Suprathreshold Auditory Cortex Activation Visualized by Intrinsic Signal Optical Imaging

The suprathreshold tonotopic organization of rat and guinea pig auditory cortex was investigated using intrinsic signal optical imaging through a thinned skull. Optical imaging revealed that suprathreshold pure sine wave tone stimulation (25–80 dB) evoked activity over large cortical areas that were tonotopically organized. Three-dimensional surface plots of the activated areas revealed "patchy" auditory-evoked activity consisting of numerous local peaks and valleys building to a maximum. Subsequent detailed electrophysiological mapping in the same subjects confirmed the localization of auditory-evoked activity based on optical imaging, including responses to a test frequency at cortical loci more than 2 octaves away from the threshold-defined isofrequency contour. The success of this technique in visualizing auditory cortex functional organization at suprathreshold stimulus levels will allow for future investigations of auditory cortex frequency representation, including representational plasticity induced by a variety of experimental manipulations.

According to the classic description of the auditory cortex, acoustic frequency is represented spatially. Threshold-based isofrequency contours, that is, theoretical lines drawn to connect recording loci of neurons with similar threshold-determined characteristic frequencies, are aligned across the cortical surface in a systematic progression of frequency, forming the tonotopic (frequency) map (Merzenich et al., 1975; Imig et al., 1977). However, since animals behave in suprathreshold auditory environments, it is important to investigate the functional organization of auditory cortex to suprathreshold stimuli, and characterize any differences between threshold-based descriptions and suprathreshold descriptions of the functional auditory cortex organization.

Recent investigations into the functional organization of the auditory cortex have begun to challenge the functional auditory cortex organization based only on threshold-level stimuli. First, as stimulus intensity increases, auditory cortex neuronal bandwidth also increases (Phillips and Irvine, 1981; Phillips et al., 1985; Schreiner and Mendelson, 1990); consequently, it is difficult to predict the range of suprathreshold stimulus frequencies to which a neuron will respond based on the value of its threshold characteristic frequency. Second, many neurons have nonmonotonic intensity/bandwidth functions, and neuronal bandwidth varies with location within the isofrequency contour (Phillips and Irvine, 1981; Phillips et al., 1985; Schreiner and Mendelson, 1990; Schreiner and Sutter, 1992); thus, concluding which neurons will respond within the frequency representation to suprathreshold stimuli is not straightforward. Third, because neurons with monotonic stimulus/intensity rate functions are segregated from neurons with nonmonotonic functions in auditory cortex, activity develops in patches rather than relatively uniformly along the isofrequency contour (Phillips et al., 1994). An unresolved important issue is the extent to which suprathreshold stimuli evoke activity across the frequency representation, that is, along the cortical surface orthogonal to the isofrequency contours. Finally, the distribution and size of these patches vary with intensity as continued increases in stimulus intensity begin to inhibit more nonmonotonic cells (Phillips et al., 1994). Together, these findings lead to the inability to accurately predict the spatial distribution of suprathreshold auditory cortex neuronal activation.

Previous descriptions of the spatial organization of frequency responses in auditory cortex were obtained using electrophysiological mapping: recording the stimulus-evoked neuronal response at tens to hundreds of cortical loci sequentially in order to extrapolate a profile of response characteristics across the entire auditory cortex. We chose to apply a new, but complementary, approach to the study of auditory cortex organization: in vivo optical imaging of intrinsic signals. Intrinsic signals are sensory-evoked changes in light reflectance related, directly and indirectly, to spiking neurons (Grinvald et al., 1986; Frostig et al., 1990). Advantages of optical imaging include (1) the ability to visualize the functional organization of a very large area of sensory cortex (e.g., with our camera/lens combination, >32 mm² in a single simultaneous measurement, (2) very high spatial resolution (~50 μm), (3) a short-duration experiment coupled with the ability to use a wide variety of stimuli (Ts' o et al., 1990), and (4) a methodology that is noninvasive to the brain, enabling repeated measurements using chronic preparations (Masino et al., 1993, 1994). This methodology, namely, optical imaging through a thinned skull, provides identical results when compared to imaging an exposed cortical surface (Masino et al., 1993). Electrophysiological mapping of auditory cortex is slow (minimum 6–18 hr; Sally and Kelly, 1988; Redies et al., 1989) and difficult to repeat in small mammals, but offers excellent temporal resolution. Similarly, optical imaging based on voltage-sensitive dyes (Orbach et al., 1985; Taniguchi and Nasu, 1993; Uno et al., 1993) offers better temporal resolution than intrinsic signal imaging, but requires the application of dyes directly to the cortex, making repeated noninvasive measurements difficult.

