Development of contralateral and ipsilateral frequency representations in ferret primary auditory cortex

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Abstract
Little is known about the maturation of functional maps in the primary auditory cortex (A1) after the onset of sensory experience. We used intrinsic signal imaging to examine the development of the tonotopic organization of ferret A1 with respect to contralateral and ipsilateral tone stimulation. Sound-evoked responses were recorded as early as postnatal day (P) 33, a few days after hearing onset. From P36 onwards, pure tone stimuli evoked restricted, tonotopically organized patches of activity. There was an age-dependent increase in the cortical area representing each octave, with a disproportionate expansion of cortical territory representing frequencies > 4 kHz after P60. Similar tonotopic maps were observed following stimulation of the contralateral and ipsilateral ears. During the first few weeks following hearing onset, no differences were found in the area of cortical activation or in the magnitude of the optical responses evoked by stimulation of each ear. In older animals, however, contralateral stimuli evoked stronger responses and activated a larger A1 area than ipsilateral stimuli. Our findings indicate that neither the tonotopic organization nor the representation of inputs from each ear reach maturity until approximately 1 month after hearing onset. These results have important implications for cortical signal processing in juvenile animals.

Introduction
A common feature of primary auditory cortex (A1) in adult animals is the topographic layout of neurons according to their preference for sound frequency (for review, see Clarey et al., 1992). The great majority of A1 neurons receive inputs from both ears (Semple & Kittzes, 1993; Kelly & Judge, 1994; Zhang et al., 2004). However, inputs from the contralateral ear dominate, resulting in excitatory responses that are more prevalent, stronger and more sensitive than those evoked by stimulation of the ipsilateral ear (Phillips & Irvine, 1983; Kelly & Sally, 1988; Kelly & Judge, 1994; Irvine et al., 1996; Rutkowski et al., 2000; Zhang et al., 2004; Mrsic-Flogel et al., 2005). Although often ineffective by itself, ipsilateral stimulation can inhibit or enhance contralateral responses, indicating that the sensitivity of A1 neurons to sound-source location depends on interactions between the two ears.

Very little is known about how the inputs to and functional organization of A1 develop. Although the tonotopic organization of auditory structures is established prior to the onset of sensory experience, not all frequencies are represented equally at the earliest ages examined (Aitkin & Moore, 1975; Romand, 1983; Brugge et al., 1988; Romand & Ehret, 1990; Webster & Martin, 1991; Ehret & Romand, 1994). Subsequent emergence of adult-like tonotopy appears to reflect a combination of cochlear maturation (Romand, 1997) and experience-dependent changes in auditory circuits (Sanes & Constantine-Paton, 1985; Poon & Chen, 1992; Stanton & Harrison, 1996; Zhang et al., 2001). Moreover, the frequency tuning of cortical neurons and their temporal and spatial response properties emerge gradually during postnatal development (Brugge et al., 1988; Eggermont, 1991, 1996; Zhang et al., 2001; Chang & Merzenich, 2003; Mrsic-Flogel et al., 2003; Bonham et al., 2004; Pienkowski & Harrison, 2005). How developmental changes in the A1 frequency representation relate to the maturation of other response properties, including aural preference, remains to be established.

Previous developmental mapping studies in mammalian A1 have relied on microelectrode recordings (Brugge et al., 1988; Blatchley & Brugge, 1990; Eggermont, 1991, 1996; Zhang et al., 2001; Chang & Merzenich, 2003; Bonham et al., 2004; Pienkowski & Harrison, 2005). This approach allows precise characterization of neuronal response properties, but it may lead to under-sampling of the area under scrutiny in young animals. By contrast, the technique of optical imaging based on intrinsic signals (Grinvald et al., 1986) allows mapping of multiple stimulus parameters with reasonable spatial resolution (100 μm) over a large cortical area (reviewed in Bonhoeffer & Grinvald, 1996). Intrinsic imaging has now been applied to various sensory areas and is being used increasingly to study the development of functional maps in the visual cortex (e.g. Chapman et al., 1996; Crair et al., 1998; Sengpiel et al., 1999; Coppola & White, 2004). Moreover, this method has been used successfully in a number of species to investigate the tonotopic organization of the mature auditory cortex (Bakin et al., 1996; Hess & Scheich, 1996; Dinse et al., 1997; Harrison et al., 1998; Harel et al., 2000; Tsytsoev & Tanaka, 2002; Versnel et al., 2002; Nelken et al., 2004; Ojima et al., 2005). The robustness and reproducibility of the optical maps and, in most cases, the similarity with the frequency representations derived from electrophysiological recordings (e.g. Versnel et al., 2002; Nelken et al., 2004) indicate that this technique should also be suitable for
studying the development of auditory cortex, where there is a need to obtain comprehensive data quickly. In the present study, we have used intrinsic optical imaging to examine the maturation of the tonotopic organization and aural preferences in A1 of the ferret.

