Characterization of functional organization within rat barrel cortex using intrinsic signal optical imaging through a thinned skull

(cytochrome oxidase/mystacial vibrissae/whiskers/rat somatosensory cortex/posteromedial barrel subfield)

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ABSTRACT We used optical imaging of intrinsic signals to characterize the functional representations of mystacial vibrissae (whiskers) in rat somatosensory cortex. Stimulation of individual whiskers for 2 s at 5 Hz resulted in a discrete area of functional activity in the cortex. Images of whisker representations were collected both through the dura and through a thinned skull. We characterized the functional representation of a whisker both spatially and temporally with two-dimensional images and three-dimensional surface plots of intrinsic signal development in the cortex in response to whisker stimulation. Single unit recordings verified that the representation of the whisker obtained with optical imaging corresponded with the electrophysiological response area of that whisker in the cortex. Lesions in the center of the functional activity were found to be in the center of the dense cytochrome oxidase patch for the corresponding whisker. In addition, a 3×3 matrix of whiskers was stimulated and the distances between the centers of the imaged representations and the distances between the centers of the layer IV cytochrome oxidase staining of the nine whiskers were found to be highly correlated ($r = 0.98$). This study shows a striking correspondence among imaging, physiology, and anatomy in the rat somatosensory cortex. Furthermore, the ability to use optical imaging through a thinned skull should allow investigations into the long-term changes in a sensory representation within a single animal.

In vivo characterization of the functional organization of a sensory system, combined with knowledge about its underlying anatomical structure, should facilitate the elucidation of structure–function relationships. This study investigates the relationship between structure and function in the adult rat somatosensory cortex and uses optical imaging of intrinsic signals to establish the normal functional organization. Our long-term goals are to investigate changes in the functional organization of the cortex (i.e., plasticity) before, during, and after manipulations to the system. The ability to use intrinsic signal imaging to investigate cortical plasticity has two requirements. (i) Optical imaging should reliably describe the normal functional organization of the cortex and its relationship to structure. (ii) The imaging should be noninvasive to the cortex to allow repeated sampling of the functional organization of the same animal over time.

The present study describes the fulfillment of both requirements in the rat primary somatosensory cortex. We focus on the representations of the mystacial vibrissae (whiskers), as this sensory system offers several unique structural and functional features. The pathways within the neuraxis that carry vibrissal information are well characterized both anatomically (1–4) and physiologically (1, 3, 5–7). The whisker area of rat somatosensory cortex contains distinct cellular

aggregates in layer IV, each corresponding to a whisker on the contralateral snout, which were termed “barrels” by Woolsey and Van der Loos (8). As a result, this cortex is often referred to as “barrel cortex,” and the pattern of the whisker representations in layer IV can be distinguished with various histochemical techniques such as succinic dehydrogenase (9) and cytochrome oxidase (10, 11). The layer IV representation forms the core of a functional column corresponding primarily to one whisker, called the “principal” whisker, which extends from the pial surface to the white matter (12). Thus, the rat somatosensory system provides a unique opportunity to stimulate a single whisker as an independent peripheral unit with a distinct structural and functional correlate in the cortex.

Optical imaging of intrinsic signals has proven to be a useful technique for the characterization of functional responses to sensory stimulation (refs. 13–16; for a review, see ref. 16) and offers advantages in studying the functional organization of a sensory representation in the cortex. *In vivo* high-resolution (50–100 μm) images of the functional organization are obtained within several minutes. Such images of functional organization permit localization of specific borders and functional landmarks within the cortex that can later be compared to structural and biochemical markers (15). Since the functional representation of a whisker is a discrete cortical area, the high-resolution image permits comparison between the functional center of the representation determined optically and the structural center of the representation determined anatomically.