Instrumental to its acceptance for use in studying other sensory cortices, an initial step was the demonstration that optical imaging could reveal the known functional organization of the sensory cortex under investigation (visual: Ts' o et al., 1990; Bartfeld and Grinvald, 1992; Bonhoeffer and Grinvald, 1993; somatosensory: Masino et al., 1993). Here we report the first successful intrinsic signal optical imaging of auditory cortex satisfying that standard.

A preliminary report of some of these data appeared in abstract form (Bakin et al., 1993).

Materials and Methods

The major features of the experimental protocol are summarized in Figure 1. Anesthetized subjects, prepared for acute experimentation, underwent several optical imaging sessions within a period of 0.5–3 hr to determine the functional organization of the auditory cortex (Fig. 1A). Immediately following the last optical imaging session, the same cortical area was mapped electrophysiologically (Fig. 1B).
Subjects and Surgical Preparation

Adult male Sprague Dawley rats (290-525 gm, 3-4 months old, n = 18; Charles River Breeding Laboratories, Wilmington, DE) were anesthetized and maintained in an areflexive state by sodium pentobarbital (initial anesthetic dose: 50 mg/kg, i.p.; subsequent continuous i.p. infusion: 0.1-0.4 ml/hr; syringe pump A99, Razel Scientific, Stanford, CT). Heart rate and temperature were continuously monitored (78354A, Hewlett Packard, Palo Alto, CA). In addition tail pinch and eyelid reflexes were assessed and additional supplements of sodium pentobarbital (20 mg/kg, i.p.) were administered as needed to ensure a satisfactory areflexive state.

Adult male Hartley guinea pigs (389-808 gm, 1.5-3.5 months old, n = 6; Hilltop Farms, Scottdale, PA) were premedicated with atropine sulfate (0.02 mg/kg, i.p.) and anesthetized with diazepam (8 mg/kg, i.p.) and sodium pentobarbital (20 mg/kg, i.p.). Additional sodium pentobarbital injections (5-10 mg/kg, i.p.) maintained surgical levels of anesthesia. Heart rate and temperature were continuously monitored as were responses to foot pinch and eyelid reflexes.

Following attainment of surgical levels of anesthesia, subjects were prepared for acute surgery. The subject’s temperature was monitored, and maintained via an adjustable heating pad. Subjects were mounted in a stereotaxic instrument using black fur. An acrylic pedestal was affixed to the surface of the skull and was anchored by several stainless steel screws, including one that would serve as a reference electrode during the electrophysiological portion of the experiment. Fixation of the subject to the stereotaxic frame via this pedestal eliminated the need for ear bars, enabling direct unobstructed access to the external auditory meatus. The tissue overlying the left auditory cortex was resected, and the exposed skull surface overlying the auditory cortex was thinned using a miniature drill bit (HP-3, SS White, Lakewood, NJ; postmortem measurements revealed the thinned skull surface to be between 100 and 200 μm thick). A well of petroleum jelly constructed around the border of the thinned skull was filled with polydimethylsiloxane silicon oil (DS Fluid, 50 cSt viscosity, Accumetric Inc., Elizabethtown, KY) and sealed with a cover glass.

A. Optical Imaging of Auditory Cortex

Technical Highlights:

- Light Source: 630 nm Filler
- Calibrated Sound Delivery System
- CCD Camera System
- Computer
- Silicon-Oil Filled Well

Timing Diagram:

- Frequency (Randomized)
- Camera Opening
- Auditory Stimuli
- Camera Opening

Optical imaging technical highlights. Following surgical preparation, subjects underwent several sessions of intrinsic signal optical imaging. A computer captured data from a CCD camera positioned perpendicular to the left auditory cortex, which was illuminated with a 630 nm light source. Bottom, Optical imaging timing diagram. Several stimulus conditions were randomly presented during a single imaging session (indicated by sine waves of different frequency). Note that the internal interval is not drawn to scale in order to conserve figure space. Actual intertrial interval was 14.5 sec. Optical data (reflected light) were collected prior to, during, and after acoustic stimulation, B. Electrophysiological mapping technical highlights. Repeated layer 3/4 multiple-unit recordings were made using an insulated tungsten microelectrode inserted normal to the cortical surface. The neural signal was amplified, filtered, and amplitude discriminated. Spike latencies were stored on computer for off-line analysis of peristimulus histograms. See Materials and Methods for further details.