Materials and methods

We collected data from 26 ferrets (Mustela putorius) of both sexes at different ages, comprising postnatal day (P) 32–36 (n = 3), P40–42 (n = 4), P49–51 (n = 3), P62–75 (n = 5) and adult P > 100 (n = 11). All animal procedures were approved by and performed under license from the UK Home Office in accordance with the Animals (Scientific Procedures) Act, 1986. The methods and materials used are essentially the same as those described in detail for adult ferrets in Versnel et al. (2002).

Surgery and animal preparation

Anaesthesia was induced (2 mL/kg, i.p.) and maintained during surgery with alphaxalone/alphadolone (Saffan; Schering-Plough Animal Health, Welwyn Garden City). During recordings, the anaesthetic regime was switched to a continuous i.v. infusion of sodium pentobarbitone at a flow rate of 2–3 mg · kg⁻¹ · h⁻¹. Atropine sulphate (0.2 mg/kg; C-Vet Veterinary Products, Leyland, UK) and dexamethasone (0.4 mg/kg; Dexamreson; Intervet UK, Milton Keynes, UK) were administered subcutaneously at 6-h intervals. In some of the adult animals, angiotensin II (3–5 mg/kg/h; Sigma, Poole, UK) was included in the infusion fluids in order to reduce vasomotion (intrinsic haemodynamic signal) by raising blood pressure. A tracheal cannula was implanted for artificial ventilation, which was provided at a rate and volume in order to maintain the end tidal CO₂ concentration in the range of 3.4–4.4%. The arterial oxygen saturation, electrocardiogram, end tidal CO₂ and the animal’s core temperature were monitored throughout the experiment.

The animals were placed in a stereotaxic apparatus. The primary auditory cortex was exposed by deflecting the temporal muscle, followed by craniotomy and durotomy. A stainless steel chamber (16 mm diameter) was cemented around the cranial window, filled with silicone oil and sealed with a glass plate, according to procedures described in detail by Bonhoeffer & Grinvald (1996).

Custom-designed earphones (M. Ravicz; M.I.T., Boston, MA, USA) were inserted into the dissected and cleaned ear canals. The earphone consisted of a radial horn tweeter (Realistic®) attached to a curved metal tube with an outer diameter of 3.2 mm. This was fitted and sealed into the ferret’s curved ear canal at a distance of about 2–4 mm from the tympanic membrane. Before each experiment the stimulus output was calibrated using an 1/8th inch microphone (type 4138, Bruel and Kjaer, Naerum, Denmark), with the microphone probe positioned 2–4 mm from the earphone tip in a closed-field configuration.

Optical imaging

Recordings were made in a purpose-built, light-proof, double-walled sound-attenuated chamber. The core of the setup consisted of the Imager 2001VSD+ (Optical Imaging, Mountainside, NJ, USA). The cortex was illuminated through the chamber with red (λL = 620 nm) or green light (λG = 546 nm) through two fibre optic light guides. A video camera (CS8310C, Tokyo Electronic Industries, Tokyo, Japan), mounted above the cortex and perpendicular to its surface, was used for grabbing images. In order to reduce artefacts due to blood vessels at the cortical surface, a macro double-lens configuration with a shallow depth of field (2 × 50 mm SLR camera lenses mounted front-to-front; Nikon, Tokyo, Japan), which was focused 400–500 μm below the cortical surface. An analogue amplifier was used to enhance the resolution of the video signal by subtracting the stimulus-evoked images from a reference image that was obtained in a non-stimulus condition before each block of stimulus trials. We used VDAQ software (Optical Imaging) for data acquisition.

A typical test consisted of 32–64 quasi-random presentations of eight or 12 different stimuli, and three no-stimulus trials. Images were acquired in 500-ms frames, starting 1 s before stimulus onset and lasting for 5.5 s, until 2.5 s after stimulus offset. The interstimulus interval was 14 s, allowing sufficient time for recovery of the haemodynamic signal.

Stimuli

Stimuli were delivered monaurally to both ears. Tones typically evoke phasic responses in A1 neurons of barbiturate-anaesthetized preparations. We therefore used periodic stimuli to evoke quasi-sustained

