Varying the Degree of Single-Whisker Stimulation Differentially Affects Phases of Intrinsic Signals in Rat Barrel Cortex

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Polley, Daniel B., Cynthia H. Chen-Bee, and Ron D. Frostig. Varying the degree of single-whisker stimulation differentially affects phases of intrinsic signals in rat barrel cortex. J. Neurophysiol. 81: 692–701, 1999. Using intrinsic signal optical imaging (ISI), we have shown previously that the point spread of evoked activity in the rat barrel cortex in response to single-whisker stimulation encompasses a surprisingly large area. Given that our typical stimulation consists of five deflections at 5 Hz, the large area of evoked activity might have resulted from repetitive stimulation. Thus in the present study, we use ISI through the thinned skull to determine whether decreasing the degree of single-whisker stimulation decreases the area of the cortical point spread. We additionally outline a protocol to quantify stimulus-related differences in the temporal characteristics of intrinsic signals at a fine spatial scale. In 10 adult rats, whisker C2 was stimulated randomly with either one or five deflections delivered in a rostral-to-caudal fashion. Each deflection consisted of a 0.5-mm displacement of the whisker as measured at the point of contact, 15 mm from the snout. The number of whisker deflections did not affect the area or peak magnitude of the cortical point spread based on the intrinsic signal activity occurring from 0.5 up to 1.5 s poststimulus onset. In contrast, the magnitude and time course of intrinsic signal activity collected after 1.5-s poststimulus onset did reflect the difference in the degree of stimulation. Thus decreasing the degree of stimulation differentially affected the early and late phases of the evoked intrinsic signal response. The implications of the present results are discussed in respect to probable differences in the signal source underlying the early versus later phases of evoked intrinsic signals.

INTRODUCTION

Using a variety of techniques in several animal models, stimulus-evoked response in primary sensory cortex has been shown to be highly dynamic, capable of varying with the parameters of stimulation (Fox and Raichle 1984; Huang et al. 1996; Leninger-Follert and Hossman 1979; Yarowsky and Ingvar 1981). The posteroomedial barrel subfield (PMBSF) of the rodent somatosensory cortex is a popular model for studying stimulus-evoked response properties because it offers two main advantages. First, the peripheral organs represented in this cortical region are large facial whiskers (vibrissae) found on the contralateral snout. As each whisker occupies a point on the somatosensory epithelium and stimuli can be delivered to a precise area of the receptor surface, the opportunity to study cortical response to a point stimulus is provided. Second, each whisker is represented in a 1:1 fashion by a discreet aggregate of cells (~0.1 mm²) in layer IV of the contralateral PMBSF called a barrel and the immediately surrounding agranular region, the septum (Jensen and Killackey 1987; Woolsey and Van der Loos 1970). Thus the discreet anatomic topography found in the periphery is maintained isomorphically in layer IV of the cortex.

In addition to serving as an anatomic correlate for each contralateral whisker, each barrel is activated preferably by stimulation of its homologous whisker, making the initial cortical response to stimulation of a single whisker readily localizable. Although the majority of afferent thalamic input from a single whisker is confined to a single barrel (for review, see Chmielowska et al. 1989; Keller 1995), each barrel initiates an extensive intracortical spread of activity through horizontal connections (Armstrong-James and Fox 1987; Armstrong-James et al. 1992). By characterizing the total response of the cortex to a point stimulation on the sensory surface, or the cortical point spread, one can incorporate both the intracortical and thalamocortically influenced response properties; this is akin to asking how much of the cortex responds to the deflection of a single whisker.

Recent research indicates that a variety of cortical response properties to stimulation of an individual whisker are dependent on the characteristics of sensory stimulation (for a review of single-unit response properties, see Armstrong-James 1995; Simons 1995). For example, the amplitude of whisker deflection is correlated positively with spiking rate of single units (Axelrad et al. 1976) as well as the magnitude and spatial spread of voltage-sensitive dye fluorescence (Orbach et al. 1985), intensity of intrinsic signal (IS) response (Peterson et al. 1998), and expression of immediate early genes (Melzer and Steiner 1997). An increase in frequency of vibrissa stimulation has been shown to increase the response magnitude of evoked potentials (Freeman and Sohmer 1996) and intrinsic signals (Blood et al. 1995) and decrease the area of evoked IS response (Sheth et al. 1998).