B. Electrophysiological Mapping of Auditory Cortex

Technical Highlights:

- Tungsten Microelectrode (1MΩ): Inserted normal to cortical surface (Layers III/IV)
- Calibrated Sound Delivery System
- Window
- Silicon-Oil Filled Well
- Computer

Electrophysiological mapping technical highlights. Following surgical preparation, subjects underwent several sessions of in vivo electrophysiology. The camera was focused 300 μm below the cortical surface, an image of these large vessels was obtained for later reference during image analysis and electrophysiology. The camera was focused 300 μm below the cortical surface to minimize contribution to the data from the surface vasculature (Grinvald et al., 1986; Ts’o et al., 1990). The cortical surface was illuminated throughout the duration of the experiment by 630 nm light delivered via two fiber-optic light guides (light source: power supply ATE 15-1S, Kepco, Flushing, NY; 30 nm bandpass, 630 nm filter, Omega Optical, Brattleboro, VT). The entire apparatus was supported by a vibration-resistant table (Research Series Plus, Newport Instruments, Irvine, CA).

Data Collection

Auditory cortex responses to two or four different frequency/intensity combinations were determined during each session. Such combinations were presented randomly until 20-40 trials of each were accumulated. One trial consisted of four to eight tone pips (50 msec duration, 250 or 500 msec intertone interval, 10 msec rise/fall time) at the particular frequency/intensity combinations being tested (Fig. 1A). During each trial, optical data were collected for a total of 4.5 sec (incorporating nine consecutive frames of 500 msec duration each) and stored on computer (MicroVax III, Digital Equipment Corporation, Maynard, MA). Auditory stimulation began at onset of the third frame and ended at onset of the sixth frame. Intertial interval was 14.5 sec. Subjects underwent several imaging sessions prior to electrophysiological mapping.

Data Analysis

The images described in this manuscript are based on a permutation of the "single condition division" as introduced by Masino et al.
In the experiments, an isointensity series of tones (50 msec tone duration, 10 msec rise/fall time, 1 sec intertone interval, 20 repetitions at 1 sec intersequence interval) consisting of those frequencies used during the auditory stimulation was divided by the combined data evoked by all of the stimulations. Each type of single division has its own advantages as discussed by Bonhoeffer and Grinvald (1993). In our permutation of single-condition division (hereby denoted the “stimulus-related blank”), frames containing stimulus-evoked data are divided by frames obtained without stimulation within the same condition (Masino et al., 1993) and not by another separate blank condition, as was the case for the genuine blank division. Although theoretically both genuine blank and stimulus-related blank analyses should produce identical results, using the stimulus-related blank for the single-condition analysis performed on the auditory data produced superior results when compared to the genuine blank condition division. The reasons for this difference are probably related to blood vessel artifacts and require further research.

An alternative to a single-condition division analysis is the differential map analysis, in which cortical activity evoked by one stimulus is divided by activity evoked by a different stimulus (Blasdel and Salama, 1986; Blasdel 1992a,b). Each approach has unique advantages. Single-condition maps require minimal assumptions about the underlying functional architecture since this map provides the location of activity evoked by one stimulus (Bonhoeffer and Grinvald, 1993). Differential map analysis highlights areas that respond preferentially to one stimulus over another, but fails to reveal cortical areas that respond equally well to both stimuli (‘common-mode’ activation). In this article we will mainly apply single-condition analysis.

Electrophysiological Mapping of the Auditory Cortex

Following completion of the last imaging session, the subject was prepared for electrophysiological mapping (Fig. 1D). The well was removed and the surface of the skull dried. A larger area of skull, including the thinned window, was removed. The dura was kept moist with warm saline while being resected. After removal of the dura, an image of the cortical vasculature was taken to aid in electrode placement and later confirmation of the optical images. A well of petroleum jelly surrounding the exposed cortical surface was filled with warm polydimethylsiloxane silicon oil. In an effort to reduce cortical pulsations after the removal of the skull overlying auditory cortex, a cisterna magna puncture was routinely performed.