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Fig. 1. Comparison of intrinsic responses under different illumination conditions. (A) Surface view of the imaged area. (B and C) Reflectance changes in response to 16 kHz tone pips acquired with either 620 (B) or 546 (C) nm illumination. The spatial extent of the activation is similar between the two conditions.
neural responses (relative to the slower haemodynamic response). Tone pip trains with equal on and off periods and a total duration of 2 s were used. In adult ferrets, the repetition rate was either 2 Hz (i.e. each set of stimuli comprised four 250-ms tone pips, which were cosine square gated with rise–fall times of 10 ms and separated by 250 ms of silence) or 4 Hz (eight 125-ms tone pips, with an interpip interval of 125 ms). Because the optical responses obtained at these two repetition rates were essentially the same (see Versnel et al., 2002), we used a single rate of 2 Hz for the juveniles. This value was chosen because it has been reported that best modulation frequencies of cortical neurons are lower in young animals than in adults (Eggermont, 1996). Tone frequencies were varied from 1 to 16 kHz at sound levels of 40–80 dB SPL. Some juveniles were stimulated with sound levels of 90–100 dB SPL. It should be noted that previous optical imaging studies have shown that the tonotopic organization of adult ferret auditory cortex does not change with sound level (Versnel et al., 2002; Nelken et al., 2004).

Image analysis

The initial processing of images was carried out with the software package ORA 2001 (Optical Imaging). Single-condition maps were obtained in the following way. Because the optical response in the juvenile animals had a latency of > 1 s, the images were first averaged over post-stimulus time frames of 1.5–4 s, and the first two frames of the recording sequence (the 1-s period before stimulus onset) were subtracted in order to remove any non-specific haemodynamic artefacts that may have been present before tone stimulation. The resulting images were then divided by the average of the ‘blank’ images acquired during no-stimulus trials, and these in turn were averaged over the number of presentations in the particular test. The pixel values therefore indicate the relative change in reflectance (ΔR/R). Preferred-frequency maps were generated by assigning to each pixel (cortical location) the frequency that evoked the strongest decrease in reflectance. For assignment to a pixel, the preferred frequency had to produce a significant response (> 25% of the maximum change in reflectance used in the single-condition maps).

Cortical areas outside the suprasylvian sulcus, which are believed from electrophysiological studies not to include auditory fields, were excluded from the functional maps for quantification of the images. Where clearly apparent, movement artefacts from surface blood vessels were excluded from the images. The single-condition maps were then scaled at 95% of the maximum reflectance in order to clip the signal maxima frequently produced by blood vessel artefacts.

We derived three parameters for quantitative assessment of single-condition maps. First, the magnitude of the reflectance changes was computed as the average value for the pixels where a response of > 50% of the maximum change in reflectance was observed. Second, response area was calculated by summing all the pixels with responses of > 75% of the maximum reflectance change (i.e. the most responsive 25% pixels). We chose this criterion value because we were interested in measuring the area of the ‘peak’ responses, so that comparisons

![Image of functional representation of pure tones in A1.](image-url)
could be made between the area of activation at different ages and following stimulation of each ear. Finally, the centroid (centre of mass) of each patch was computed from all pixels with a response of > 50% of the maximum. The location of the centroid was reduced to one coordinate by minimizing the correlation of the second coordinate with the stimulus frequency by rotation. This parameter was useful for maps with a single activation patch, as a positional index for the activation. The square of the correlation coefficient ($R^2$) for the regression of the centroid location with the logarithm of the stimulus frequency (thus expressed in octaves) was used as a measure of tonotopic order: high $R^2$ values indicate a systematic shift in preferred frequency with cortical location, while values near 0 indicate an absence of tonotopy. The slope (mm/octave) of this regression, referred to as the cortical magnification factor (CMF), gives an indication of the amount of cortical territory representing an octave. Local CMF (mm/octave) was also calculated between centroids of neighbouring patches in order to determine whether distances between the representation of adjacent sound frequencies change with age. These measures were implemented for comparisons of maps of different stimulus conditions (tone frequency, intensity and ear of stimulation) in juvenile and adult animals.

**Electrophysiology**

Recordings were made extracellularly from neurons in A1 from one juvenile and one adult ferret using tungsten microelectrodes under the same anaesthesia conditions and the same set of stimuli (2-s trains of 250 ms tones, 40–80 dB SPL) used for optical imaging. Data acquisition and stimulus generation were controlled using TDT System II and BrainWare (Tucker-Davis Technologies, Alachua, FL, USA). Each penetration site was assigned a preferred or best frequency based on the tone frequency that elicited the greatest number of spikes. Data were analysed blindly without knowledge of the location of each recording site. The map of penetration sites and

![Diagram](image)

**Fig. 3.** Comparison of optical and multiunit responses. (A) A map of ferret A1 (F9906, aged P51) showing the relationship between the preferred frequencies found with the imaging and electrophysiological methods. The colours indicate the most effective tone frequency from the imaging experiments. Each electrode recording site (circle) is labelled with the tone frequency that elicited the strongest firing rate. Crosses indicate non-responsive sites. Raster plots on the right show the responses to the same stimuli as those used for intrinsic imaging (contralateral 1, 4, 8 and 16 kHz tone trains at 80 dB SPL). The preferred frequency (arrows) and stimulus duration (grey bar) are indicated. (B) Histogram comparing the preferred sound frequency for the optical and electrophysiological in two animals (F9906, F9914), expressed as the difference in octaves.

