Optical Imaging of Intrinsic Signals in Ferret Auditory Cortex: Responses to Narrowband Sound Stimuli

Huib Versnel, Jennifer E. Mossop, Thomas D. Mrsic-Flogel, Bashir Ahmed, and David R. Moore

University Laboratory of Physiology, University of Oxford, Oxford OX1 3PT, United Kingdom

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INTRODUCTION

A main theme of studies of the auditory cortex has been the mapping of various acoustic features including frequency, level, and source position. The spatial organization of sound frequency, representing place coding in the cochlea and tonotopy in the brain stem and midbrain, has been reported in auditory primary field (AI) of several mammals (e.g., macaque: Merzenich and Brugge 1973; cat: Merzenich et al. 1975; ferret: Kelly et al. 1986; see overviews in Clarey et al. 1992; Schreiner et al. 2000). Other response properties, such as level sensitivity (Heil et al. 1994; Schreiner et al. 1992; Suga 1977), frequency tuning bandwidth (Schreiner and Mendelson 1990; Versnel et al. 1995), and binaural interaction (Imig and Adrián 1977; Kelly and Judge 1994; Middlebrooks et al. 1980) have been found to be mapped roughly orthogonal to the tonotopic organization. However, the organization of these other properties appears to be less robust and to vary substantially from animal to animal. The cortical mapping of further features [e.g., frequency modulation (FM) direction selectivity] may depend on the stimulus paradigm and/or species (Nelken and Versnel 2000).

In the mapping studies referred to above, recordings were made with single microelectrodes. These have two inherent disadvantages: the number of cortical loci is limited and recordings must be made serially over a long period of time (typically more than 24 h). Consequently, the spatial resolution is limited, and data may be affected by the changing conditions of the cortex or of other aspects of the animal’s physiology. Optical imaging of intrinsic signals, introduced by Grinvald et al. (1986), is a technique that allows simultaneous recording from a relatively large cortical surface and is therefore well suited to cortical mapping. Indeed, optical imaging has proven to be successful in mapping studies, particularly on the visual and somatosensory cortex (see overview in Bonhoeffer and Grinvald 1996). The optical imaging studies of visual cortex have confirmed ideas based on electrophysiology and anatomy, such as the organization of ocular dominance and orientation preference (Blasdel and Salama 1986; Grinvald et al. 1986; Ts’o et al. 1990). Importantly, they have also provided further insights on the spatial organization of these features, including the radial arrangement of the orientation map (Bonhoeffer and Grinvald 1993).

The results in visual and somatosensory cortex motivated the application of optical imaging to the auditory cortex. Initial reports have confirmed the tonotopic organization in the primary field and bordering areas (Bakin et al. 1996; Harel et al. 2000; Hess and Scheich 1996; Spitzer et al. 2001). However, the optical signals in auditory cortex appear to be weaker than in the visual cortex, and consequently, it has been more difficult to obtain functional maps (Gochin et al. 1992; Harrison et al. 1998; Spitzer et al. 2001). Illumination wavelength is a key factor affecting auditory imaging. The wavelengths used for imaging in other sensory systems are typically 600–700 nm (orange-red light) because vascular artifacts are relatively small and well-reproducible maps can be derived (Bonhoeffer and Grinvald 1996; Frostig et al. 1990; McLoughlin and Blasdel 1998). By contrast, in auditory cortex, several studies suggest that maps were only obtained consistently when a shorter wavelength (550 nm, green light) illumination was employed (Bonham et al. 1997; Dinsel et al. 1997; Harel et al. 2000; Spitzer et al. 2001). However, apart from vascular artifacts, the drawback of green light is that the...
spatial correlation of the optical activity with neural activity is smaller and the activated area is larger (Hess et al. 2000; Malonek and Grinvald 1996). Given these limitations, it is particularly important to seek further improvements in auditory optical imaging through stimulus optimization, noise reduction and image analysis.

In this paper, we present results obtained with optical imaging of auditory cortex of the ferret using short-wavelength illumination, particularly addressing stimulus variations. In previous reports the principal stimulus used was a train of pure-tone pips. However, dynamic stimuli, such as FM tones, have been found in electrophysiological studies to be more effective in activating most AI neurons (Mendelson and Cynader 1985; Whitfield and Evans 1965). Thus we have compared optical responses to three narrowband sound stimuli: pure-tone trains, sinusoidal amplitude modulated (SAM) tones, and sinusoidal frequency modulated (SFM) tones. We have also varied sound level and repetition rate or modulation frequency (MF) of the stimuli. A primary aim was to find an effective narrowband stimulus for imaging auditory cortex.

