps, subbarrel structure similar to that observed in the LI map of Fig. 2 was visible. For example, the inset below the 200-ms map shows the averaged signal from the two halves of the D3 barrel, in which supralinear (left) and linear (right) interactions were observed. The same time course of recovery of suppression was observed in the LFP and MUA responses recorded from the electrode located between the C3 and B2 barrels (not shown).

The time course of recovery of the second response was sensitive to the distance between deflected whiskers. Figure 6 shows the result from an experiment in which the deflection of B3 (piezoelectric ramp, 1,300°/s, 8-ms rise time) was preceded at various intervals by deflection of C1 (air puff, 10 ms, 4 PSI). The greater interwhisker distance resulted in single-whisker responses with much less initial overlap than that of those shown in Fig. 5, in which the whiskers were nearest neighbors. As in Fig. 5, the spatial extent of activation is shown by overlying isofluorescence contours on the barrel grid (top left; C1 in blue, contour drawn at 0.09% ΔF/F, or 9 SD above baseline noise; B3 in red, contour at 0.04% or 4 SD). Single-whisker responses were almost entirely circumscribed to their main barrel at 24 ms (thick contours) and first invaded common territory at 40 ms (thin contours), in contrast to the example of Fig. 5 in which common territory was invaded by 28 ms poststimulus. The second response was first visible at the shorter interdeflection interval of 50 ms, but it was circumscribed to the second whisker’s barrel and highly suppressed.

**FIG. 4.** Relationship between linearity and control response amplitude. A: raw residual from linearity (ΔF/F) vs. normalized amplitude of response 1 (left, “vs r1”) and response 2 (right, “vs r2”), for 0-ms (left) and 10-ms (right) intervals. Dotted line indicates linearity. B: normalized residual from linearity (equivalent to LI) vs. normalized amplitude of response 1 (left, “vs r1”) and response 2 (right, “vs r2”), for 0-ms (left) and 10-ms (right) intervals. Each symbol is data from one barrel-column. Each color-symbol combination represents data from one experiment.
20% of control, LI map at left). Example barrel traces of the obtained response (black traces, second row) showed a small response in the B3 and B4 barrels (90% suppression of peak amplitude, measured from departure point, see METHODS) and complete suppression in C1 and D3 barrels when compared with the predicted linear sum (brown traces). At the 100-ms interval, the LI map shows values closer to linearity in the area responding to B3 than in the D1 barrel at 100 ms in Fig. 5. The responses in the B3 and B4 barrels had recovered to 60 and 80% of their control peak amplitudes, respectively, whereas the second response remained highly suppressed over the majority of the cortical area (C1 and D3 columns). At the 200-ms interval, limited areas of supralinearity and a large area of linear summation could be observed (LI map at left). The observed response (black traces) in the B3 column to the deflection of the B3 whisker had fully recovered to its control peak amplitude and time course, whereas the response in B4 was 125% of control, that in C1 had recovered to only 40% of control, and the response in the more distant D3 column to only 30% of control. Thus the response to the second stimulus (B3) became visible at a shorter interdeflection interval than in experiments in which the deflected whiskers were nearest neighbors. As in the previously described experiments, for a given time interval, recovery of the second response was greater in the columns closer to the second whisker’s main barrel.

In addition to the LI discussed above, we calculated a response ratio (RR) analogous to that used for extracellular (Simons 1983) and intracellular (Higley and Contreras 2003, 2005) recordings of single neurons. The RR (see METHODS)
measures the effect of response 1 on response 2 rather than linearity of summation. Specifically, RR measures how much the signal level at the time of the peak of the second response increased or decreased with respect to baseline when preceded by another response. Reductions in the peak response to the second deflection as reflected in the RR might be caused either by a decrease in the second response amplitude (divisive reduction) or by a lowering of the baseline of the second response due to the decay kinetics of the first response (subtractive reduction). In both situations, the functional significance of the reduction is that cells are kept farther from spike threshold during the second response, although the underlying mechanisms are different in each case. Throughout our data set we observed both divisive and subtractive effects.