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their frequency preference was then superimposed onto the preferred-frequency map obtained by intrinsic optical imaging.

Results
We used intrinsic imaging under green illumination ($\lambda = 546$ nm) to map A1 in 26 ferrets at different postnatal ages (P32–adult). Previous optical imaging (Versnel et al., 2002; Nelken et al., 2004) and electrophysiological mapping (Kelly et al., 1986; Phillips et al., 1988; Bizley et al., 2005) studies in this species have established the location of A1 in adult ferrets. We therefore sampled an equivalent area of the middle ectosylvian gyrus in the juvenile animals. Ferrets provide a robust surgical preparation at the age when they start responding to airborne sound (approximately 1 month after birth, Moore & Hine, 1992). A1 is located on the surface of the ectosylvian gyrus throughout postnatal development and therefore accessible for study with intrinsic optical imaging. We found that auditory stimulation evoked changes of the reflectance signal in A1 of but one of the youngest animals. We did not detect any activity consistently evoked in cortical regions outside the suprasylvian sulcus.

Comparison of green and red light imaging
Most (4/5) intrinsic recordings attempted under longer wavelength illumination (red light, $\lambda = 620$ nm) did not show significant stimulus-related reflectance changes over A1. In the other experiment,

Fig. 4. Development of functional tonotopy. (A) Age in postnatal days (P) is indicated to the left of surface views of the imaged area. Each subsequent column illustrates single-frequency maps derived from contralateral 1, 4, 8 and 16 kHz tone stimulation at 80–90 dB SPL, and from the no-stimulus condition, as indicated. (B) Plot of patch centroids vs. stimulus frequency, with fitted regression lines. Cortical magnification factor values (slope in mm/octave) and $R^2$ values are indicated.
however, a similar spatial activation pattern was observed with red and green light (Fig. 1). In both cases, a blurred and relatively broad area of activation was apparent following stimulation with 16 kHz tones. The remainder of this paper is therefore concerned only with intrinsic optical signals obtained using green light (λ = 546 nm).

**Tonotopic organization of adult A1**

Figure 2 shows images of the left A1 of an adult ferret (F9837) following stimulation with four tone frequencies (1, 4, 8 and 16 kHz at 80 dB SPL), demonstrating an ordered, latero-medial progression of activation patches (from low to high frequency) running roughly orthogonal to the caudal edge of the ectosylvian gyrus (Fig. 2B and C). A similar tonotopic arrangement was found in each of the adult ferrets (see Versnel et al., 2002), and subsequently confirmed using a different approach for intrinsic optical imaging by Nelken et al. (2004). The data obtained from the individual frequency maps were combined to produce a map of preferred sound frequency (Fig. 2C), with each pixel colour-coded with the frequency that produced the greatest reflectance change. As a measure of tonotopy (Fig. 2D), we plotted the centroids (denoted with a white cross in Fig. 2B) for each single-frequency map as a function of log stimulus frequency. The large $R^2$ value of the regression fitted to this plot indicates high tonotopic order, whereas the slope of the fit estimates the CMF, defined as the amount of cortical space spanning an octave, which in this example was 0.51 mm/octave.

**Comparison of intrinsic and multiunit responses**

At the end of two of the imaging experiments (F9906, P51; F9914, P139), microelectrode penetrations were used to assess the spatial correspondence between the frequency preferences estimated from multiunit spike counts and from the optical responses. This is shown for case F9906, aged P51, in Fig. 3A. Despite the patchy nature of the frequency map, possibly due to the weaker responses recorded in juvenile animals, we usually found that the tone frequency (at 80 dB SPL) that evoked the highest neuronal firing rate was also the frequency that evoked the greatest reflectance change in the corresponding regions of the optical map. This correspondence was observed for 68% (15/22) of the responsive sites in the two animals (Fig. 3B). The correlation between the tone frequency driving the strongest multiunit and optical responses was significant: $R^2 = 0.66$ in ferret F9914 ($n = 15; P < 0.001$) and $R^2 = 1$ in ferret F9906 ($n = 7; P < 0.001$).

**Development of tonotopy**

A set of frequency maps recorded in six ferrets of different ages is shown in Fig. 4. The first column shows surface images of the cortex. Each of the subsequent columns illustrates single-frequency maps derived from contralateral stimulation with 1, 4, 8 and 16 kHz tones at 80–90 dB SPL, together with the no-stimulus condition.