Multi-unit and single-unit recordings from the middle cortical layers (rat, 300–600 μm; guinea pig, 400–1000 μm) were made using Parylene-C-insulated tungsten microelectrodes (tip impedance, 1.0–1.5 MΩ; Microprobe, Inc., Clarksburg, MD). Neural activity was amplified (DAM-80, World Precision Instruments, Sarasota, FL) and filtered (bandpass, 500–3000 Hz; KH-3550, Khron-Hite, Cambridge, MA). Neural spikes were amplitude discriminated (Frederick Haer, Ann Arbor, MI) and the latencies recorded on computer (Apple Ile with software written in house).

Every cortical locus with satisfactory neural activity (spike signal: noise ≥ 2:1) was characterized with the identical frequency/intensity combination used during the optical imaging session (50 msec tone duration, 10 msec rise/fall time, 20 repetitions). In addition, in some experiments, an isointensity series of tones (50 msec tone duration, 10 msec rise/fall time, 1 sec intertone interval, 20 repetitions at 1 sec intersequence interval) consisting of those frequencies used during the optical imaging session, as well as additional frequencies, was presented in order to determine the loci’s best frequency (the frequency that evokes the greatest discharge spike rate). Either approach enabled an answer to whether or not a cortical locus responded to any of the frequencies used during optical imaging. A cortical locus was denoted as having a positive response to a frequency when the tone-evoked neural discharge rate was at least twice the pretone (background) discharge rate (Bakin and Weinberger, 1990).

At the conclusion of the electrophysiological mapping session, the subjects were euthanized with an overdose of sodium pentobarbital (100 mg/kg, i.p.).
Tonotopic Organization of Auditory Cortex

Successful intrinsic signal imaging revealing evoked acoustic activity enabled investigation of whether or not it was possible to detect evidence of tonotopic organization. In this section we describe the tonotopic organization of rat and guinea pig auditory cortex to suprathreshold stimuli. In a later section we explicitly address the large sizes of the activated cortical areas elicited by acoustic stimulation.

Rat

The rat auditory cortex contains a single tonotopically organized field with a rostral-to-caudal progression of high to low frequencies (Fig. 5A; Sally and Kelly, 1988). Figure 6 illustrates two points: (1) optical imaging can reveal the tonotopic organization of the rat auditory cortex, and (2) there is a close correspondence between the optical determination and the electrophysiological determination of areas activated by a particular frequency/intensity combination. In this case, two frequencies (30.0 kHz and 6.5 kHz) were randomly presented during the imaging session. The images are of the identical surface area of auditory cortex and are aligned. The 30.0 kHz stimulus evoked activity over a large area in the rostral portion of the cortex imaged (Fig. 6A). In agreement with the known organization of rat auditory cortex, the 6.5 kHz stimulus evoked activity in a large region more caudal to the 30 kHz patch (Fig. 6B).

Subsequent to imaging, the same cortical area was mapped electrophysiologically at 107 loci. The symbols overlaying the images in Figure 6 illustrate the high degree of agreement between the spatial localization of auditory cortex evoked activity from the two different methodologies. Crosses indicate loci with electrophysiologically measured responses to the frequency used to generate the optical image. Circles are sites that responded to both frequencies (30.0 and 6.5 kHz). Open squares are nonresponsive loci. Note the correspondence between the area of activation and the responsive loci (crosses and circles), illustrating the close agreement between the optically determined active areas and electrophysiologically determined loci responsive to the same frequency. This is true for both 30.0 kHz and 6.5 kHz (Fig. 6A, B, respectively). Also, note the close alignment of the loci with responses to both frequencies (circles) within the overlap region of the evoked areas measured optically.

Samples of electrophysiological responses obtained from three cortical loci investigated in this experiment are presented in Figure 7. Here, the responses of cells to both 6.5 and 30.0 kHz stimuli are depicted. The cells were recorded in three areas with distinct optical responses: an area that responds strongest to 6.5 kHz area, an area that responds strongest to 30.0 kHz area, and an area that responds well to both. Cells recorded at loci within the area preferring 6.5 kHz area (Fig. 7, top) were responsive to 6.5 kHz but not to 30.0 kHz; that is, their electrophysiological responses matched their intrinsic signal optical imaging responses. Similarly, cells recorded from loci within the area preferring 30.0 kHz as measured optically (Fig. 7, bottom) were responsive to 30.0 kHz but not to 6.5 kHz. In contrast, cells that responded electrophysiologically to both frequencies were primarily localized within areas that optical measurements revealed responses to both frequencies.