We quantified LI and RR for the whole population of barrel-column recordings (n = 374, Fig. 7A, brown and blue histograms, respectively). The average LI values for the population at 0- and 10-ms intervals were 0.74 ± 0.31 and 0.67 ± 0.41, respectively. At the 50-ms interval they were 0.10 ± 0.28 (mean and SD indicated below the histogram). At 100 ms, the average LI was 0.21 ± 0.30, whereas by 200 ms it had recovered to 0.83 ± 0.52. The average RR value for the population (n = 374, red histograms) was >1 (summation) for the 0-ms (1.46 ± 0.30) and 10-ms (1.30 ± 0.41) intervals. However, for the longer intervals of 50, 100, and 200 ms the RR was mainly <1 (0.72 ± 0.56, 0.35 ± 0.83, and 0.52 ± 0.80, respectively), indicating that in most cases, not only was there no summation of the second response, but the peak signal level after the second deflection was below the peak of the second response alone. The RR values were highly variable at all ISIs and at ISIs ≥50 ms were more variable than the corresponding LI distributions (error bars below histograms). This indicates that although sublinear interactions were omnipresent at these ISIs, the strength of the suppressive effect on the response to whisker 2 was variable. The way in which the two measures diverge is illustrated with an example recording (Fig. 7B, identical color scheme), in which the peak of response 2 was 50% of the linear prediction (LI = 0.5), but close to the response 2 control peak (RR ≈ 1).

In most of our experiments neighboring pairs of whiskers were deflected; therefore the locations of earliest recovery of linearity were close to both the first and the second whiskers’ main barrels. We addressed the question of whether proximity to its main barrel was critical for recovery of the second response by studying experiments in which the deflected whiskers were separated by at least two barrels (n = 5, see Fig. 6 for an example). For each barrel column showing a response (n = 93), we computed the difference in its distance to the two main barrels, or differential distance. This value is positive if the barrel is closer to whisker 2 and negative if the barrel is closer to whisker 1 (Fig. 8). The plot of LI at 100 ms against differential distance for all data points showed a positive correlation, indicating greater recovery in barrels that were closer to the main barrel of the second whisker.
In five preparations, after a complete whisker interaction experiment, we replaced the first whisker deflection with an electrical stimulus delivered through one of the cortical electrodes, while continuing to record LFP and MUA activity from the others. The second whisker stimulus (piezoelectric ramp) remained identical. In the experiment presented in Fig. 9A (same preparation as in Fig. 5), we used an electrical stimulus with the minimum amplitude necessary to evoke an observable LFP response on the neighboring electrode. In the experiment described in Fig. 9B (same preparation as in Fig. 6), the...
electrical stimulus evoked a response comparable in amplitude to that of the whisker stimulus, but was delivered to a topologically distant part of S1 (outside of the PMBSF in the “face barrels”) and propagated horizontally across most of the PMBSF (Fig. 9B montage, “elec”). The properties of electrically evoked responses in cortex are dictated by the underlying circuitry and resemble whisker-evoked responses in amplitude, spatial extent, rise time, and decay time (Civitico and Contreras 2005). Nevertheless, in both experiments, electrically evoked responses did not suppress subsequent whisker responses. In addition, the agreement between the expected linear sum (brown traces) and the observed response (black traces) for barrel-column–averaged VSD signals was remarkable, contrasting sharply with the results of the whisker–whisker experiments. The LI maps for the 100-ms interval show a variety of linear, sublinear, and supralinear interactions at the single-pixel level, rather than the spatial restriction and strong suppression of the second response observed with whisker–whisker stimulation. This result is in agreement with electrical–whisker response interaction results obtained with intracellular recordings in the rat in vivo (Higley and Contreras 2005).

The interval-dependent suppression by preceding whisker responses, and the lack of such suppression after electrical stimulation, could also be observed in MUA activity recorded from tungsten electrodes. Figure 10 shows a typical example, recorded simultaneously with the data shown in Fig. 6 (whisker–whisker, left side) and Fig. 9B (electrical–whisker, right side). The electrode was located in the A row, beyond A5, and is visible in the center of the top edge of the VSD images in Figs. 6 and 9B. Deflections of both B3 and C1, as well as stimuli delivered to the cortex from the neighboring electrode, produced robust multunit responses on this electrode. Single-trial examples are shown in Fig. 10B. Significant suppression of the B3 response was observed at 50- and 100-ms intervals after C1 stimulation. In contrast, an almost exact linear summation was observed after electrical stimulation.