Although the temporal and spatial characterization of intrinsic signals has been reported in the rat barrel cortex (13) and images of forepaw digit stimulation have been obtained from the rat somatosensory cortex (17), images have now been obtained and analyzed from the rat barrel cortex. We describe both the spatial and temporal features of intrinsic signals in somatosensory cortex in response to whisker stimulation. Finally, we compare functional representations determined with imaging to representations obtained with single-unit recordings and cytochrome oxidase staining in cortical layer IV.

MATERIALS AND METHODS

Subjects. Male and female adult (3–9 months) Sprague–Dawley rats (Charles River Breeding Laboratories) were anesthetized with intraperitoneal sodium pentobarbital (Nembutal, 50 mg/kg). Animals were maintained in an areflexic state with a continuous intraperitoneal infusion of Nembutal (0.1–0.4 ml/hr, Razel syringe pump) and additional supplements when necessary. Throughout the experiment vital signs such as heart rate, oxygen saturation, and temperature were constantly monitored with a patient monitor (Hewlett–Packard 78354A). Temperature was maintained between 36.5 and 37.5°C with an adjustable heating blanket

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(Baxter Health Care, Mundelin, IL; K-module). At the end of the experiment, the subjects were sacrificed with an overdose of Nembutal.

Surgery. In all experiments we outlined an 8×8 mm area overlying left somatosensory cortex (antero-medial corner of the opening 1 mm rostral and 2 mm lateral to bregma). In most experiments we thinned the skull over this area with a drill bit (HP-3, SS White). Postmortem measurements determined this skull thickness to be 150–200 μm . In some experiments a craniotomy exposed the left somatosensory cortex but left the dura mater intact. In both experimental preparations, and as in previous optical recordings of rodent somatosensory cortex (13, 18), a wall of petroleum jelly was built around the border of the drilling and filled with silicon oil (200 fluid; viscosity, 50 cs; Accumetric, Elizabethtown, KY). The petroleum jelly wall was covered during imaging with a 22×22 mm coverglass.

Whisker Stimulation. In all experiments whiskers were singly deflected with a 5 cm length of polyethylene tubing placed orthogonal to the whisker shaft at a distance of either 10 or 15 mm from the snout. The tube was not touching any other whiskers at any time and only deflected the principal whisker during the stimulation period. The stimulator was positioned such that during deflection of the principal whisker no other whisker movement was observed. Whiskers were moved rostro-caudally with a 0.5- to 1.0-mm deflection. A modified dc-meter activated by a computer-controlled pulse generator (Master-8; A.M.P.I., Jerusalem) delivered the whisker deflections. After a 700-ms baseline period, the whisker was stimulated continuously at 5 Hz for 2.0 s during each 4.5-s trial. During each experiment, the computer randomly interlaced delivery of stimulation and nonstimulation (control) trials.

Data Collection. Most of the details of the data collection used in these experiments were developed and described by Ts'o *et al.* (15) and Frostig *et al.* (14) for intrinsic signal imaging of cats and monkeys. Briefly, a slow-scan charge-coupled device camera (Photometrics, Tucson, AZ), equipped with either a 50-mm AF Nikkor lens (Nikon 1:1.8) combined with an extender (Nikon, PK-13) or a macroscope (19), was positioned over the somatosensory cortex. The first configuration accumulates data over a 6.7×4.9 mm area whereas the macroscope accumulates over a 3.4×2.6 mm area. In both cases a 192×144 pixel array was collected. Initially, an image of the blood vessel pattern at the surface of the cortex was obtained to serve as a reference. The camera was then positioned to focus 300 μm below the surface for the data collection. Two fiber-optic light guides illuminated the cortex only during data collection with a light driven by a Kepco power supply (ATE 15-15M) that passed through a 630-nm light filter (Omega Optical, Brattleboro, VT; bandpass, 30 nm). Each trial of data collection totaled 4.5 s with an intertrial interval of 7 s. The camera continuously collected data during the 4.5 s with a frame duration of 500 ms, resulting in nine frames of data for each trial. Whisker stimulation started 200 ms after the beginning of frame 2 and continued until 200 ms after the beginning of frame 5.