With the use of intrinsic signal optical imaging (ISI), we previously have shown that five deflections of a single whisker (5 pulse) evokes a point spread encompassing a surprisingly large area of cortex, often on the order of several millimeters (Chen-Bee and Frostig 1996; Masino and Frostig 1996). Given that cortical response properties can depend on stimulus parameters, we wondered whether the area of the point spread, as well as the magnitude of maximum activity within the point spread, was specific to five-pulse stimulation. By assessing differences in the point spread elicited with five deflections of a single whisker to that elicited with one deflection of the same whisker (1 pulse), this study determined how properties of the
point spread changed by reducing the amount of peripheral stimulation. Our choice of one pulse was an attempt to resolve an apparent contradiction in the literature. Studies interested in single-unit response properties typically rely on one pulse stimulation, one deflection per second, whereas studies employing ISI have detected either a weak, nonlocalized response (Sheth et al. 1998) or no physiological response to a 1-Hz stimulus (Blood and Toga 1995; MacVicar and Hochman 1991). Because successful imaging of one pulse stimulation should enable more direct comparisons between optical imaging and electrophysiological results, we also were interested in determining whether ISI is sensitive enough to detect the effects of one pulse stimulation.

This study also compared changes in the time course of the evoked intrinsic signal response over cortical distance between five and one pulses. Optical imaging with voltage-sensitive dyes has shown that the latency to peak for the fluorescence signal increases with distance from the peak of activity (Orbach et al. 1985), and single-unit studies have demonstrated an increase in response latency with increasing distance from the peak of the response (Armstrong-James and Fox 1987; Armstrong-James et al. 1992). Additionally, analysis of the time course of intrinsic signals in visual cortex has shown that increasing the number of stimuli (Malonek et al. 1997) or duration of the stimulus (Frostig et al. 1990; Grinvald et al. 1986) results in a slower return to baseline reflectance values. Thus we were interested in determining whether changes in time course across cortical distance also can represent changes in the amount of peripheral stimulation.

The present study used ISI to compare the cortical response to five- and one-pulse stimulation within the same animal. By quantifying the amplitude and areal extent of the point spread as well as investigating changes in the temporal characteristics of the intrinsic signals, this study sought to demonstrate that ISI can detect cortical activity evoked by one deflection of a single whisker, determine if the area of, and maximum activity within, the point spread differed between five and one pulses, and establish the extent to which the time course of the intrinsic signals reflect differences between five- and one-pulse stimulation. The results of this study were presented previously in abstract form (Polley et al. 1997).

METHODS

A detailed account of the experimental procedure has been reported previously by Masino et al. (1993). Although a brief summary of the procedures for surgery and data collection used in this study is provided, the methods of intrinsic signal quantification and analysis are described in more detail.

Subjects and anesthesia

Each of the 10 male Sprague-Dawley rats (385–575 g) used in this study was housed in gang cages in 12-h light/12-h dark cycle with food and water ad libitum. The animal was removed from the gang cage on the experimental day and was administered an initial intraperitoneal injection of pentobarbital sodium (Nembutal, 55 mg/kg). The heart rate, respiratory rate, and body temperature were monitored constantly (Hewlett-Packard Patient Monitor 7835A, Hewlett-Packard, Andover, MA), and body temperature was maintained at 37°C with an adjustable heating blanket. Additional supplements (0.5–1 ml) were given throughout the course of the experiment to maintain a relatively constant level of anesthesia as assessed by respiration rate, heart rate, body temperature, corneal reflex, and color of the extremities (an indicator of blood oxygenation).

Surgery

A 5 mm² area of skull overlying the primary somatosensory cortex (anteromedial corner positioned 1 mm caudal and 2 mm lateral to bregma) was exposed and then thinned with a dental drill to ~150 μm. To maintain the transparency of the skull throughout the experiment, a wall of petroleum jelly was built around the border of the thinned skull, forming a well, which then was filled with silicon oil (200 fluid, viscosity, 50 cs; Accumetric, Elizabethtown, KY) and covered with a glass coverslip (thickness 1–1.5 mm).