The earliest age at which tone-evoked signals were observed in A1 was P33 (F9844; Fig. 4A, first row). Only 4, 8 and 16 kHz (at 90 dB SPL) tone stimulation elicited responses, with an extensive overlap between the 8 and 16 kHz activation areas. The 4 kHz stimulus activated a smaller area of cortex than 8 or 16 kHz tones. In the other animal of a similar age (P32, F9836; data not shown), tone stimuli of up to 100 dB SPL failed to evoke any significant responses.

At all subsequent ages, we found a consistent latero-medial shift of activation patches with increasing sound frequency, resembling the tonotopic arrangement in the adult animals (Fig. 4A). Despite the larger vascular artefacts in P36 and P41 animals, the same basic tonotopic organization was observed. In most examples, the tone-evoked activations in older animals (P > 60) appeared more elongated, resembling stripes rather than patches, as seen in younger animals (Fig. 4).

CMFs (regression slopes of the centroid plots in Fig. 4B) from all animals were plotted as a function of age (Fig. 5A). Despite the variability in CMF values, there was a gradual age-dependent increase in the amount of cortical territory representing an octave (Fig. 5A; ANOVA, $P < 0.001$). In order to assess whether an equivalent age-dependent expansion in the cortical representation occurred for all four sound frequencies, CMFs were calculated from centroid coordinates of neighbouring patches (Fig. 5B). From P36...
onwards, the largest CMFs were found between the 4 and 8 kHz patches (comparison between frequency, ANOVA, \( P = 0.02 \)), indicating that more cortical territory is devoted to the representation of those and intermediate frequencies. The 1–4 kHz CMF values initially increased and then declined by adulthood, whereas 8–16 kHz CMFs increased dramatically only after the second postnatal month (t-test, \( P < 0.001 \)). The latter result implies that an expansion of cortical territory representing higher tone frequencies occurs late in postnatal development.

There was a parallel age-dependent increase in the cortical area activated by tone stimulation (Fig. 5C; ANOVA, \( P < 0.001 \)). Although this increase was proportional between different tone frequencies (ANOVA, between-frequency comparison, \( P = 0.23 \)), higher-frequency tones (8 and 16 kHz) tended to activate more area in ferrets older than P60.

**Contralateral and ipsilateral maps**

Optical preferred-frequency maps in A1 were obtained following stimulation of each ear separately. Figure 6 compares the contralateral and ipsilateral single-condition images from the cortex of a P40 (Fig. 6A) and an adult (Fig. 6B) ferret (1–16 kHz tone stimuli presented at 80 dB SPL). Both contralateral and ipsilateral stimuli produced similar latero-medial shifts in the location of the activation patches (and in the location of their centroids) with increasing tone frequency (compare rows in Fig. 6A and B). However, the patterns of activation were not identical, particularly in the adult ferret (Fig. 6B). This was to be expected given the differential responsiveness of individual neurons to contralateral and ipsilateral stimulation in adult ferret A1 (Kelly & Judge, 1994). Nevertheless, a substantial overlap between the contralateral and ipsilateral activation areas for each frequency was found at all ages and is further exemplified for two more juvenile animals in Fig. 7.

Figure 8 compares the extent of the cortex (averaged across frequency) that was activated by contralateral and ipsilateral stimulation as a function of age. In keeping with the individual frequency data in Fig. 5C, we observed a significant age-dependent increase in the size of the cortical representation evoked by contralateral stimuli (ANOVA, \( P < 0.001 \)). However, this area increase was not found for ipsilateral stimulation \( (P = 0.15). \) In the first few weeks after hearing onset, no difference was observed in the cortical areas activated by stimulation of each ear, whereas a spatially more extensive representation of contralateral stimuli emerged over the course of postnatal development (Fig. 8; comparison of adult maps: paired t-test, \( P = 0.008 \)).

**Optical response magnitude increases with age**

The strength of the intrinsic optical responses to contralateral ear stimulation (80 dB SPL) increased with age (Fig. 9A; ANOVA,
In juvenile ferrets (< P60), the average responses were similar in magnitude for all stimulus conditions, whereas above P60, the higher sound frequencies (8 and 16 kHz) evoked significantly stronger responses than lower frequencies (1 and 4 kHz), with mean $D_R/R$ values of $4.8 \pm 0.3$ and $3.2 \pm 0.4$, respectively (mean ± SEM; $t$-test, $P < 0.001$). Ipsilateral responses also increased in strength with age, but the difference between low- and high-frequency responses was less pronounced (Fig. 9B).

Figure 9C shows that, averaged across frequencies, the magnitude of the optical signals recorded from juvenile ferrets was similar following stimulation of each ear in juvenile animals (P33–P51), whereas in the adults, contralateral stimuli elicited a significantly larger response area.