Data Analysis. A typical image was obtained by averaging 7–32 trials of data collection. To visualize the discrete functional representations in barrel cortex, images of whisker activation were generated by using a single condition analysis. In a single condition analysis, data obtained during stimulation is divided by data obtained during no stimulation. Generally, images of intrinsic signal activity in visual (14, 15) and somatosensory (17) cortices have been obtained by a differential analysis of dividing the response to one stimulus by the response to an opposing stimulus. For example, previous studies divided the response to right eye stimulation by the response to left eye stimulation to obtain a map of ocular dominance columns in the visual cortex of a monkey

(14, 15). In our experiments, division of intrinsic signal activation in response to stimulation of one whisker by activation of another whisker rarely produces an image of the functional representation.

Single-Unit Recording. After imaging, the thinned skull and/or dura overlying somatosensory cortex was removed from those animals selected for single unit recording. Tungsten microelectrodes (Microprobe, Clarksburg, MD; WE5003 (20–30)H, 0.7–1.5 M Ω) and a DAM 80 amplifier (WPI Instruments, Waltham, MA) were used to isolate and amplify multiunit clusters in the cortex. Spike waveforms of multiunit recordings were separated (MultiSpike Detector; Yissum, Jerusalem) and peristimulus time histograms were created using HIST software (Spike Systems, New York). Lesions for histological verification were made using an Iso-Flex stimulator (A.M.P.I.) at 5 μA for 5 s.

Cytochrome Oxidase Staining. At the end of the experiment some rats were perfused transcardially with 0.1 M Sorenson's phosphate-buffered saline followed by ice-cold 2.5% (wt/vol) paraformaldehyde/0.5% glutaraldehyde/2% (wt/vol) sucrose in 0.1 M phosphate buffer. The cerebral cortex was dissected after perfusion and flattened between glass slides to a thickness of ≈ 2 mm (20). The tissue was cryoprotected in 30% sucrose and sectioned tangential to the pial surface (50- μm sections). Cytochrome oxidase histochemistry was performed according to Wong-Riley and Welt (21).

RESULTS

Any of the large mystacial vibrissae are routinely stimulated and imaged as a discrete representation in the cortex. Clear images of activation are obtained in response to whisker deflections both through the dura and through a thinned skull. Since the intrinsic signal response varies according to variables such as wavelength of illumination (14), type of stimulation, and depth of anesthesia (S.A.M., M.C.K., and R.D.F., unpublished observations), in this study the intrinsic signal response is characterized under one condition: 2 s of 5-Hz stimulation while illuminating the cortex with 630-nm light. There is high correlation between the functional area determined both optically and electrophysiologically and the structural representation of the whisker determined anatomically.

A typical profile of the intrinsic signal array and the resulting image of whisker activation as detected through the dura is shown in Fig. 1. The image shown is of activation in the cortex in response to a 1-mm deflection of whisker C3 at 5 Hz for 2 s. The black patch seen in Fig. 1B, defined as the functional representation of the whisker, has a diameter of ≈ 900 μm . The total area of activation exhibiting a stimulus-related change in the intrinsic signals includes both the functional representation and an area surrounding the functional representation. Outside the area of activation, the intrinsic signal traces are relatively flat (Fig. 1A). An exception to this is seen in several areas outside the area of activation where an artery or a vein causes a change in the intrinsic signals. Previous intrinsic signal recording in barrel cortex described strong responses from the arteries appearing within the recorded area (13). We found, however, that such signals can originate from both the veins and the arteries, and most commonly originate from the vessels entering or exiting the activated area. The intrinsic signals arising from the blood vessels are usually either distinctly slower (veins) than the activated cortical area or have a distinctly different shape (arteries) than the intrinsic signals of the activated area. The amplitude of the largest fractional change in Fig. 1A is 7×10^{-4} . In general, the fractional change of the intrinsic signals upon whisker stimulation ranged from 1×10^{-4} to 3×10^{-3} .