Data collection

Light reflectance from a 6.8 × 5.1 mm area of somatosensory cortex, converted to a 192 × 144 pixel array, was collected with a slow-scan charge-coupled device camera (Photometrics, Tuscon, AZ), equipped with an inverted 50-mm AF Nikon lens (1:1.8) combined with an extender (Nikon, PK-13) that was positioned to focus 300 μm beneath the cortical surface. The cortex was illuminated with a stable 630-nm light source (Kepco, ATE 15–15 M). Data were acquired continuously for 4.5 s in nine 500-ms frames, constituting one trial. Prestimulus activity of 1,000 ms was collected before the onset of whisker stimulation. A computer-controlled whisker stimulator (Bakin Systems 2, Irvine, CA) deflected the C2 whisker contralateral to the thinned skull. In each trial, the point of contact occurred 1.5 cm from the surface of the snout and displaced the whisker 0.5 mm (2°) one or five times per second in a rostral-caudal fashion. The amplitude of whisker deflection caused no movement of adjacent whiskers. Each pulse deflected the whisker for 100 ms with a 100-ms interpulse interval for the five-pulse condition. In addition to maintaining a constant level of anesthesia throughout the experiment, we randomly interlaced delivery of either one or five pulses such that any fluctuations in the animal’s state of arousal would have a similar impact on both stimulus conditions. Data collection included both stimulation and nonstimulation (control trials) interlaced randomly by the computer with an intertrial interval of 15 s. A complete data session contained a summation of 64 trials for each stimulus condition.

Image quantification

VISUALIZATION OF STIMULUS EVOCTED ACTIVITY. Figure 1A depicts a ratio image created by dividing activity occurring 0.5 s to ≤1.5 s after stimulus onset by the 1 s of activity immediately preceding stimulus onset. Although dividing poststimulus activity by prestimulus activity is the intuitive way to characterize any stimulus evoked response, it is apparent that employing such a method to visualize the evoked IS response results in an image dominated by the stimulus-evoked blood vessel activity. Using an intratrial division analysis (see Chen-Bee et al. 1996 for extensive discussion of rationale and methods) enables one to create a ratio image that effectively minimizes the presence of blood vessel activity while accentuating cortically derived IS activity (Fig. 1B). Because IS originating from cortical tissue exhibit a shorter latency to peak than vessel-derived IS, allocating IS activity occurring 0.5 s to ≤1.5 s after stimulus onset to the numerator and both the 500 ms of prestimulus activity immediately preceding stimulus onset and 500 ms of poststimulus activity occurring between 1.5 and 3.5 s to the denominator removes much of the blood vessel activity from the image. Therefore, using the intratrial division analysis to minimize the presence of the vascular response is particularly important for obtaining an accurate estimate for the area of the point spread based on cortically derived intrinsic signals as quantifying the area of the cortical point spread can be influenced erroneously by IS originating from stimulus-dependent vessel activity.

The intratrial analysis used in this study differs from those used in
previous studies from this lab (Chen-Bee et al. 1996; Masino and Frostig 1996) in one respect: previously, the same poststimulus frame for each data session was placed in the denominator. In this study, we used a more adaptive criterion for establishing the ratio image in which activity occurring 0.5 s to \( \leq 1.5 \) s after stimulus onset (referred to as the rising phase hereafter) was divided by 500 ms of prestimulus activity and the 500 ms of poststimulus activity that contained the raw reflectance value closest to that of the prestimulus activity (at 2.0 and 3.0 s after stimulus onset for 5 and 1 pulses, respectively). Because denominator values were kept constant, a difference in ratio values between data sessions could be attributed only to differences in the rising phase of the IS, independent of differences in the time course of falling phase activity. Thus the adaptive intratrial analysis is designed not only to highlight IS activity of cortical (and not vascular) origin, but even more specifically to emphasize only the rising phase of that signal. Assuming that the poststimulus time point most similar to the prestimulus time point is the same for all locations within the evoked IS response, each pixel of the 192 \( \times \) 144 pixel array was processed using this ratio and assigned a value indicative of the evoked activity level. A gray scale mapping function then was applied to the ratio values so that the rising-phase point spread function of a single whisker is visualized as a coherent dark area (see Fig. 1B).

**PEAK HEIGHT AND AREAL EXTENT ANALYSIS.** These analyses are useful in determining if the maximum magnitude of the IS rising phase varies between stimulus conditions and if the area of the point spread varies between stimulus conditions. For each data session in 8 of 10 rats, the ratio values first were processed with a Gaussian filter (half-width = 5) to reduce the high-frequency noise component. A computer algorithm then located the pixel with the lowest ratio value within the coherent stimulus-evoked point spread, corresponding to the point of greatest stimulus-dependent rising-phase activity, defined as the peak of the rising-phase point spread. Because of potential fluctuations in spontaneous activity caused by changes in cortical excitability and anesthesia levels, the peak of the rising-phase point spread is most meaningful when considered in relation to the level of nonstimulus-evoked activity, indicated by the median ratio value. Thus the maximum rising-phase magnitude, or the peak height, was calculated by subtracting the median value from the peak of the rising-phase point spread value.