By dividing the data obtained by activation of the auditory cortex evoked by one frequency by activation evoked by another frequency (differential condition analysis; see Materials and Methods) we can highlight the areas that prefer one frequency over the other and cancel out areas that were activated by both frequencies (“common-mode” activation; see Materials and Methods). In this analysis, the cortical activity evoked by one condition was divided by the activity evoked by a second condition. The subject presented in Figure 8 received 8.0 kHz and 28.0 kHz (40 dB) stimuli. This division results in a light patch indicating cortical tissue preferentially activated by 28.0 kHz and a dark patch indicating cortical tissue preferentially activated by 8.0 kHz. The bordered gray area between the two patches was not silent; rather, it re-

Figure 3. Imaging rat auditory cortex through a thinned skull. A. An active area produced by a 26.0 kHz, 50 dB auditory stimulus. B. The area of cortex that was imaged in A. Note that in this case, the largest dural and surface vasculature is visible through the approximately 100 μm thick skull window.

Patchiness in the magnitude of response over the spatial extent of the responsive area was observed in each case investigated. Since blood vessel artifacts may appear as peaks in a three-dimensional image, we correlated the optical location of peaks with blood vessel artifacts. The analysis of the extent of the responsive area was observed in each case in three areas with distinct optical responses: an area that responds strongest to 6.5 kHz area, an area that responds strongest to 30.0 kHz area, and an area that responds well to both. Cells recorded at loci within the area preferring 6.5 kHz area (Fig. 7, top) were responsive to 6.5 kHz but not to 30.0 kHz; that is, their electrophysiological responses matched their intrinsic signal optical imaging responses. Similarly, cells recorded from loci within the area preferring 30.0 kHz as measured optically (Fig. 7, bottom) were responsive to 30.0 kHz but not to 6.5 kHz. In contrast, cells that responded electrophysiologically to both frequencies were primarily localized within areas that optical measurements revealed responses to both frequencies.

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sponded equally well to both frequencies, resulting in an area of no preference. Had these data been presented using the single-condition division described previously, this gray area would have been the area of overlap between the areas activated by each of the two frequencies. The localization and extent of the optical active areas were confirmed electrophysiologically (71 penetrations), including the recording of loci with responses to both frequencies within the bordered gray area.

**Guinea Pig**

The primary auditory cortex of the guinea pig consists of several fields, including two major tonotopically organized fields arranged in mirror images (Fig. 5B; Hellweg et al., 1977; Redies et al., 1989). The anterior field has a rostral-to-caudal progression of increasing best frequencies. The frequency organization of the posterior field is reversed; that is, there is a rostral-to-caudal progression of decreasing frequencies.

An example of imaging the guinea pig’s anterior field with three frequencies is presented in Figure 9. The area of activation evoked by the lowest frequency (1.0 kHz, 70 dB; Fig. 9A) was located rostrally; an intermediate frequency (4.7 kHz, 70 dB; Fig. 9B) evoked activity from cortical tissue located caudal to the low-frequency area; finally, a higher frequency (16.5 kHz, 70 dB; Fig. 9C) evoked activity from an area of auditory cortex more caudal than the other frequencies. Thus, increasing the stimulus frequency shifted the active areas caudally across the cortical surface. This suggests that this experiment imaged the guinea pig’s anterior field. Subsequent electrophysiological recording confirmed that these images were indeed taken from the anterior field. Finally, note the patchiness evident in the active area labeling. Imaging of the posterior field in additional experiments resulted in a caudal progression of active areas when a decreasing series of frequencies was used (see Fig. 5B).

Comparison of images obtained from the rat and guinea pig auditory cortices reveals potential differences in active area characteristics. For example, it appears that tone-evoked activity in rat auditory cortex is less defined than in the guinea pig auditory cortex (compare Figs. 3, 6 to Fig. 9). Further research is required to determine these issues.