A more complete spatio-temporal profile of a typical cortical response to the type of stimulus given in these experi-

FIG. 1. Composite image of the blood vessel pattern overlying somatosensory cortex with intrinsic signals superimposed (*A*) and the image of functional activity obtained from analyzing these intrinsic signals (*B*). (*A*) Blood vessel pattern as imaged through the dura when the camera was focused at the surface of the brain. Intrinsic signals shown are an average of 24 trials, and each trace corresponds to the spatial average over a 10×10 pixel array. [*x* axis = time (s); *y* axis = fractional change (arrow length = fractional change of 1×10^{-3}); by convention the intrinsic signal traces are shown as up-going although cortical activation actually causes a decrease in light reflectance.] The large changes in intrinsic signals are clearly localized to the area of the functional representation of the principal whisker (black patch corresponding to whisker C3 activation seen in *B*). There is an area of intrinsic signal activity that extends beyond the borders of the functional representation but the signals are flat in the surrounding areas. An exception to this is seen on some of the blood vessels that also show a change in intrinsic signals upon activation, particularly if they are flowing in or out of the area of the principal whisker. (*B*) Image of functional activation obtained from the intrinsic signals seen in *A*. Although the blood vessels can be seen on the image, they are clearly distinguishable from the strong continuous activation area of the whisker. Actual cortical area of the images shown above is 4.9×3.8 mm.

ments is seen in Fig. 2. This figure further examines a 1.4-mm^2 area selected from Fig. 1 that contains the functional representation of the whisker at its center. Six sequential 500-ms frames show the rise and fall of the activation with respect to starting (Fig. 2*a*) and stopping (Fig. 2*d*) 2 s of continuous 5 Hz stimulation of the whisker. This typical activation profile illustrates that the intrinsic signal activation in the cortex undergoes three characteristic phases. The first phase constitutes a general increase in cortical activity that appears over the selected area (Fig. 2*b*). By the next frame (Fig. 2*c*), the activity becomes localized to an area within the functional representation. This localized activity is the second phase of intrinsic signal activity in barrel cortex. During the third phase a sharp peak of activation rises out of the center of this localized area and lasts for two frames (Fig. 2*d* and *e*). Finally, the peak disappears, and the initial localized area of activity corresponding to the second phase is present again (Fig. 2, compare *f* to *c*) before cortical activity returns to baseline. The second phase of activity that lies within the functional representation (Fig. 2*c*) is typically ≈ 400 μm in

FIG. 2. Three-dimensional representation of a 1.4-mm^2 area of cortex selected from Fig. 1 during six successive 500-ms frames. (*a*) Frame 1, collected 300 ms after the onset of stimulation. Given that intrinsic signals are not detectable in <300 ms, no activity is obvious in this frame, but an overall rise above baseline is apparent by the next frame (*b*). (*b*) An overall increase in brain activity can be seen in this first phase of brain activation. (*c*) A more localized increase constitutes the second phase of intrinsic signal activation, diameter ≈ 400 μm . (*d*) During the third phase of activity a sharp peak rises out of the center of activation. Although the stimulation ends during this frame, the sharp peak of activity remains through the next frame (*e*). (*f*) Within 1 s after the end of stimulation the third phase has disappeared and activation similar to the second phase is again present before the cortex returns to baseline activity.

diameter when stimulating whisker C3 in this manner. The sharp peak of activity that constitutes the third phase (Fig. 2*d* and *e*) is more variable between experiments. Interestingly, the size of the localized peak (second phase) of intrinsic signal activation during stimulation of whisker C3 as viewed on a surface plot (Fig. 2*c*) corresponds with the size of the cytochrome oxidase-rich area in layer IV of the C3 whisker representation (10, 11). Generally, the intrinsic signal activity starts as early as 300 ms, and peaks within 1–3 s after the onset of stimulation.