To quantify the areal extent of the rising-phase point spread for each data session, the peak height was normalized to 100%, and an algorithm enclosed the cortical area exhibiting rising-phase activity that met any desired threshold percentile of the normalized peak height with an areal extent border. This study compared differences in the size of the rising-phase point spread when thresholds were set at 50, 70, and 90% of maximum activity (see Fig. 1C).

Although processing the images with a Gaussian filter was effective in reducing the high-frequency noise component of the unfiltered image, it influenced the areal extent estimation. To obtain an estimate of the rising-phase point spread at each of the aforementioned thresholds independent of filtering, we applied a regression-based correction protocol described in Masino and Frostig (1996). Briefly, each data session was processed with increasing degrees of Gaussian filtering.

**FIG. 1.** The 5 \( \times \) 5 mm\(^2\) images summarize the analysis of intrinsic signal activity evoked by whisker stimulation (1 pulse stimulation of whisker C2 is shown here) through the thinned skull of the same animal. Medial is toward the top and rostral is toward the left for all images. A: evoked intrinsic signal (IS) response obtained by dividing poststimulus activity by prestimulus activity. Image is processed with a Gaussian filter (half-width = 5) and converted to gray-scale values. Note that the evoked response obtained using this division is predominantly comprised of blood vessel activity (black streaks). B: including poststimulus values in the denominator effectively minimizes blood vessel activity. Point spread of the rising-phase activity is visualized as a dark coherent area. Intrinsic signals are processed with an adaptive intratrial division and filtered with a Gaussian filter (half-width = 5) before conversion to gray-scale values. C: areal quantification of the rising-phase point spread. Activity borders based on the normalized threshold analysis are superimposed on the same image as in A. Starting with the smallest one, each activity border encloses an area containing activity of \( \geq 90, 75, \) and 50%, respectively, of peak rising-phase activity (designated with a cross). D: determining the location of sampling for temporal analysis of intrinsic signals. Activity borders shown in B are superimposed on the surface vasculature of the same animal. Activity was sampled from 5 areas (each 12 \( \mu \)m\(^2\)) along each of 10 activity borders (3 are shown here) and averaged for each border. Locations of the sampled areas for a given border were calculated with respect to distance away from the peak of the rising-phase point spread. In addition, as many as 4 larger areas (46 \( \mu \)m\(^2\) each) were sampled and averaged per animal at far distances away (3.2–3.8 mm) from the peak of the rising-phase point spread.
The y intercept of the linear regression drawn for each data session provided an estimate of the areal extent expected in the absence of filtering for that data session. All descriptive statistics provided in the present investigation were based on the corrected areal extent values.

Quantifying the temporal characteristics of the intrinsic signal at various distances

The intratrial division analysis is customized to assign the reflectance value from the poststimulus time point that is most similar to the prestimulus reflectance value to the denominator regardless of when it occurs. Thus this division analysis is designed to highlight differences in the rising phase of activity. To establish if differences in the IS existed at time points later than 1.5 s and to analyze differences in all phases of the IS at a higher temporal resolution, this study also characterized differences in the amplitude and time course of intrinsic signals at increasing distances from the peak of the rising-phase point spread.

For each data session in the same eight rats, we used areal extent borders to measure distance away from the peak of the rising-phase point spread. Activity was sampled at 11 such borders from the 100th (peak of the rising-phase point spread) to the 50th (area at half-height) percentile border in 5% decrements. Because the borders were roughly circular, the radius of its area estimated the average distance between a given point on that border and the location of peak of the rising-phase point spread. Five discreet 12-μm² circular areas were sampled along a given border and averaged so that a representative average of activity was determined per border. In all cases, sampling locations avoided surface blood vessels (Fig. 1D).

In addition, in four of eight animals, the size and transparency of the thinned window was sufficient to enable sampling of activity from 46 μm² circular areas located 3.2–3.8 mm away from peak of the rising-phase point spread to provide an estimate of nonevoked activity. If multiple areas at this distance were sampled in the same animal, they were averaged in order for all animals to be equally represented in the final average. Again, sampling locations avoided blood vessels (Fig. 1D).