We have also addressed the issue of noise reduction. A major part of the noise in the optical signal consists of an oscillatory signal with a frequency around 0.1 Hz (Mayhew et al. 1996). This noise component is referred to as cerebral vasomotion. Mayhew and colleagues found that vasomotion is related to arterial blood pressure. The noise can therefore be reduced by, for example, enhancing blood pressure with angiotensin (Berwick et al. 1998). This method was tested in the current study.

The ferret was considered a suitable animal for our study since AI is located on a gyrus and can be imaged over its entire extent. In addition, the electrophysiology of the ferret auditory cortex has now been described comprehensively, and various mapping studies have been performed (Kelly and Judge 1994; Kelly et al. 1986; Moore et al. 1997; Mossop et al. 1997; Nelken and Versnel 2000; Phillips et al. 1988; Shamma et al. 1993; Versnel et al. 1995).

**METHODS**

**Surgery and animal preparation**

The data presented here were collected in 18 ferrets (*Mustela putorius*), 12 females and 6 males. Most of the ferrets were older than 6 mo (≤4 yr), and three were 3- to 5-mo-old. Data from five other ferrets were discarded because of an insufficient signal-to-noise ratio. All animal procedures were approved by and performed under license from the U.K. Home Office in accordance with the Animals (Scientific Procedures) Act 1986.

In all ferrets, the main anesthetic agent during optical recording was pentobarbital sodium. The anesthetic protocol was sometimes modified in other respects because of the effect of anesthetics on the crucial hemodynamics for optical imaging (e.g., coupling of blood flow and neural activity, vasomotion). In 10 ferrets, anesthesia was induced with pentobarbital (40 mg/kg, ip), and during surgery, was maintained by continuous infusion of pentobarbital (6 mg/kg/h) in Hartmann’s solution with 5% glucose. In three of these ferrets, anesthesia was maintained during recording by continuous infusion of pentobarbital at a gradually slower flow rate. In 7 of the 10 ferrets, a neuromuscular blocker was administered (20 mg/kg gallamine, iv) at the start of the recording to aid stability, and anesthesia and paralysis were maintained with continuous infusion of pentobarbital (1.5–2 mg/kg/h) and gallamine (20 mg/kg/h). The animals were ventilated with room air and added O<sub>2</sub> and CO<sub>2</sub>. A sufficient anesthetic level was maintained, judged on the basis of slow-wave EEG. In the remaining eight ferrets, anesthesia was induced with saffan (alphaxalone/alphadalone; 2 ml/kg, ip), and during surgery, additional doses of saffan were given intravenously if necessary. During recording, anesthesia was maintained by continuous intravenous infusion of pentobarbitone at a flow rate of 2–4 mg/kg/h. The choice of anesthetic protocol is important with respect to vasomotion, which distorts the optical response and increases with decreasing blood pressure (Berwick et al. 1998). Vasomotion appeared more prominent with pentobarbitone, which is known to induce cardiovascular depression, than with saffan. In eight animals, angiotensin II (3–8 μg/kg/h) was included in the infusion fluids to reduce vasomotion by increasing the blood pressure.

For all 18 ferrets, atropine sulfate (0.2 mg/kg, sc) and dexamethasone (0.4 mg/kg, im) were administered at 12-h intervals. The expired carbon dioxide was monitored and maintained in a range of 3–5%.

The arterial oxygen saturation and the arterial blood pulsation were monitored in all experiments.

The primary AI of the ferret is located on the ectosylvian gyrus, as shown in numerous electrophysiological experiments (e.g., Kelly et al. 1986). The left AI was exposed by craniotomy and the overlying dura was incised and reflected. A stainless steel chamber (16-mm diam) was cemented around the cranial window, the dura was removed, and the chamber was filled with silicone oil and sealed with a glass plate according to procedures described by Bonhoeffer and Grinvald (1996).

The right ear canal was dissected and cleared of any debris. An earphone, custom-designed to fit the ferret’s curved ear canal (M. Ravicz, Massachusetts Institute of Technology, Boston, MA) was inserted. The earphone consisted of a radial horn tweeter (Realistic) attached to a curved metal tube with a diameter of 2.3 mm. The tip of the earphone tube was 2–3 mm from the tympanic membrane.