Similar spatio-temporal aspects of activation in the cortex were obtained through a thinned skull. The image shown in Fig. 3 is a 3.1×2.2 mm area of cortex as imaged through a thinned skull (150 μm) with the microscope during deflection of whisker B2. In a previous study (14), designed to image cat visual cortex through a thinned skull, it was found that near infra-red illumination (930 nm) was optimal for penetrating through the skull, while red (630 nm) illumination was used when the skull and dura were removed. In the present study, however, the same wavelength of illumination (630 nm) was used for all of the imaging experiments. This wavelength is optimal for localizing the intrinsic signals to the area of the functional representation (14). Although the skull increases the scattering of reflected light, which should deteriorate the image, there was no need to change any parameters of stimulation, data collection, or data analysis to obtain discrete images of the sensory representation through a thinned skull. Thus, a thinned skull preparation is now routinely used for intrinsic signal optical imaging of the rat barrel cortex.

After imaging, the dura mater and/or the thinned skull were removed from those animals selected for electrophysiology. Recordings within the optically determined functional representation always confirmed this area to be the best electrophysiological response area of the principal whisker

FIG. 3. Activation of a whisker through a thinned skull. Image shown is an average of 32 trials of 0.5-mm rostro-caudal stimulation of whisker B2 at 15 mm from the snout. All parameters of collection and analysis were identical to imaging done through the dura as in Fig. 1. In this experiment the skull was thinned to 150 μm . The grainy appearance of this image compared to Fig. 1 is the result of the higher magnification obtained when using the macroscope. Actual size of the cortical image shown above is 3.1×2.2 mm.

stimulated. Responses in the center of the functional representation were always robust, and radial penetrations moving away from the center recorded decreased responses as the distance from the center increased. Similarly, there is a gradual decrease in the intrinsic signals as the distance from the center of the functional representation increases (see Fig. 1A).

To compare our imaged representation of the whiskers with the cytochrome oxidase staining of the barrels in layer IV, nine whiskers were imaged during one experiment to generate a map of their functional representations. Imaging was performed through the skull, and all whiskers were deflected 0.5 mm at 5 Hz in the rostro-caudal plane at a distance of 15 mm from the snout. Whiskers were always stimulated individually since a previous imaging study using single versus simultaneous stimulation of whiskers reported functional interactions between adjacent whiskers as well as partial overlap in their functional representations.[†] The center of the activation of each whisker was determined on each image, and all nine whisker representations were compiled into the composite image shown in Fig. 4A. After imaging, the cortex was flattened and cytochrome oxidase histochemistry was performed (Fig. 4B). Notice the resemblance of the shape of the imaged representations to the anatomic representations found in layer IV (Fig. 4A and B). The relative distances between centers of the whisker representations were calculated by selecting a whisker representation at a corner of the 3×3 matrix (B1) and measuring the distance to the center of all other whiskers. The distances between the centers of the representations visualized with imaging and the distances between the centers of the representations stained with cytochrome oxidase were highly correlated ($r = 0.98$, see Fig. 4C).

In another experiment, designed to relate the images of discrete representations to the anatomical representations in layer IV, six whiskers were imaged and then lesioned in the center of their optical representations at a depth corresponding to layer IV. Again, the lesions were in the dense cytochrome oxidase staining of the corresponding whisker representations (data not shown).

[†]Masino, S. A., Chen, C., Dory, Y. & Frostig, R. D., Fifth Conference on the Neurobiology of Learning and Memory, October 1992, Irvine, CA, p. 57 (abstr.).

FIG. 4. Comparison and correlation between imaging and anatomy of 3×3 matrix of whiskers. (A) Nine whiskers (B1, B2, B3, C1, C2, C3, D1, D2, and D3) were individually stimulated and imaged through the skull (thinned to 150 μm). Elongations in the representations of B1 (lower right) and D1 (upper right) stem from intrinsic signal changes in veins flowing out of these areas. (B) Photomontage of cytochrome oxidase staining of the same nine whisker representations in layer IV. (A and B, bar = 500 μm .) (C) The relative distances between the centers of the whisker representations on the maps obtained optically and anatomically were compared and correlated ($r = 0.98$; see text for more details).