IS activity was expressed as fractional change relative to prestimulus activity according to the following formula: FC = (R_t − R_s)/R_s, where FC indicates fractional change, R_s is equal to the raw reflectance value for any point in time, and R_t refers to the raw spontaneous reflectance value 500 ms before stimulus onset. Note that the ratio used for expressing FC is different from that used for creating the image of the point spread in that it includes only prestimulus values in the denominator. Because raw IS activity was sampled locally only from small areas of cortex not obscured by blood vessels, the contribution of vessel derived IS was minimal, and we therefore did not have to use poststimulus values in the denominator as we did to create the image of the cortical point spread.

Extended frames analysis

Post hoc analysis of eight rats revealed that the trial duration of 4.5 s was not sufficient to characterize completely the differences in IS time course between one and five pulses. Thus we collected data in two additional animals for a duration of 9 s poststimulus onset, 5.5 s longer than the previously described analysis. Activity was sampled at the peak of the rising-phase point spread within 50 μm² circular area. Peak height and areal extents were not analyzed for these animals and were therefore not included with the initial eight rats. All other details regarding the subjects, anesthesia, surgery, and whisker stimulation were identical to the methods described in the previous sections.

RESULTS

Area and peak height of the point spread for the rising phase of IS activity

The area and height of the rising-phase point spread elicited by five- or one-pulse stimulation was calculated for eight rats. Qualitatively, the size of the rising-phase point spread appears similar for both five- and one-pulse stimulation based on the...
spread of raw IS (Fig. 2A) and appearance of the images (Fig. 2B). Areal extent analysis determined that the rising-phase point spread for five-pulse stimulation ranged from 0.62 to 2.82 mm² when rising phase activity was thresholded at 50%, from 0.15 to 1.17 mm² at 70%, and from 0.03 to 0.30 mm² at 90% of maximum rising phase activity. In accordance with our results, a large degree of variability can be found at the level of barrel cortex anatomy (Riddle and Purves 1995). However, the range of functional variability reported here is approximately two times larger than that reported for anatomic variability yet is in agreement with previous findings from our laboratory (Chen-Bee and Frostig 1996; Masino and Frostig 1996). The area of the one-pulse rising-phase point spread ranged from 0.38 to 2.88 mm² at the 50%, from 0.11 to 1.37 mm² at the 70%, and from 0.02 to 0.33 mm² at 90% of maximum rising phase activity. Thus both the means and the variability of the areal extent values are similar between five and one pulses. This large degree of variability only partially can be attributed to the experimental protocol and alternatively can be explained by sources of variability that are inherent to the animals such as differences in peripheral features, differences in early life experiences, and differences in the use of peripheral sense organs throughout the course of an animal’s lifetime (for further discussion, see Chen-Bee and Frostig 1996). The means ± SE for the area of the point spread at each threshold for five and one pulses are illustrated in Fig. 3A. No significant differences were found between five- (1.44 ± 0.22 mm²) and one-pulse (1.42 ± 0.26 mm²) stimulation at the 50% (paired t-test, P = 0.941), 70% (0.63 ± 0.10 mm² vs. 0.56 mm² ± 0.13 mm²; paired t-test, P = 0.640), or 90% (0.13 ± 0.03 mm² vs. 0.11 mm² ± 0.03 mm²; paired t-test, P = 0.69) thresholds, suggesting that, within a time window of 0.5 s to ≤1.5 s, a similar area of cortex responds to both types of stimulation.

Using the peak height analysis described in the previous section, we could determine whether the peak of rising-phase point spread differed between stimulus conditions. The means ± SE of the peak height are illustrated in Fig. 3B. No significant difference was found (paired t-test, P = 0.503) between five- (mean ratio value of 5.44 ± 0.44 × 10⁻⁴) and one-pulse (mean ratio value of 4.52 ± 0.54 × 10⁻⁴) stimulation, indicating that the magnitude for the peak height of the rising-phase point spread was similar for both stimulus conditions.

**Temporal characteristics of the IS**

Areal extent analysis is designed to detect differences in the cortical point spread for the rising phase of evoked activity and therefore does not address potential differences in IS activity occurring at later time points. To determine whether similarities in the point spread were present at later points in time and to analyze the properties of the IS with greater temporal precision, a detailed analysis of the IS time course was required. Intrinsic signal profiles at the peak of rising-phase point spread and 11 increasing distances from this point were compared between one and five deflection pulses, with the 11th distance located outside the area of evoked activity.