**DISCUSSION**

Our results show that whisker response interactions were consistently sublinear over the population of 25 experiments, despite variability in response size, kinetics, and distance between stimulated barrels. At 0- and 10-ms intervals we observed a single response to paired stimulation. The amplitude of this response was larger than that of either response alone but less than the predicted linear sum. The magnitude of sublinearity was not correlated with the amplitude of the predicted linear sum nor with the amplitude of either individual response at any given location. At intervals of 50 and 100 ms, sublinearity was greatest, with the degree of suppression highly variable, but consistently weakest in the second stimulated whisker’s main barrel. For a given interdeflection interval, the degree of recovery was highly variable over space.

These observations are consistent with a triphasic profile of response interaction in supragranular cortex, in which a window of summation for multiwhisker responses extends from 0 (simultaneous) to 20–50 ms, followed by a period of suppression lasting until the 100- to 150-ms interdeflection interval, after which recovery or even facilitation is possible. We could discern no barrel-associated spatial patterns in the sublinearity of responses at short interdeflection intervals, nor in the recovery at longer intervals, beyond the tendency of the home barrel to participate in the earliest recovery.

**Mechanisms of cross-whisker suppression**

There are several mechanisms by which the suppression observed at intervals of 50 to 100 ms might be mediated. Suppression of the second response might be attributable to intracortical inhibition accompanying the spread of excitation in response to a whisker deflection, in which case inhibition in a column should be proportional to the excitation evoked by the first response. We observed that a large first response was not necessary at a given cortical location for complete suppression of the second response at that location (e.g., Fig. 5, column B1 at 100 ms), although we note that, depending on the network architecture, the first response need only spread as far as the second whisker’s barrel to suppress the second response in distant columns by preventing its normal spread. Further evidence against intracortical suppression is provided by our observation that activation of the cortex by electrical stimuli (Figs. 9 and 10) did not consistently suppress responses to subsequent whisker stimuli. If excitation of local cortical networks is always followed by proportional local feedforward and feedback inhibition, then a volley of corticocortical synaptic input should produce inhibition of subsequent whisker
responses, regardless of the source, particularly in cases when the observed response to electrical stimulation was large, as in Fig. 9B. In addition, detailed intracellular analysis has provided strong evidence against intracortical inhibition as the predominant mechanism for whisker-to-whisker suppression (Higley and Contreras 2003, 2005).

Another possibility is that suppression is caused by presynaptic mechanisms such as short-term depression of thalamocortical synapses (Chung et al. 2002; Castro-Alamancos and Connors 1997) or presynaptic γ-aminobutyric acid type B (GABA_B) inhibition. The latter possibility is supported by the results of Porter and Nieves (2004), who found that the GABA_B antagonist CGP35348 reversed the baclofen-induced results of Porter and Nieves (2004), who found that the GABA_B antagonist CGP35348 reversed the baclofen-induced.

Because our optical data show that suppression is largely independent of response amplitude at any given location in cortex, but critically dependent on the timing of inputs relative to one another, we favor a mechanism based on subcortical interactions. A mechanism of intrathalamic inhibition mediated by GABA_A receptor activation is consistent with the time course of the suppression reported here. In this scenario, “first whisker” input to the thalamus generates inhibition in surrounding barreloids by activation of the peri-VC sector of the reticular nucleus (RE). Because the inhibition decays after ≥100 ms, a time course similar to the period of spindle oscillations in our mice (23 spindles over 10 mice, average period 235 ± 67 ms) and thus compatible with GABA_A RE input, thalamocortical transmission recovers, although attenuated at first. The attenuation of thalamocortical transmission during the early recovery decreases propagation of the response in cortex, producing spatially limited responses. Additional evidence for strong intrathalamic suppression is provided by the recent finding that cortical inhibition of adjacent whisker responses is strongly dependent on thalamic input (Kwegyir-Afful et al. 2005). Indeed, shunting inhibition of trigeminothalamic input in VB cells recorded intracellularly in vivo, caused by a prior whisker deflection (Higley and Contreras, unpublished observations), supports the notion that cross-whisker suppression strongly relies on intrathalamic inhibitory mechanisms (Lee et al. 1994a,b). The data presented here is consistent with the known effects of intrathalamic inhibition on single-unit responses under anesthesia (Castro-Alamancos 2004).