**Recordings**

Optical recordings were made in a purpose-built, light-proof, double-walled sound-attenuated chamber. The recording protocol was based on that described in detail by Bonhoeffer and Grinvald (1996). The core of the set-up consisted of the Imager 2001 (Optical Imaging Inc.). The cortex was illuminated by green light (wavelength, 546 nm; 50%-bandwidth, 10 nm; Coherent-Ealing Ltd.) directed through two fiber optic light guides. A video camera, mounted above the cortex and perpendicular to its surface, was used for grabbing images. The selected region of interest, covering AI, had a size of about 6 × 4 mm. To reduce artifacts due to blood vessels at the cortical surface, a macro double-lens configuration with a shallow depth of field (2 Nikkor, 50-mm SLR camera lenses mounted front-to-front) was attached to the camera and the camera was focused 400–500 μm below the cortical surface. An analog amplifier was used to enhance the resolution of the video signal by subtracting the stimulus-evoked images from a reference image. The reference image was obtained in a nonstimulus condition before each block of stimulus trials. We used VDAQ data acquisition software (Optical Imaging).

A typical experiment consisted of 32 or 48 presentations of randomly varied stimulus blocks, each having 8 or 12 different sound trials and 3 no-stimulus trials. Images were acquired in 0.5-s temporal windows (Fig. 1A). The recordings started 0.5 or 1 s before stimulus onset and lasted for 5–5.5 s, until 2–2.5 s after stimulus offset. The interstimulus interval was 14 s, sufficiently long to allow recovery of the hemodynamic signal (Bonhoeffer and Grinvald 1996). In several experiments, recordings were made of spontaneous (i.e., without experimental stimuli) optical activity. Images were then acquired in 1-s temporal windows and the duration was 31 s. These recordings allowed us to examine slow optical signals in the range of 0.03–0.5 Hz.
Stimuli

Stimulus levels were calibrated using a 3.2-mm Bruel and Kjaer microphone (4138), with the microphone probe positioned 2–3 mm from the earphone tip in a closed-field configuration.

The ferrets were stimulated at the contralateral ear. As responses of auditory cortical neurons in anesthetized preparations are typically phasic, we used periodic stimuli to evoke quasi-sustained (relative to the slow hemodynamic response) neural responses. The stimuli were narrowband: trains of tone-pips, SFM tones, or SAM tones. The tone-pip trains had equal on and off periods (Fig. 1). Repetition rate can thus be referred to as MF (e.g., 4 Hz refers to 125-ms tone-pip and 125 ms of silence). All stimuli had a total duration of 2 s, and they were cosine square gated with rise-fall times of 10 ms. In each experiment, the center frequency was varied, and in several experiments, one or two of the following parameters were also varied: the sound level, stimulus type, and MF. Modulation depths of SFM sounds were 10%, corresponding to about 0.15 octave, and modulation depths of SAM sounds were 90%. Center frequencies were in the range 1–16 kHz, sound levels were in the range 50–80 dB sound pressure level (SPL), and MF was varied from 1 to 16 Hz.

Image analysis

The initial processing of images was done with the software package ORA 2001 (Optical Imaging). The images were averaged over the number of presentations (16–64) and over four poststimulus time frames from 1.5 to 3.5 s. The choice of the time window is motivated by the time course of the stimulus-evoked optical response that shows the strongest activity between 1.5 and 3.5 s (see Fig. 1, further discussed in Results). Stimulus-related images were divided over images acquired during silence. They were scaled at the 99.5% positive pixel values. A change is reversed to have stimulus-evoked activity be reflected by positive pixel values.

Two types of maps were obtained. Single-condition maps (e.g., Fig. 3A) were obtained by dividing the images for a particular stimulus over images acquired during silence. They were scaled at the 99.5% positive pixel values. Frequency preference maps (e.g., Fig. 3B) were generated by computing, at each pixel, the frequency that evoked the largest reflectance change. A pixel was assigned a no-response when any stimulus-triggered signal was below the background noise level. This background level was estimated from the magnitude of images recorded before stimulus onset.

We derived three response parameters from the single-condition maps. First, a magnitude was computed as the average pixel value for values >50% of the scaling amplitude. The magnitude, like the pixel values, is a relative measure, and it is always greater than or equal to zero (it is zero if no pixel in the entire region of interest has a positive value). Second, a center of gravity was computed for pixels with a response >50% of the scaling amplitude. This parameter was used as an index of the location of activation. Third, the area of activation was computed for frequencies including that shown in Fig. 1, A and B (8 kHz). Figure 1C shows that the time courses of the magnitudes did not differ significantly with stimulus frequency: the onset and peak latencies were very similar (1 and 2.5 s, respectively). Note that the magnitudes before stimulus onset had significant values above zero (0.002–0.004), reflecting background reflectance changes (ventral-caudal corner in images before stimulus onset, Fig. 1A). Figure 1D shows that activation patches, as defined by the centers of gravity, shifted by about 0.5–2 mm along both dorsoventral and rostrocaudal dimensions over a course of 3 s. The early and late images, with relatively small signals (and consequently smaller signal-to-noise ratios), contributed to a large extent to these considerable shifts. Spatial shifts of images used for the functional maps (1.5–3.5 s) were <0.5 mm, except for the caudal shift of the optical response to 1 kHz. Shifts larger than 0.5 mm (but smaller than in Fig. 1D), were found in some of the other experiments, and as in Fig. 1D, only for 1-kHz patches in the caudal direction.