Figure 4 illustrates IS activity at 4 of the 12 distances sampled, 3 within the evoked response area and 1 location 3.2–3.8 mm from the peak of the rising-phase point spread. In agreement with the results from the peak height and areal extent analyses, the rising phase of the IS exhibits a high degree of similarity between stimulus conditions at each of the three distances sampled within the evoked response. In con-
In contrast, the falling phase of the IS, defined as fractional change values collected at 1.5 to 3.5 s poststimulus onset, varied across stimulus conditions and distance away from the peak of the rising-phase activity. As illustrated in Fig. 4, the IS activity evoked by one-pulse stimulation returned to prestimulus levels within 3.5 s poststimulus onset at all three locations sampled within the area of evoked response. In contrast, the IS activity evoked by five-pulse stimulation exhibited a faster return to prestimulus levels within 2.0 s poststimulus onset for the same locations. Furthermore, the five-pulse IS activity decreased below prestimulus levels, with the maximum decrease occurring within 3.5 s poststimulus onset. The amplitude of this maximum decrease appeared greatest at the location of the peak rising-phase activity and declined with increasing distance away from this peak. Because the decrease below prestimulus levels observed for the five pulses varied over distance, the falling phase of IS activity for one- and five-pulse conditions became more similar with increasing distance away from the peak of the rising-phase point spread.
from the peak of the rising-phase point spread. By focusing on activity collected at 1 to 1.5 s and 3 to 3.5 s poststimulus onset as representative of the rising and falling phases, respectively, we could compare between one and five pulses with higher spatial resolution. As illustrated in Fig. 5A, the similarity in the rising-phase activity between one and five pulses was observed at higher spatial resolution. Furthermore, for both one and five pulses, a subtle decrease in rising-phase activity was observed with increasing distance away from the peak of rising-phase point spread. The disparity in the falling phase activity between one and five pulses was still evident at higher spatial resolution (Fig. 5B).

By following activity for only a duration of 3.5 s poststimulus onset, it was unclear whether the falling phase activity for one pulse was slower and eventually would decrease below prestimulus levels or if this characteristic was a property of the five-pulse signal only. Furthermore, it was unclear whether the falling phase activity for five pulses decreased maximally below prestimulus levels within 3.5 s poststimulus onset or whether it would continue to decrease. To address these questions in two rats, we sampled IS activity for an additional 5.5 s sampled over the location of the peak of the rising-phase point spread. As illustrated in Fig. 6, a decrease in the falling phase activity below prestimulus levels existed for one pulse. With respect to the five-pulse signal, the falling phase activity continued to decrease beyond 3.5 s poststimulus onset. Although the initial decrease below prestimulus levels occurred later for one pulse, the maximum decrease occurred at the same time (within 4.5 s poststimulus onset) for both one and five pulses. However, the magnitude of the decrease was always larger for the five-pulse signal at all time points sampled.

As a technical aside, we also addressed an inherent assumption of the adaptive intratrial assumption of the adaptive intratrial analysis for areal extent determination; namely, that the poststimulus time point most similar to the prestimulus time point is the same for all locations within the evoked response. It appears that such an assumption holds true for one-pulse stimulation as the poststimulus time point chosen for its intratrial analysis (3.0 to 3.5 s poststimulus onset) is most similar to the prestimulus time point at all distances sampled within the evoked response (Figs. 4 and 5B). The assumption also appears to hold true for five-pulse stimulation as its poststimulus time point (2.0 to 2.5 s poststimulus onset) is most similar to the prestimulus time point at all distances sampled (Fig. 4). Thus the areal extent quantification of the rising phase point spread for five-and one-pulse stimulation (Fig. 3) does not appear to violate any assumptions of the adaptive intratrial analysis.