**Particular aspects of mouse barrel responses**

The latencies to onset of our supragranular responses recorded with VSD are longer than those reported for single units in layer 4 of rat barrel cortex. They are, however, consistent with the concurrently recorded LFP and MUA responses, as well as with latencies reported for unit responses in layer 2/3 in the rat (Brumberg et al. 1999; Wilent and Contreras 2004) and in layers 4 and 2/3 in the mouse (Welker et al. 1993). The latter study provided evidence for unique processing pathways in the mouse in the form of subpopulations of short- and long-latency cells in layer 4. There has been no further exploration of this possibility in the literature, nor have there been any single-unit studies of response interactions in the mouse. Response interaction studies in the rat allow for the possibility of summation or facilitation followed by suppression, at a timescale considerably compressed from that reported here. This is expected given the shorter latencies to response observed in the rat.

**Whisker interactions in the rat**

In a brief treatment of whisker response interactions, the first VSD study of whisker-evoked responses reported a 35% reduction of the response to deflection of D3 when preceded by deflection of D1 at an interval of 60 ms (Orbach et al. 1985). A previous VSD study in the rat barrel cortex (Kleinfeld and Delaney 1996) described the steady-state response averaged over hundreds of milliseconds to trains of paired whisker deflections with a 25-ms offset between the stimuli, and reported that the peak amplitude of the VSD signal was much less than the linear sum of the individual response amplitudes and less than the amplitude of either response alone. At the same time, in half of their experiments they observed a significant increase in the area occupied by the response. Direct comparison of our results with theirs is problematic because of the significantly slower frame rate and the use of stimulus trains as opposed to pairs. Another study addressed the question of multiwhisker response integration using intrinsic metabolic signals (Goldreich et al. 1998), integrated over several seconds, and found that the intensity of the response in a single barrel column was greater for a single whisker than for paired deflections. Peristimulus responses to paired whisker deflections have thus far been recorded only at high temporal resolution with single electrodes. A study of single units in the rat barrel cortex (Shimegi et al. 1999) found instances of facilitation of combined whisker responses in layer 2/3 at intervals between −5 and 5 ms (their Fig. 11). In addition, a later study (Shimegi et al. 2000) found that directional agreement and barrel location significantly affected the presence and magnitude of facilitation, quantified by an LI index. We observed limited supr linearity in the present study. However, VSD signals mostly reflect postsynaptic potentials (PSPs) and it is likely that suprlinear spike output might arise from population PSPs summing linearly or sublinearly. Furthermore, in 5 of 10 simultaneous deflection experiments, we observed an initial supralinearity (see, e.g., Fig. 2) during the early part of the response, which could be the subthreshold correlate of the suprlinear summation observed in previous
The study of such supralinear interactions, which requires a much higher temporal resolution, is currently ongoing. Previous intracellular studies in the rat (Higley and Contreras 2003, 2005), which used a response ratio, demonstrated a primarily suppressive effect. This suppression was weak at 3 ms and maximal at interdeflection interval of 20 ms, with complete recovery by 100 ms, in full agreement with the original extracellular studies by Simons (1985).