Background noise, as seen in curve a in Fig. 1B, was observed in most experiments. Figure 2A presents an example of such background activity, recorded during 32 s in the absence of acoustic stimuli. Typical of the noise often encountered, it revealed slow fluctuations with distinct periodic patterns (with a frequency of 0.1 Hz). The phase of this periodic noise shifted across the surface. For instance, when the reflectance was at a maximum at site c, it was at a minimum at site b (see Fig. 2A). The noise amplitudes could be greater than the amplitude of a strong stimulus-evoked signal (about 0.01, see Fig. 1B). Figure 2B demonstrates the effect of the noise on stimulus-evoked signals. Some of the traces deviated markedly from the base-
line before stimulus onset, and the fluctuation of the trace corresponding to a nonauditory site was relatively large (amplitude $>0.005$).

Berwick et al. (1998) found that slow oscillations, associated with vasomotion, are inversely related to arterial blood pressure and therefore can be reduced by a blood pressure enhancing drug, angiotensin II. Figure 2, C and D, demonstrates the effect of continuous intravenous infusion of angiotensin II. The oscillatory signals (Fig. 2C) were reduced by more than 50%, and the stimulus-evoked time courses (Fig. 2D) contained less noise. For instance, $d$ (corresponding to a nonauditory site) was almost flat and near zero. Recording of the stimulus-evoked signals (Fig. 2D) were started 10–15 min after infusion of angiotensin, when typically the effect on the optical signal was first observed.

**Tonotopicity and general results**

Figure 3 demonstrates the tonotopic organization of ferret AI. The activation patches in single-frequency maps shifted with frequency from ventral to dorsal (Fig. 3A). The activation patches ran approximately parallel to each other and perpen-
dicular to the posterior suprasylvian sulcus. The no-stimulus map indicated two typical patterns of background noise: stripes corresponding to vessel artifacts and patches probably related to the 0.1-Hz signal. Vessel artifacts were also visible in the frequency maps, in particular in the areas outside of AI (dorsal, and dorso-caudal). The frequency preference map (Fig. 3B) confirms the clear tonotopic organization. The distinct borders between the frequency regions are striking, considering no image smoothing has been applied. A comparison to the vascularization pattern (Fig. 3A) indicates that the borders do not correspond to large blood vessels. Only toward the edges of AI (indicated by the solid contours) was the BF map noisy. Figure 3C shows the center of gravity found for each single-frequency map as a function of frequency, again demonstrating a high degree of tonotopic order. The square of the linear correlation coefficient, $R^2$, and the slope are further used as tonotopicity parameters.

The similarity of maps and the consistency of tonotopic order are demonstrated in Fig. 4, where frequency preference maps and tonotopicity plots of five more ferrets are presented. In all maps (Fig. 4A), the tonotopy was evident, with the preferred frequency progressing from ventral to dorsal, even in a case where the cortex was severely swollen by edema (F9704). Tonotopic reversals that would be indicative of secondary auditory fields were not found, but slight disorders of tonotopy occurred in the ventral region in some animals (Fig. 3B, F9405; Fig. 4A, F9802). In some animals (e.g., F9755), strong optical activity was found in the pseudosylvian sulcus, located rostro-ventrally from AI, but without any systematic tonotopic organization. The size of the region showing tonotopically organized activity varied from animal to animal. For instance, F9716 had a smaller map and F9802 had a larger map. The variability of cortical size per frequency band is reflected by the variability of slopes found in the tonotopicity plots (Fig. 4B).

Figure 5 summarizes the frequency mapping data for each animal where the optical signal was above background noise. In each animal, representative maps showed a significant tonotopic order: the tonotopicity parameter $R^2$ was $>0.7$ (averaged per animal) and the grand average of $R^2$ was 0.9 (Fig. 5A). The cortical distance per octave varied considerably from animal to animal (Fig. 5B), as was shown in the frequency preference maps (Fig. 4A). For comparison purpose, we derived values from electrophysiology experiments (Mossop 1999; Versnel et al. 1995) by assessing the distances between isofrequency contours that were at least one octave apart. The cortical distance per octave found in most optical imaging experiments appeared to be in the range observed in electrophysiology mapping experiments (average value: 0.8 mm/octave).

As described earlier, we observed shifts with time of the centers of gravity (e.g., Fig. 1D). While the cortical expanse of the full tonotopic representation sometimes changed, as seen in the dorsal dimension in Fig. 1D, the tonotopic order was well preserved from one time frame to the next, and the positional change of the centers of gravity was not systematic.