**Spatial Distribution of Suprathreshold Activation**

The extent to which suprathreshold activation overlaps threshold isofrequency contours is unknown. To approach this problem it is preferable to describe both the suprathreshold stimulus-evoked active areas and the threshold-based isofrequency contour maps within a single animal. As the optical imaging experiments could not be carried out in an acoustic chamber, it was impossible to obtain threshold-level responses due to a background ambient noise level of 20–35 dB. However, it was possible to compare the suprathreshold stimulus-evoked active areas determined optically with the best-frequency (the frequency that elicits the greatest response at a given intensity; by definition, the characteristic frequency is the threshold best frequency) isofrequency contours determined electrophysiologically. To accomplish this, we characterized the best frequency of each cortical locus by presenting a wide range of stimulus frequencies (0.5–40 kHz), as well as characterizing the response of each cortical locus to the frequencies used during the optical imaging sessions. Using the same descriptive tool used in traditional threshold-based electrophysiological mapping experiments, namely, connecting the loci with a line indicating those with the identical best frequency, we could then compare the extent of activation of loci of a particular best frequency with the area activated by that same frequency. This comparison is illustrated in Figure 10 for two frequencies (30.0 kHz and 6.5 kHz; data are from the same subject illustrated in Figs. 6, 7). There are two features in Figure 10 worth highlighting: (1) best-frequency contours tend to be colocalized with the areas of greatest intrinsic signal activation (darkest portion of the activated areas), and (2) the spatial extent of cortical activation in response to suprathreshold stimulation is quite large, when measured either optically or electrophysiologically.

Figure 10A compares several isofrequency contours with the 30.0 kHz active area determined optically. Of interest, note the solid white isofrequency contour that corresponds to those loci with the best frequency of 30.0 kHz. It is located within the cortical area activated by a 30.0 kHz stimulus measured optically (dark areas), and in particular, it includes the area of greatest activation. In addition, the area activated by the suprathreshold stimulus, when measured either optically or electrophysiologically (see Fig. 6A), extends spatially far.
Figure 5. Tonotopic organization of rat and guinea pig primary auditory cortex. A, Rat auditory cortex has a single tonotopically organized field. Characteristic frequencies are organized such that high frequencies are located rostrally and low frequencies caudally (adapted from Sally and Kelly, 1988). Numbers indicate frequency in kHz. B, Guinea pig auditory cortex has two tonotopically organized auditory fields arranged in mirror fashion. The rostral portion of the anterior field contains low-characteristic-frequency cells. Characteristic frequencies increase in a caudal direction until reaching a high-frequency border. At this border, the posterior field begins, and characteristic frequencies decline until reaching a low-frequency area located most caudally (adapted from Hellweg et al., 1977; Redies et al., 1989).

Beyond this isofrequency contour. In fact, the isofrequency contour of best frequencies more than 2 octaves lower (6.5 kHz) still lies within the 30.0 kHz active area, illustrating that best frequencies alone are inadequate predictors of the range of suprathreshold stimuli to which cells might respond. The same conclusions are true for the 6.5 kHz active area and its isofrequency contour (Fig. 10B): the solid white isofrequency contour is found within the zone of greatest intrinsic signal magnitude, and the active area includes cortical tissue typically described with an isofrequency contour almost 2 octaves away (2.0 kHz).

While octave measurements enable direct comparisons across different frequency ranges, an alternative measure for within-frequency-range comparisons is the amount of cortical tissue responsive to a particular frequency (Fig. 11A,B). Combining data available in the published literature, as well as the present electrophysiological mapping data, and at threshold, electrophysiological responses to a given frequency can be recorded from a strip of cortical tissue approximately a few hundred micrometers wide. In contrast, loci covering more than 1 mm of cortical tissue are electrophysiologically responsive to the same frequency at suprathreshold intensity. This holds true for both rat (Fig. 11A) and guinea pig (Fig. 11B) auditory cortex. Thus, the extensive spatial spread of suprathreshold auditory cortex activation orthogonal to the isofrequency contours observed optically was also supported by measurements of the cortical distance over which it is possible to detect electrophysiological neuronal responses to a given frequency.