In juvenile ferrets (< P60), the average responses were similar in magnitude for all stimulus conditions, whereas above P60, the higher sound frequencies (8 and 16 kHz) evoked significantly stronger responses than lower frequencies (1 and 4 kHz), with mean $\Delta R/R$ values of $4.8 \pm 0.3$ and $3.2 \pm 0.4$, respectively (mean ± SEM $\times 10^{-3}$; $t$-test, $P < 0.001$). Ipsilateral responses also increased in strength with age, but the difference between low- and high-frequency responses was less pronounced (Fig. 9B).

Figure 9C shows that, averaged across frequencies, the magnitude of the optical signals recorded from juvenile ferrets was similar following stimulation of each ear. As expected from the individual frequency response magnitudes shown in Fig. 9A and B, after ~P50, contralateral stimulation resulted in much stronger responses. This can also be seen in the individual examples presented in Fig. 7 by comparing the white scale bars superimposed on each image, which represent the response magnitude for that stimulus condition.

Mean ratios of contralateral to ipsilateral response strength were calculated for every stimulus (tone frequency) condition and plotted as a function of age (Fig. 9D). There was a significant effect of age on this ratio (ANOVA, $F = 12.5; P < 0.001$). In the youngest age groups, P33–36 and P40–42, the mean ($\pm$ SEM) contra-ipsi ratios were $0.71 \pm 0.12$ and $0.89 \pm 0.14$, respectively, indicating that ipsilateral stimuli were better than or at least as effective as contralateral stimuli in activating A1. The mean contra-ipsi ratio increased significantly by P62 ($1.7 \pm 0.3; P < 0.001$), and doubled by adulthood ($2.1 \pm 0.2; P < 0.001$). For comparison, there was no change in the response ratio evoked by 80 and 60 dB SPL contralateral stimuli (Fig. 9D; $P > 0.05$). This suggests that processing of contralateral and ipsilateral stimuli does not reach maturity until after approximately 1 month post-hearing onset.

### Time course of intrinsic optical signals

The typical time course of the tone-evoked reflectance signal recorded from the cortex of an adult ferret is shown in Fig. 10A–C (ferret F9837). The signal appeared after approximately 1 s post-stimulus onset, as seen by an increase of light absorption (black pixels) by the fifth frame, reached its peak between 2 and 3 s post-stimulus onset, and then returned to baseline. Different locations across A1 surface (pixels #2–5) elicited different reflectance magnitudes (Fig. 10B and C, and 8 kHz stimulus), but similar onset latencies. The signals were flat for locations outside the ectosylvian gyrus (pixel #6) or on the dura (pixel #1).

The reflectance signal in juvenile animals had a longer latency than that in adults (Fig. 10D–F). Figure 10D depicts sequential images recorded from a P41 cortex (F9845). Here, the signal appeared by 1.5 s after stimulus onset, and then rose to a maximum at about 4 s.

Figure 11 summarizes the average time course of the response from the centres of the activation patches in A1 at different ages. Each curve represents the average time course from three different frequency

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**Fig. 7.** Comparisons of contralateral and ipsilateral maps (16 kHz tone stimuli) from a P42 (A) and P63 (B) animal. The spatial extent of the activations is very similar but not identical for the two ears. The white scale bars indicate reflectance change values for each stimulus condition.

**Fig. 8.** Comparison of the mean cortical area activated by contralateral and ipsilateral stimuli that were presented within the same recording session. A very similar area was activated by stimulation of each ear in juvenile animals (P33–P51), whereas in the adults, contralateral stimuli elicited a significantly larger response area.
conditions in two animals at each age. Two age-related trends were apparent in the time course of the optical signal: a reduction in onset latency and, as described above, an increase in amplitude. These results confirm that the optical signals evoked by acoustic stimulation undergo a marked maturational change over the second postnatal month.

Discussion

Using the technique of intrinsic optical imaging, we examined the development of the tonotopic organization of A1 in the ferret with respect to contralateral and ipsilateral ear stimulation. Our main findings are: (i) tonotopic organization can be observed as early as P36; (ii) the magnitude of the optical signals increased and their time course decreased with age; (iii) the cortical area activated by tonal stimulation and the magnification factor of the tonotopic map increased as the cortex expanded, with a disproportionate increase in the representation of high frequencies (> 4 kHz); (iv) stimulation of each ear produced optical signals with a similar magnitude and spatial extent in juvenile ferrets, whereas these parameters were significantly larger following contralateral stimulation in older animals.

Our results show that it is possible to obtain reliable tonotopic maps with a high rate of success, despite the reported difficulties in imaging A1 (Harrison et al., 1998; Spitzer et al., 2001). We found that imaging with green light generally resulted in much larger and more robust changes in stimulus-related reflectance than with red light. It is generally thought that haemodynamic artefacts are more prominent when imaging with shorter light wavelengths (e.g. 546 nm; Frostig et al., 1990; McLoughlin & Blasdel, 1998), at which the cortical reflectance change is primarily attributed to changes in blood volume (Bonhoeffer & Grinvald, 1996). However, in one experiment in which both red and green illumination conditions were used, we observed very similar patterns of cortical activation (Fig. 1). Moreover, we noticed that the juvenile cortex consistently exhibited negligible cerebral vasomotion, an oscillatory low-frequency signal attributed to arterial blood pressure, which normally provides a major source of noise in imaging studies (Mayhew et al., 1996). As a consequence, we
Shamma et al.