**Sound level**

Most experiments were performed at fixed sound levels of 70 or 80 dB SPL, well above threshold. In five animals, the
optical signals were examined as a function of sound level. Figure 6 shows images for two frequencies (8 and 16 kHz) over a range of 30 dB, illustrating the general result in one ferret (F9802). Generally, the signal magnitude (Fig. 6B) increased or remained stable with level. Decreases of magnitude with level were not found in any experiment. The patches did not shift spatially with level to any marked extent (Fig. 6A and C). The area of activation, defined by the red and orange regions, was also similar at different levels, although, sometimes, the area increased slightly (Fig. 6A). These general findings are supported quantitatively by paired comparisons in all five animals between responses to 8 kHz (most applied frequency) at 60 and 80 dB SPL (magnitude: $t(4) = 7.0, P < 0.01$; center of gravity: both coordinates: $df = 4, P > 0.1$; area: $df = 4, P > 0.1$).

**Type of narrowband stimulus (tone-pip trains, SFM, SAM)**

In search of an effective narrowband stimulus, we compared optical responses to three types of periodic narrowband tone stimuli: tone-pip trains, SFM tones, and SAM tones. Figure 7 shows series of single-frequency maps for the three stimuli. The maps appeared to be very similar. For instance, the 1- and 16-kHz maps for tone-pip trains and SFM tones were almost identical. The centers of gravity confirm the spatial similarity (Fig. 7C). As would be expected, differences appeared larger when the response magnitude was relatively small (and the signal-to-noise ratio low), which is the case for the 2.8-kHz maps. The spatial spread of activity was larger for tone-pip trains than for SFM tones in the 2.8- and 8-kHz maps. However, this reflected only a small overall trend, and the activation area did not vary significantly with stimulus type (paired $t$-tests at 5 frequency-intensity combinations; $P > 0.05$; $df = 3–5$). The magnitudes clearly differed: the largest signals were found for tone-pip trains and the smallest for SAM sounds (Fig. 7B). This trend was similar for all carrier frequencies.

The comparison between the stimulus types was made in four animals. Figure 8 shows the animal-averaged results. The magnitudes to tone-pips and SFM tones were larger than for SAM tones, and the tone pips and SFM tones produced very similar response magnitudes (Fig. 8A). At 80 dB SPL, differences were statistically significant between responses to tone-pip trains and those to SAM tones (paired $t$-test, $t(3) = 3.7; P < 0.05$); at 60 dB, SFM tones produced significantly larger responses than SAM tones [$t(3) = 5.1, P < 0.05$]. The centers of gravity for the three stimulus types were generally close to each other (within 0.4 mm). Figure 8B demonstrates the lack of systematic differences at four stimulus conditions. Statistical
comparisons confirmed the similarity at all of eight frequency-intensity combinations (paired t-tests; \( P > 0.1; \) \( df = 3–5 \)).

Additional experiments were performed in which only the two most effective stimuli, tone-pip trains and SFM tones, were compared. We examined whether stimulus preference, measured by the ratio of response magnitudes, depended on the stimulus parameters MF, sound level, and center frequency. Multiple regression analysis, with the logarithmic ratio of magnitudes as the dependent variable, showed a significant correlation with sound level (\( P < 0.001, n = 68; 6 \) ferrets, 33 combinations of stimulus parameters), but none with MF or carrier frequency (\( P > 0.1 \)). The preference for tone-pip trains over SFM tones increased with sound level. In effect, at 60 dB, SFM tones were preferred to tone pips, and at 80 dB, vice versa.

**Modulation frequency**

In six ferrets, the optical responses were examined as a function of modulation frequency (or repetition rate) of tone-pip trains or SFM tones in a range of 1–16 Hz (7 recording sessions) or 2–8 Hz (6 sessions). Figure 9 presents examples of single-frequency maps in one ferret (F9837). For both the low and the high center frequency (1 and 8 kHz), the maps did not vary substantially with MF (Fig. 9A), in particular, the locations were robust (Fig. 9C). The spread of activation varied somewhat, e.g., the maps at 16 Hz had smaller patches (the orange and red regions); such variations were generally found, but they were insignificant [analysis of variance (ANOVA), \( df = 4, P > 0.1 \)]. The largest activity in these examples was found at 4 Hz (Fig. 9B).