Figure 6. Electrophysiological confirmation of auditory cortex optical imaging. Active areas produced by stimuli of 30.0 kHz (A) and 6.5 kHz (B), each 50 dB contralateral stimulation, presented in random fashion. Both images are of the same cortical surface. Symbols indicate subsequent electrophysiological confirmation of the active areas: crosses, loci that responded to the same frequencies used to activate the cortex optically; circles, loci that responded to both frequencies (30.0 and 6.5 kHz); small open squares, nonresponsive loci. Note the close correspondence between the responsive electrophysiological recordings (crosses/circles) and the optical measurements of frequency-evoked activity (dark areas). This cortex was electrophysiologically mapped at 107 loci. (The diagonal light streaks in the images are blood vessel artifacts.)
Discussion

Tonotopic auditory cortex is typically described as a series of isofrequency contours: cells with similar characteristic frequencies are arranged in contours orthogonal to the gradient of characteristic frequencies. This description is based largely on neuronal responses in deeply anesthetized subjects given threshold-level stimuli. However, behaving animals usually experience suprathreshold stimulus levels (e.g., Recanzone et al., 1993). Using intrinsic signal optical imaging, we were able to demonstrate the tonotopic organization of both rat and guinea pig auditory cortex with suprathreshold stimulus levels.

While the active areas progressed in a predictable fashion along the cortical surface when frequency was changed, three findings were not expected given the threshold-based auditory cortex descriptions: (1) the active areas observed were large, often extending over several millimeters (Figs. 3, 4, 6, 8–10); (2) highly disparate frequencies could activate areas that overlapped (Figs. 6, 8, 9); and (3) activity levels within active areas were not uniform, but consisted of numerous local “peaks” and “valleys,” that is, areas of high activation intermixed with areas of low activation (Figs. 3, 9).

One possible explanation of these results is that the large size of the active area is biased by the technique of intrinsic signal optical imaging. This is unlikely for a variety of reasons. First, our within-subject electrophysiological mapping in layers 3/4 of an active area invariably resulted in recordings with responses to the same frequency used during the optical session. These recordings were obtained over large areas identical to those that generated responses visualized optically (Figs. 6, 7). Second, measuring the distance of the rostral-caudal domain over which neuronal responses can be electrophysiologically recorded revealed that suprathreshold stimuli activated a much larger cortical area than threshold stimuli (Fig. 11). Finally, Malonek et al. (1990) reported that they could faithfully image between 700 and 1000 μm below the cortical surface in the monkey (layers 3–4C). Assuming the optical properties of primate and rodent cerebral cortex to be similar, then these depths would include signals from layers 4–5 of rat (600–1025 μm). Thus, optical imaging records activity generated in the same cortical layers as those mapped.
et al., 1994). The authors reported that suprathreshold stimuli cat primary auditory cortex (Heil et al., 1992, 1994; Phillips findings are consistent with a recent electrophysiological study of the distribution of response magnitude across the cortical surface in the isofrequency contours. Further research is required to determine if the peaks and valleys observed in the rat and guinea pig optical recordings correspond to this segregation of the electrophysiological response types described in the cat.

Spatial Distribution of Suprathreshold Stimulus-Evoked Activity
As previously mentioned, recent work has been directed toward understanding the spatial distribution of suprathreshold responses. Heil et al. (1992, 1994) found that within an isofrequency contour, increasing intensity increased the spatial extent of activation for localized areas of the isofrequency contour. As previously mentioned, Phillips et al. (1994) found that while threshold stimulation elicits activity in patches along the isofrequency contour of the test frequency, increasing stimulus intensity produces silent areas along the isofrequency contour due to local clustering of suppressed nonmonotonic cells. In addition, patches along different isofrequency contours develop, reflective of cells with different characteristic frequencies but with bandwidths that include the original testing frequency. Our present results are in agreement with these findings. Heil et al. (1994) predicted that an expansion in the spatial distribution of evoked activity would be found orthogonal to the isofrequency contours at high intensity. A comparison of the optically defined active areas with isofrequency contours has confirmed this prediction (Fig. 10). Phillips et al. (1994) found evidence of spatial spread across the isofrequency contour on the scale of about ±1.0–1.5 octaves in cat primary auditory cortex. In comparison, the potential for spatial spread in the rat and guinea pig primary auditory cortex was greater than 2.0 octaves. In addition, previous workers, using optical imaging of voltage-sensitive dyes applied directly to guinea pig auditory cortex (Taniguchi and Nasu, 1993; Uno et al., 1993), have also reported large frequency-evoked areas. However, as they did not provide electrophysiological confirmation, the question about the correspondence of the widespread activation to discharging neurons has remained open.