DISCUSSION

Spatial distribution of the evoked rising-phase activity

We found the area of rising-phase activity when thresholding at 50% of maximum activity was 1.44 mm² in response to five-pulse stimulation of the C2 whisker, an area roughly encompassing nine barrels and septa surrounding C2. This area is larger than the area of the C2 barrel (0.14 mm²) (D. Riddle, personal communication) by an order of magnitude. Although a discrepancy in size between a single whisker’s barrel and the cortical area responsive to that whisker has been reported (Armstrong-James and Fox 1987; Axelrad et al. 1977; Kleinfeld and Delaney 1996; Kossut 1988; Orbach et al. 1985) the magnitude of discrepancy is in agreement with our previous studies that employed five-pulse stimulation (Chen-Bee and Frostig 1996; Masino and Frostig 1996). We also found that a similarly large area of rising phase activity was evoked with one-pulse stimulation using various activity thresholds (Fig. 3A). The maximum magnitude of the rising phase activity did not differ between five- and one-pulse stimulation (Fig. 3B) either.
Thus one-pulse stimulation evokes a similar area and maximum magnitude of the rising phase activity as does five-pulse stimulation, suggesting that the large point spread of a single whisker previously reported by our laboratory (Chen-Bee and Frostig 1996; Masino and Frostig 1996) cannot be attributed to the use of five-pulse whisker stimulation. Furthermore, we provide evidence that ISI can detect a highly localized cortical response to one-pulse stimulation, which is in contrast to previous attempts by several imaging techniques [MacVicar and Hochman 1991 (ISI); Blood et al. 1995 (ISI); Fox and Raichle 1984 (PET)]. It recently has been reported that stimulation of a single whisker at 1 Hz yields a larger, more diffuse area of activity when compared with activity evoked by 5 and 10 Hz stimuli, but no difference in the magnitude of the peak optical signal (Sheth et al. 1998). Although the data reported here are in agreement with the similarity of the peak signal, we found a highly localized response to a one-pulse stimulus. The discrepancy between the area of the evoked response in the aforementioned study versus the present study may be due to the number of averaged trials, efforts to isolate different phases of the intrinsic signal, and delivery of a repeated versus a single 1-Hz stimulus.

It is possible that the rising-phase point spread for the C2 whisker does not differ between five and one pulses because its area and maximum magnitude are not susceptible to change. However, this possibility is unlikely given that the point spread of an individual whisker has been shown to differ over time (Masino and Frostig 1996), in response to sensory deprivation (Masino and Frostig 1995; Polley et al. 1998), and on acute neurotrophin administration (Prakash et al. 1996). Thus our results suggest that similarities in the rising-phase point spread for one and five pulses faithfully represent the cortical response to different degrees of whisker stimulation. However, it does not exclude the possibility that the rising-phase point spread cannot vary with other aspects of whisker stimulation such as changes in frequency, amplitude, or duration of whisker stimulation. Further research is necessary to address these issues.

**Spatial distribution of the evoked falling phase activity; comparison to rising-phase activity**

In contrast to the rising phase activity (0.5 to ≤1.5 s post-stimulus onset), the falling phase activity (1.5 s and later, poststimulus onset) behaved differently between five- and one-pulse stimulation. The falling phase activity for five-pulse stimulation was faster to return to prestimulus levels (Fig. 4) and exhibited greater decreases below prestimulus levels (Fig. 5B and 6). Thus our results revealed an uncoupling between the rising and falling phases of IS activity evoked by whisker stimulation, suggesting that the falling phase is not solely due to a passive return of mechanisms underlying the rising phase. The possibility that different mechanisms may underlie the rising and falling phases of evoked IS activity is plausible given that the signal measured with ISI originate from various sources, namely changes in the oxidation state of hemoglobin, light scattering, and changes in blood volume, and that these sources can follow different time courses (Frostig et al. 1990). Depending on the wavelength of light used for imaging, a particular source becomes the dominant source of intrinsic signals. For example, 630-nm illumination (used in the present study) is optimal for collecting intrinsic signals dominated by changes in the oxidation state of hemoglobin. This particular signal source can be subdivided further into two components, the increase in deoxygenated hemoglobin resulting from local delivery of oxygen from the capillary bed followed by an increase in oxygenated hemoglobin and blood flow. The increase in IS activity due to a dominant increase in deoxygenated hemoglobin (Hbr) appears to peak 1–2 s after stimulus onset, which typically is followed by a decrease in IS activity that peaks 4–6 s after stimulus onset predominantly resulting from the delivery of oxygenated hemoglobin (HbO2) (Frostig et al. 1990; Malonek and Grinvald 1996; Malonek et al. 1997). However, the time course of the two signal sources overlap in time ~1–3 s poststimulus onset.