Figure 10A shows the magnitude as a function of MF for several ferrets. For tone-pip trains (top), all magnitude curves (modulation transfer functions) had a band-pass character and a best MF (2–4 Hz). The modulation transfer functions for SFM sounds (bottom) were broader than for tones, but in most cases a best MF could be defined (2–8 Hz). The animal-averaged curves of center of gravity (Fig. 10B) confirm the observation of Fig. 9 that patches did not systematically shift with MF. Statistical tests did not reveal significant effects on either center-of-gravity coordinate (linear regressions of individual or averaged data: \( P > 0.1, n = 5 \); paired t-test comparisons between animal-averaged data at 2, 4, or 8 Hz: \( P > 0.1, df = 3–4 \)).

Figure 11 presents the mean best MFs obtained in each ferret. It confirms that, generally, the modulation frequency evoking the strongest optical response was in the range 2–4 Hz. Occasionally, and only for SFM sounds, a best MF of 8 Hz was recorded. The average best MF was significantly higher for SFM sounds than for tone-pip trains (t-test on paired data: \( t(3) = 3.2, P < 0.05 \)). Multiple regression analysis showed that the best MF did not depend on sound level or carrier frequency (\( P > 0.1, n = 30, 6 \) ferrets, 17 combinations of stimulus parameters).

**DISCUSSION**

We demonstrated that consistent tonotopic maps can be obtained by optical recordings of AI in the anesthetized adult
ferret. These maps are robust with respect to different stimulus parameters, including modulation frequency and sound intensity, and they are in good agreement with the electrophysiological literature. Provided background fluctuations are small, and appropriate stimuli are used, a frequency map of AI can be acquired in each ferret. We found that appropriate narrowband stimuli to produce intrinsic signals in ferret auditory cortex are a tone-pip train or SFM sound with a modulation frequency of 2–4 Hz at a high sound level. The 0.1-Hz vasomotion activity that is the principal form of background fluctuations can be reduced by administration of angiotensin.

**Short-wavelength imaging**

Wavelengths of light in the range of 620 or 700 nm are the standard used in intrinsic optical imaging of visual cortex (Bonhoeffer and Grinvald 1996); these did not yield consistent results for auditory cortex in our set-up. Instead, green light (546 nm) illumination was used because this significantly improved both signal strength and signal-to-noise ratio. Several other laboratories applying intrinsic-signal imaging of auditory cortex have used green light illumination (Bonham et al. 1997; Dinse et al. 1997; Harel et al. 2000; Spitzer et al. 2001), and two auditory laboratories have successfully used red light (Bakin et al. 1996; Hess and Scheich 1996). The set-ups of these two groups of labs additionally differ in species (ferret, cat, and chinchilla vs. rat, guinea pig, and gerbil) and in the method of craniotomy and cortical exposure (excised dura vs. thinned skull and intact dura). It is unclear whether the differences in these species and methodologies explain the differences in the success of red light imaging.

The underlying sources of the optical signal vary with wavelength (Bonhoeffer and Grinvald 1996; Frostig et al. 1990; Malonek et al. 1997). Specifically, it is thought that deoxyhemoglobin (HbR) changes significantly contribute to the red light reflectance changes, and blood volume changes to the green light reflectance changes. The long response latency we found (Fig. 1), which was about 1 s longer than generally found in red light optical imaging, corroborates this view, given that the HbR component has an early onset.

The area of optical activity exceeds the area of neural spiking activity, an effect thought to be due to subthreshold activity (Gilbert et al. 1996; Toth et al. 1996). The area of green-light reflectance changes is even larger than that of the red-light signal (Hess et al. 2000; Malonek and Grinvald 1996). Indeed, the single-condition maps in our experiments (Figs. 3A, 6, 7, and 9) showed a large spread of activity (e.g., several octaves along the tonotopic axis), exceeding the bandwidths of multiple-unit clusters (mostly <2 octaves, Versnel et al. 1995). However, maps in which different stimulus conditions are compared, such as ocular dominance and orientation prefer-
ence maps in visual cortex, were found to be similar for short and long wavelengths (Frostig et al. 1990; own data: we compared orientation maps in cat visual cortex in our lab for wavelengths of 546, 600, 620, and 700 nm). Frequency preference maps as presented in this paper (Figs. 3B and 4) might therefore not depend critically on illumination wavelength.

Reflectance noise

Vasomotion causes a 0.1-Hz background oscillation of the reflectance, which can be a major source of noise in optical imaging (Mayhew et al. 1996). A 0.1-Hz oscillation has occasionally been reported in previous studies (e.g., Frostig et al. 1990; Harel et al. 2000; Spitzer et al. 2001). We found in our experiments that the vasomotion amplitude could vary in the course of an experiment and could be even larger than the response magnitude. According to J.E.W. Mayhew and J. Berwick (personal communication: they recorded vasomotion in rat barrel cortex; Berwick et al. 1998), vasomotion increases with decreasing blood pressure and thus can be counteracted by a blood-pressure enhancing drug, such as angiotensin. Indeed, we found that intravenous infusion of angiotensin II does lead to a reduction of 0.1-Hz oscillations. It should be noted that blood pressure should not be too high because it can lead to strong vascular pulsations, causing large vessel artifacts.