The present results, along with previous studies involving voltage-sensitive dye imaging or electrophysiology, suggest that the conceptual framework of spatially constrained small groups of cells, that is, isofrequency contours, may be accurate only for threshold and near-threshold data, but requires modification to account for suprathreshold auditory cortex processing. These characterizations of the suprathreshold active areas are not directly predicted from the threshold-based findings, though they can probably be derived from two findings: (1) bandwidth typically increases with intensity, and (2) there is an overall limit to the size of the auditory cortex. Together, one could infer that at suprathreshold stimulus intensities, a given frequency could evoke activity over a large patch of auditory cortex, and conversely, a given patch of cortical tissue could be activated by a wide range of frequencies. Both of these hypotheses have been suggested in other experiments. The present study demonstrated each of these findings directly using intrinsic signal optical imaging, and the optical imaging-based conclusions were also supported by the subsequent electrophysiological findings.
A. 30.0 kHz

B. 6.5 kHz

Figure 10. Optically measured suprathreshold response areas versus electrophysiologically measured iso-frequency contours. Plotted on the optically measured suprathreshold area of activation are iso-frequency contours determined electrophysiologically: A, 30.0 kHz; B, 6.5 kHz same subject presented in Figs. 6, 7). In both A and B, the solid white iso-frequency contour is the same frequency as that used to generate the active area. Dashed white lines indicate iso-frequency contours of other characteristic frequencies. In A, the active areas encompass contour lines describing frequencies more than two octaves away (30.0 kHz area includes the 6.5 kHz contour line). Similarly, in B, the active areas encompass contour lines describing frequencies almost two octaves away (6.5 kHz area includes the 2.0 kHz contour line). Thus, the area extent of the cortical active area evoked by a suprathreshold stimulus is much greater than the area of loci with best frequencies identical to that stimulus.

Is Intrinsic Signal Optical Imaging Dependent on Sensory Modality?

There is a report of intrinsic signal optical imaging failing to reveal auditory cortex activation. In an article describing intrinsic signal imaging of somatosensory cortex, Gochin et al. (1992) reported the inability “to detect acoustic activation of rat auditory cortex.” Without a thorough description of their protocol, it is difficult to speculate why they were unable to observe activation. Unfortunately, these authors concluded that this failure might “foreshadow limitations, or at least difficulties, that may be encountered in the use of particular optical imaging methods.” We believe that the present report should remove this concern. Intrinsic signal optical imaging is based on activity-dependent intrinsic signals; wherever optical imaging has been applied to sensory cortex, the basic organization as previously determined by electrophysiological techniques has been confirmed. Moreover, in some systems, new insights were obtained by intrinsic signal optical imaging and were subsequently confirmed by electrophysiology (reviewed by Frostig, 1994).

Interestingly, early attempts at visualizing auditory cortex activity using another functional mapping technique, 2-deoxyglucose (2DG), also reported failure and proposed that either something was unique about auditory cortex, or there was a limitation to the technique (Webster et al., 1978; Hungerbuhler et al., 1981). Later work successfully mapped auditory cortex with 2DG using frequency-modulated stimuli, and suggested that previous failures were due to the use of nonoptimal stimuli (Scheich and Bonke, 1981; Gonzalez-Lima and Scheich, 1986a). It should be noted that optical imaging of intrinsic signals does not require the use of such complex stimuli, as auditory cortex activation following pure tone stimuli was visualized.

Future Directions

This initial inquiry has demonstrated the effectiveness of intrinsic signal optical imaging to reveal the large spatial extent of suprathreshold stimulus-evoked auditory cortex activation, but was by no means a definitive analysis of auditory cortex activation and intrinsic signal generation. Important next steps include determining the specific relationships between
the precise size of the areas activated and major stimulus parameters, such as frequency, intensity, and binaural interaction patterns, as well as quantifying the relationship between electrophysiological and intrinsic signal response magnitudes (Frostig et al., 1994). This information on the spatial functional organization of the auditory cortex will be extremely helpful in the investigations of the development and nature of auditory cortex plasticity, such as age-related hearing loss (Willott et al., 1993), the effects of peripheral damage or denervation (Robertson and Irvine, 1989), and learning (Gonzalez-Lima and Scheich, 1986b, Weinberger et al., 1990; Recanzone et al., 1993). The difficulty in making repeated within-subject measurements, because of either cortical trauma inherent in electrophysiological mapping or the terminal nature of the 2DG functional imaging methodology, has constrained inquiry into each of these areas. Optical imaging through a thinned skull obviates these problems while providing for numerous, rapid, high-spatial-resolution measurements of the functional organization of the auditory cortex.

Notes
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