Animals, intrinsic optical imaging is a suitable method for studying the responses observed in the present study at P32. Nevertheless, in older kittens (Romand, 1983). Studies of the development of orientation selectivity in ferret visual cortex have also reported differences in the age at which cortical responses mature between the two techniques and could also explain the lack of auditory neuronal activity. Finally, the tonotopic arrangement obtained with intrinsic imaging is in agreement with previously reported microelectrode studies of adult ferret A1 (Kelly et al., 1986; Phillips et al., 1988; Shamma et al., 1993; Versnel et al., 1995; Nelken et al., 2004; Bizley et al., 2005).

Development of tonotopic maps

Ferrets begin to hear airborne sounds by the end of the first postnatal month (Moore & Hine, 1992). We were able to record sound-evoked optical responses in one animal at P33, but this activity was not tonotopically organized. The lack of tonotopy cannot be attributed to the relatively weak optical signals recorded from this animal, as clear tonotopy was observed at P36 with very similar response magnitudes (Fig. 11). Although 4, 8 and 16 kHz stimuli were effective in activating A1 at P33, no response was obtained to 1 kHz tones, which might reflect disproportionately high thresholds for low frequencies in the youngest animals, as has been found for auditory-nerve fibres in kittens (Romand, 1983).

Despite the apparent lack of tonotopy at P33, we have previously reported that ferret A1 neurons are selective for sound frequency at this age (Mrsic-Flogel et al., 2003). Studies of the development of orientation selectivity in ferret visual cortex have also reported differences in the age at which cortical responses mature between these two techniques. Whereas the earliest optical maps of orientation selectivity can be observed by P31–35 (Chapman et al., 1996), microelectrode recordings have revealed the presence of orientation tuning a week earlier (Krug et al., 2000). This lag between the development of orientation tuning at the single cell level and that seen with optical imaging can be attributed to a difference in sensitivity between the two techniques and could also explain the lack of auditory responses observed in the present study at P32. Nevertheless, in older animals, intrinsic optical imaging is a suitable method for studying the development of functional maps in both the visual and auditory cortices.

We observed a gradual expansion in the amount of cortical tissue representing an octave, as indicated by an increase in the CMF (mm/octave) from P33 until adulthood (Fig. 5A). Similarly, the average patch size in response to tonal stimuli increased as a function of age (Fig. 5C). This expansion is expected given the substantial increase in size of the ferret ectosylvian gyrus during the second postnatal month (Smart & McSherry, 1986).

The age-related increase in the extent of A1 representing an octave was, however, anisometric for different frequencies. The cortical territory responding to higher frequencies (8–16 kHz) underwent a dramatic expansion after the second postnatal month, with a parallel contraction in the magnification factor for lower frequencies (1–4 kHz) (Fig. 5B and C). This developmental progression led to the over-representation of high frequencies, as observed previously in the mature ferret A1 (Kelly et al., 1986; Phillips et al., 1988). These late developmental changes are likely to be independent of the maturation in the cochlea, which is complete at an earlier stage of development (Romand, 1997), and may therefore reflect functional changes driven by acoustic experience. These results are consistent with previous electrophysiological studies reporting a differential emergence in the representation of different frequencies in cortical and subcortical structures after hearing onset (kitten: Aitkin & Moore, 1975; Brugge et al., 1988; Blatchley & Brugge, 1990; Webster & Martin, 1991; Ehret & Romand, 1994; mouse: Romand & Ehret, 1990; rat: Zhang et al., 2001).

Comparison of contralateral and ipsilateral maps

In adults, the magnitude of the optical signals evoked by contralateral ear stimulation was approximately double that produced by stimuli delivered at the same sound level to the ipsilateral ear. This is consistent with the results of previous electrophysiological studies, showing that contralateral responses in A1 have lower thresholds and higher spike counts and that the proportion of units (and therefore cortical area) responding to contralateral stimulation is much larger (Phillips & Irvine, 1983; Kelly & Judge, 1994; Mrsic-Flogel et al., 2005). An intrinsic optical imaging study in adult rats has also shown that contralateral signals are about twice as large as those evoked by ipsilateral stimulation (Tsytsoarev & Tanaka, 2002).