Tonotopicity

The frequency maps showed a consistent order, with frequencies increasing from ventral to dorsal, in good agreement with the tonotopic organization found with electrophysiological recordings in AI (Kelly et al. 1986; Phillips et al. 1988; Shamma et al. 1993; Versnel et al. 1995). The isofrequency contours derived from electrophysiological recordings run roughly orthogonal to the posterior ectosylvian sulcus. The frequency regions and the borders between them of the optically recorded frequency maps confirm this orientation.
A comparison of cortical distance per octave (Fig. 5B) also showed that optical data were similar to electrophysiological data (Mossop 1999; Versnel et al. 1995). On the average, the distance per octave found in optical data were shorter. This difference may be explained by a skewness in the spatial distribution of optical activity toward the center of AI (often observed in the 16-kHz images of Fig. 6). Consequently, for each frequency, the centers of gravity are pulled toward the center of AI, causing a smaller distance per octave. However, sampling errors cannot be fully excluded. It is impossible to distinguish the well-known interanimal variability in the tonotopicity of AI (Merzenich et al. 1975) from the largely unknown interanimal variability of cortical hemodynamics and microvasculature.

While the tonotopic organization revealed by microelectrode and optical recordings is similar in several aspects, neural best frequencies (BFs) are typically obtained at low sound levels, 0–20 dB above the response threshold. In ferrets, that would be 20–60 dB SPL in the range of 1–16 kHz (Kelly et al. 1986; Kowalski et al. 1995; Phillips et al. 1988). In contrast, the core
of the optical data were obtained at higher sound levels, 60–80 dB SPL. Because a substantial percentage of AI units (around 50%) show reduced response rates at high levels (cat: Schreiner et al. 1992; Heil et al. 1994; Phillips et al. 1994; ferret: Kowalski et al. 1995), the tonotopic order deteriorates with increasing sound level (Phillips et al. 1994). Thus the optical maps at high and low levels should not show identical tonotopic characteristics. The finding that they were very similar

FIG. 8. Magnitudes (A) and centers of gravity (B) for the 3 stimulus types, averaged over 4 ferrets (including the one shown in Fig. 7). The histograms show the animal-averaged values with SDs. In each of the 4 ferrets, magnitudes have been averaged across center frequency.

FIG. 9. A: frequency maps are compared for various modulation frequencies (1–16 Hz) at 2 center frequencies (1 and 8 kHz). Stimuli: tone pip trains at 80 dB SPL. Number of trials: 32. For further details, see legend of Fig. 3A. B: magnitudes corresponding to maps of A. C: centers of gravity corresponding to maps of A.
may be explained by two aspects. First, nonmonotonic responses in the ferret occur at higher levels than in the cat, the species used in the study of Phillips et al. (1994). Comparing the data of Kowalski et al. (1995) with those of Schreiner et al. (1992) suggests that best tone levels are 20–30 dB higher in the ferret than in the cat. Thus reduction of response rates in ferret AI at 80 dB SPL might not be prominent and may not have a significant effect on the tonotopic order. Second, optical activity reflects the metabolic demand of both excitatory spiking activity and of excitatory and inhibitory subthreshold activity (Gilbert et al. 1996; Toth et al. 1996). At high levels, subthreshold activity of cells with low best levels might cancel the reduction of their neural spiking activity.

**Secondary auditory fields**

The good agreement between the tonotopic organization found in our imaging data and in the electrophysiological literature on AI suggests that the maps we found mostly or exclusively represent the primary field. Activity from secondary fields might have been included in border regions of AI. Only a few secondary auditory fields in ferret have been described in literature: the anterior auditory field (AAF; Kowalski et al. 1995) and the ventroanterior and ventroposterior areas (VA and VP; Wallace and Bajwa 1991; Wallace et al. 1997). Dorsally, the 16-kHz activation patches described here might have included activity from AAF, and ventrally, the 1-kHz patches might have included activity from VP. Evidence for a tonotopic maps corresponding to secondary fields was not found. This can be attributed to a position down a sulcus (AAF) or simply outside of the imaged area (VP), or in cases where a substantial region ventrally from AI was imaged, a relatively weak hemodynamic activity to narrowband sounds (VP).

Interestingly, a region down the pseudo-sylvian sulcus, anterior to AI and ventral to AAF, showed activity of similar strength to that in AI. This region could be part of area VA or it could represent another area not previously described. Like AAF, it cannot be studied well with optical imaging because of its position in a sulcus.