DISCUSSION

In this study we characterize the relationship among intrinsic signals, electrophysiology, and anatomy in the adult rat barrel cortex. Using optical imaging, we describe reproducible spatio-temporal activation in rat barrel cortex in response to a behaviorally relevant (22) stimulus frequency of 5 Hz. This study was designed to demonstrate that (i) optical imaging of intrinsic signals provides a reliable high-resolution

image of the functional organization of barrel cortex and (ii) such functional imaging can be obtained in a manner that is noninvasive to the brain.

We verified the accuracy of our imaging results in barrel cortex both physiologically and anatomically. Postimaging electrophysiology confirms that the optically determined functional representation corresponds with the electrophysiological response area of the principal whisker stimulated. The imaged response area in the cortex is discrete and localized, but the spread of the intrinsic signals does overlap into the functional representations of neighboring whiskers. Electrophysiological recordings also show responses to adjacent whiskers in the superficial layers (23), and the size of the functional representations we obtain with intrinsic signal imaging corresponds well with 2-deoxyglucose results in the rat barrel cortex (24).

Perhaps more notable than the electrophysiological confirmation is the relationship and correspondence between the optical map and the cytochrome oxidase map when the representations of nine whiskers are compared. The general shape of the representations and the relative distances between the representations are consistent when comparing imaging and anatomy. Moreover, lesions in the center of the optical activity correspond with the center of the dense layer IV cytochrome oxidase patch for a given whisker. These correspondences between imaging and anatomy hold even with optical distortions caused by the curvature of the cortex and distortions introduced by the flattening and histological processing of the tissue.

The general shape of the intrinsic signals and the spatial and temporal properties observed is similar for all of the whiskers and corresponds well with intrinsic signal characterization previously reported in barrel cortex (13) and other cortical systems (14). Furthermore, the signals are identical whether collected through the dura or through a thinned skull. A thinned skull preparation is virtually noninvasive to the cortex and aids in the stability of the preparation during the course of the experiment. Variables that might influence the experimental results, such as changes in intracranial pressure (usually manifested as gradual herniation of the cortex), mixing of biological fluids with the silicon oil, and sporadic bleeding, are eliminated. In addition to these advantages gained within a single experiment, this preparation provides the opportunity to repeatedly sample the functional organization of the same animal over time.

Although the areal extent of the functional representation coincides with the electrophysiological response area, the time course of the signal in general is quite different from the latencies and durations detected electrophysiologically in the barrel cortex. The reason for this is 2-fold. (i) Intrinsic signals rely primarily on metabolic activity, which has a slower time course than electrical activity. The wavelength used in this study (630 nm) selects for intrinsic signals primarily arising from activity-dependent oxygen delivery mechanisms from capillaries (14). (ii) The type of stimulation that we deliver in this experiment is very different from the stimulation used in the electrophysiological studies (5–7, 23). Ours is a continuous stimulation for 2 s, whereas most electrophysiological experiments analyze the response to a single deflection of a whisker lasting from a few milliseconds up to 200 ms.

In conclusion, we have found a striking correspondence among imaging, physiology, and anatomy in the representation of the whiskers in rat somatosensory cortex. Previously used primarily in the visual system, optical imaging of intrinsic signals corroborated the existence of and relationship among ocular dominance columns, orientation columns, and cytochrome oxidase blobs (25). Furthermore, it illumi-

nated properties of the functional organization of the visual cortex, such as the organization of orientation columns into pinwheels (25, 26). The rat somatosensory system affords a unique opportunity to study the organization of the sensory representation of a distinct peripheral unit, the whisker, which has a discrete functional and structural correlate in the cortex. These advantages, along with the ability to regularly and reliably image through a thinned skull, make this system well-suited for long-term investigations of plasticity in the adult somatosensory cortex.

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