By contrast, we did not observe any consistent differences in the pattern of activation produced by stimulation of the left and right ears. This is to be expected given that the majority of neurons in A1 are binaurally sensitive (Kelly & Judge, 1994; Irvine et al., 1996; Rutkowski et al., 2000; Zhang et al., 2004). Because we did not stimulate the two ears together, our data do not shed light on the issue of whether neurons exhibiting different types of binaural interaction are organized in bands or clusters.

In contrast, we found that both contralateral and ipsilateral responses were of similar magnitude in juvenile animals (P33–P51; Fig. 9). Because of the weaker optical signals, it is possible that haemodynamic artefacts could have contributed more significantly to the calculation of the response magnitudes in younger animals than in the adults. However, this explanation for the change in aural preference is unlikely, because the ratio of signal magnitudes in response to contralateral stimulation at 80 dB and 60 dB SPL remained relatively constant with age (Fig. 9C and D), indicating that the optical imaging technique is sensitive enough to detect differences in reflectance signal values in response to stimulation at different sound levels, even in the youngest ages examined.
The contralateral dominance of the adult A1 emerges not because ipsilateral response magnitudes decrease over development, but because contralateral signals increase disproportionately (Fig. 9). It is important to note that the intrinsic signal does not necessarily correlate with excitation, but rather with neuronal activity (subthreshold or spiking) that can be either excitatory or inhibitory (Toth et al., 1996). The increasing signal magnitude with age could therefore be associated with maturational changes in a range of cortical properties, as well as greater recruitment of neurons and increases in firing rate, or an improvement in neurovascular coupling. Moreover, changes in neuronal connectivity and neurotransmitter receptor expression within A1 have been found to occur late in development. For instance, the number of γ-aminobutyric acid (GABA)-positive neurons in ferret A1 increases after hearing onset, peaking at about 2 months after birth (Gao et al., 1999), while the spatial arrangement of callosally projecting neurons in the kitten does not reach maturity until well into the fourth month of age (Feng & Brugge, 1983). Finally, because ipsilateral ear stimulation can strongly inhibit cortical neurons (Phillips & Irvine, 1983; Kelly & Sally, 1988; Kelly & Judge, 1994; Irvine et al., 1996; Rutkowski et al., 2000; Zhang et al., 2004; Mrsic-Flogel et al., 2005), it is therefore possible that the balance between cortical excitation and inhibition is refined following the onset of hearing, as appears to be the case in developing ferret visual cortex after the onset of vision (Chen et al., 2005). These developmental changes in turn may contribute to the reported refinement of spatial, spectral and temporal tuning of neurons in A1 after hearing onset (Eggermont, 1991; Zhang et al., 2001; Mrsic-Flogel et al., 2003). In addition to age-related alterations within the cortex itself, we cannot rule out the possibility that maturation of subcortical circuits (e.g. Sanes & Rubel, 1988; Sanes & Frauf, 2000; Kapfer et al., 2002; Leake et al., 2002; Kim & Kandler, 2003; Awatramani et al., 2005) may contribute to the changes observed within A1.

Interestingly, developmental change in contralateral and ipsilateral processing has also been observed in the visual system, although in this case contralateral eye orientation maps are initially stronger (at 2 weeks following birth), while the ipsilateral eye maps reach equivalence 2 weeks later (Crair et al., 1998).

The time course of intrinsic signals

The age-dependent changes in optical response parameters were paralleled by the maturation of the time course of the intrinsic signal. Optical signals in juvenile animals were both smaller and had longer latencies than those recorded later in development, with adult values acquired by the end of the second postnatal month. The time course observed in our adult recordings (onset time ~ 1 s and peak 2.5–3.5 s) is similar to that found by Harrison et al. (1998), who also used short wavelength illumination, although shorter latencies have been reported using longer wavelength illumination (within 0.5 s of stimulus onset; Bakin et al., 1996; Hess & Scheich, 1996). Single-unit recordings from A1 in juvenile ferrets have also shown that response latencies decrease with age (Mrsic-Flogel et al., 2003). However, the magnitude of the latency reduction in the electrophysiological responses is insufficient to explain the changes observed in the present study in the time course of the optical signals. It is therefore possible that coupling between the neuronal responses and cortical blood flow becomes tighter during development as capillary density matures (Tuor et al., 1994).

In summary, our results show that while the coarse tonotopic organization is established at an early stage, adult-like processing of tonal stimuli from the two ears emerges a month after the onset of hearing. Although the development of the auditory periphery clearly contributes to the maturation of certain cortical response properties (Mrsic-Flogel et al., 2003), others are shaped more by central changes and by auditory experience (e.g. Zhang et al., 2001; Chang & Merzenich, 2003). It remains to be seen how these maturational changes in cortical function influence the development of auditory perception.

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Abbreviations

A1, primary auditory cortex; CMF, cortical magnification factor; P, postnatal day.

References