**Sound parameters**

We studied the dependence of optical responses on the type of narrowband stimulus, sound level, and MF. The results contained two major findings. First, the tonotopic maps did not vary markedly with variation of any of the three sound parameters. Second, the magnitude of the optical signals did vary with each of these parameters. The intrinsic signal increased with level; it was maximal for an MF of 2–4 Hz, and it was larger for tone-pip trains and SFM tones than for SAM tones. Accordingly, an optimal stimulus to obtain frequency maps in the anesthetized ferret using optical imaging would be a tone-pip train or an SFM tone with a 2–4 Hz MF and a level of 70–80 dB SPL. Bakin et al. (1996) and Hess and Scheich (1996), who reported successful imaging of auditory cortex...
under red light illumination, had used 2–4 Hz tone-pip trains. These were optimal stimuli, assuming our results apply to species used in those studies (rat, guinea pig, and gerbil). Harrison et al. (1998) and Spitzer et al. (2001) reported relatively low success rates for imaging the tonotopy of auditory cortex. Our MF results indicate that these low success rates might have been caused, at least partly, by the use of tone-pip trains with suboptimal MFs (Harrison et al.: 10 Hz; Spitzer et al.: 1.25 Hz).

Tone-pip trains are quasi-rectangular AM sounds and differ from SAM tones only in the shape of the temporal envelope. A rather straightforward explanation may be offered for the greater effectiveness of the tone-pip trains. The trains have temporally steeper rise flanks than SAM stimuli and this will lead to stronger neural responses in AI (Heil 1997). Consistent with this idea, Eggermont (1994) found that exponential AM stimuli were more effective in driving AI units than were SAM stimuli. The SFM stimuli differ from either of the other two stimuli in that they have constant amplitude and a time varying spectrum. It is therefore not as straightforward to predict the effectiveness of SFM tones. Whitfield and Evans (1965) found AI neurons responding to SFM while they were unresponsive to pure tones. They also reported wider frequency tuning to SFM than to pure tones. Units can also respond selectively to SFM while they were unresponsive to SAM and SFM stimuli in cat AI, in contrast to our imaging data. This discrepancy could be because Eggermont recorded only from a restricted region of AI. Second, his SFM and SAM stimuli were always centered around a unit’s best frequency, whereas responses to both BF and off-BF tones will contribute to the optical signal. Third, a contribution of subthreshold activity might be larger for SFM responses; lateral inhibition is thought to play a role in FM responses (Shamma et al. 1993).

The range of best MFs reported for AI neural responses to SAM and SFM tones in anesthetized mammals is 4–20 Hz (cat: Eggermont 1994; Schreiner and Urbas 1988; rat: Gaese and Ostwald 1995). The range of best ripple velocities found in ferret AI for broadband sound stimuli with rippled spectra (8–12 Hz; Kowalski et al. 1996) indicates that the ferret has a similar range of best MFs as the cat and rat. The best MFs we found were on the low side of the electrophysiological range. In particular, the modulation transfer functions we found for SFM agreed rather well with the average-rate transfer function shown by Eggermont (1994) in terms of shape and best MF.

Implications

This study has demonstrated the feasibility of optical imaging for deriving tonotopic maps in ferret auditory cortex. The tonotopic organization might not be very interesting in itself, because it has been well established in the primary cortical fields of several mammals. However, a complete map of tonotopicity in AI could be used as a template for electrode recordings or anatomical tracer injections, for example, where there is a need to identify certain frequency regions. Optical imaging can also be usefully applied to studies of the development and plasticity of tonotopic organization, where the need to obtain comprehensive data quickly is imperative. This approach has been effective in studies of visual cortex development (e.g., Chapman et al. 1996) and plasticity (e.g., Sengpiel et al. 1999). Accordingly, we have used optical imaging to study development of the tonotopic organization in ferrets (Mrsic-Flogel et al. 1999).

Optical imaging relates to other hemodynamic based techniques, such as functional magnetic resonance imaging (fMRI) (Hess et al. 2000). For instance, a good correspondence between fMRI studies (Hall et al. 2001) and our study is found in the monotonic level-dependence of the response magnitude. Thus our results regarding the stimulus parameters such as stimulus type and repetition rates may be relevant for stimulus paradigm choices in fMRI.

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Present addresses: H. Versnel, Dept. of Otorhinolaryngology, University Medical Centre Utrecht, P.O. Box 85500, 3508 GA Utrecht, The Netherlands; T. D. Mrsic-Flogel, Max Planck Institute of Neurobiology, Am Klopferspitz 18A, 82152 Martinsried, Germany.

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