**Behavioral relevance**

In our results and those of others, the nature of the interaction between whisker responses is primarily determined by their temporal separation. At short interdeflection intervals (0–10 ms), whisker responses may produce a combined signal that is larger than that of either of them alone (but rarely larger than their sum). At intervals between 10 and 50 ms, the second whisker response is largely abolished. Recovery from this suppression window proceeds from 100 to 200 ms, and the recovered second whisker response may exceed the original in amplitude and may have modified kinetics. These observations are consistent with a clocking mechanism for whisker inputs that would operate as follows: within the summation window, the first whisker response to object contact may be modified (summation) by contacts with subsequent whiskers. Within the suppression window that follows, additional whisker inputs do not reach the cortex. After the suppression window, inputs may be facilitated. This arrangement may serve to group whisker inputs into periods according to the mouse whisking frequency of 10–20 Hz [which is faster than in the rat (Jin et al. 2004)], possibly with amplification after the first cycle. Detailed study of mouse-whisking behavior along the lines of previous studies in the rat (Bermejo et al. 1996; Harvey et al. 2001a,b)—but more ideally during exploration—is needed to assess the behavioral significance of these time intervals, although we should not expect exact quantitative agreement because of possible anesthesia effects, differences between natural and experimental stimulation, or modification of primary sensory inputs by input from motor cortex (Ganguly and Kleinfeld 2004; Veinante and Deschenes 2003).

**Remaining questions**

All whisker deflections in this study were in the ventral direction. In light of the well-documented directional sensitivity of barrel cortex neurons as well as the obvious behavioral significance of the direction of whisker movement, it is reasonable to assume a directional effect on whisker interactions, and such an effect has been demonstrated at the single-cell level (Shimegi et al. 2000). A future study will examine the relative magnitude of whisker interaction effects when the whiskers are deflected in opposite directions instead of in the same direction.

In 20 of 25 experiments described in this study, the whiskers deflected were nearest neighbors. Pairs of whiskers located more caudally (arcs 1–3) were favored because of the larger size and more medial location of their barrels, which made them more accessible for imaging. The nearest-neighbor experiments were divided roughly evenly between caudal–rostral and rostral–caudal ordering. Single-unit studies in the rat (Brumberg et al. 1996; Simons and Carvell 1989) described a spatial gradient of inhibition in which caudally adjacent whiskers suppress their neighbors more strongly than rostrally adjacent ones, and ventrally adjacent whiskers more strongly than dorsal ones. At the spatial and temporal resolutions used here, we did not observe these gradients. Therefore L1 and RR values were pooled for the population data in Figs. 3, 4, and 7. The goal of this study was not to test for a spatial gradient, but to describe the complete time course and polarity of the interaction between responses at the level of population-averaged columnar PSPs.

**Advantages of the mouse model**

For imaging of whisker responses over the entire barrel cortex, the mouse model has some advantages over the rat, including a more medial, flatter barrel cortex, smaller barrels that allow more of the network to be visible at one time, greater mechanical stability, and the possibility of examining the alteration of whisker responses in genetically modified mice with informative molecular or behavioral phenotypes. In addition, in both mouse and rat, it is now possible to use genetic techniques to deliver fluorescent indicators to specific subpopulations of cells, as has been accomplished with enzyme-activated voltage-sensitive dyes in vitro (Hinner 2004) and with lentivirus-delivered fluorescent indicators in vivo (Dittgen et al. 2004).

**VSD response kinetics**

Previous VSD studies of whisker-evoked responses showed both monophasic, depolarizing-only responses (Kleinfeld and Delaney 1996; Orbach et al. 1985; Petersen et al. 2003a,b) and multiphasic responses with a long hyperpolarization and rebound (Derdikman et al. 2003). In this study, we observed both (see, e.g., Fig. 2 for monophasic and Fig. 5 for multiphasic). The presence of a hyperpolarizing phase in the VSD signal was highly variable from trial to trial, but it was observed in response to both mechanical and air-puff stimuli (see, e.g., Fig. 5), contrary to Derdikman et al. (2003). We attribute the variability in response kinetics between reports to the effects of different anesthetics and the intertrial variability within our data set to the fluctuating distribution of baseline $V_m$ values observed under ketamine–xylazine anesthesia.

The correspondence in time course between VSD signals averaged over small areas and intracellular recordings from single cells is remarkable, given that the VSD signal from a pixel includes different types of processes from many different types of cells. Apparently, the subthreshold behavior of neurons over large areas of tissue is remarkably consistent. The role of spike generation in transforming this spatiotemporally uniform subthreshold signal into a presumably more granular output pattern must be thoroughly investigated.

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**References**