When considered with regard to the biphasic IS response we report here, these findings have implications for understanding the relationship between stimulus parameters and the compo-
ments of the IS. The rising phase of the IS activity (0.5 to ≈1.5 s poststimulus onset) collected in the present study most probably corresponds to a dominant increase in Hbr, whereas the later time points of the falling phase (3 to ≈4.5 s) most probably corresponds to a dominant increase in HbO₂. Because of the overlap in time between the two signals, activity occurring 1.5 to ≈3 s poststimulus onset can contain a combination of both a decline in Hbr and an initial increase in HbO₂. Thus the similarity in rising-phase activity observed for one- and five-pulse stimulation (Figs. 3, 4, and 5A) may be interpreted as Hbr-dominated mechanisms responding similarly to both types of stimulation. Furthermore, the dissimilarity in the later time points of the falling phase (Figs. 5B and 6) may be interpreted as HbO₂-dominated mechanisms responding differentially to five-and one-pulse stimulation. Mechanisms underlying IS activity occurring ≈4.5 s poststimulus onset are poorly understood at this time and are beyond the scope of the present study.

Malonek et al. (1997) recently have demonstrated that increasing the number of visual stimuli does not affect the rising phase of the oximetric signal but results in an attenuation of the falling phase and a slower time course for cerebral blood flow. Similar effects on the falling, but not rising phase, also have been found when the duration of the visual stimulus has increased (Frostig et al. 1990; Grinvald et al. 1986). Although the similarity of the rising phase is consistent with these data, the opposite effects on the falling phase/blood flow component is consistent with the recent finding that increasing the frequency of vibrissa movements causes a linear increase in blood flow (Gerrits et al. 1998). Furthermore, in each of the experiments conducted in the visual system, the stimulus still was being delivered during the falling phase of the IS response. In the present study, both the five- and one-pulse stimulus deliveries were terminated before the onset of the falling phase, therefore the magnitude of the falling phase was not compromised by delivery of a protracted stimulus.

The apparent discrepancy between the regulation of Hbr and HbO₂ delivery in response to whisker stimulation has several implications for the supply and demand of oxygen in the cortex: 1) the finding that varying the degree of single-whisker stimulation has a similar effect on the Hbr-dominated epoch of the IS response but a greatly different effect on the HbO₂-dominated epoch of the IS further supports the findings that the initial oxygen consumption (presumably in the capillary beds) need not predict the amount of resupply of HbO₂ (Fox and Raichle 1986; Leninger-Follert and Lubbers 1976; Lou et al. 1987; Malonek and Grinvald 1996; Yarowsky and Ingvar 1981). 2) The Hbr dominant source of intrinsic signals is more responsive to reduced levels of sensory stimulation. Although an HbO₂-dominated undershoot phase was observed for the one pulse, it was noticeably slower and weaker in amplitude compared with five pulses (Fig. 6). This in part may explain the past inability of various imaging techniques to detect a localized cortical response to 1-Hz stimulation as most of them do not isolate the Hbr-dominant response (Blood et al. 1995; Fox and Raichle 1984; Sheth et al. 1998). Because the Hbr-derived signal exhibits a greater spatial correspondence to the underlying neuronal activity than the blood-volume-derived signal (Frostig et al. 1990; Malonek and Grinvald 1996; Malonek et al. 1997) and occurs more rapidly than vascular components (Chen-Bee et al. 1996; Grinvald et al. 1986), it is an optimal signal source to assess the presence of stimulus-dependent cortical activity. On the basis of the similarity of the Hbr-dominated signal over cortical distance, one could hypothesize that the both the functional recruitment of local capillary oxygen supply and the neurophysiological response occur over a similar area of cortex irrespective of the amount of peripheral stimulation. However, the finding that the delivery of HbO₂, which is tightly coupled with cerebral blood flow (CBF) differs between stimulus conditions suggests that the delivery of five pulses is more capable of invoking the action of vasoactive metabolites than is one-pulse stimulation. Such putative candidates include pCO₂, K⁺, adenosine, acetylcholine, and nitric oxide, all of which are known to dilate arterioles, directly increasing the rate of CBF (for review, see Lou et al. 1987; Villringer and Dirnagl 1995).

The authors thank S. Schlocker and C. Yap for assistance with data analysis and N. Prakash for insightful comments.

This research was supported by National Institute of Mental Health National Research Service Award MH-14599 to D. B. Polley, National Institute of Neurological Disorder and Stroke Grant NS-34519, and National Science Foundation Grant IBN 9507936 to R. D. Frostig.

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Received 29 June 1998; accepted in final form 29 October 1998.

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