Postnatal Maturation of Primary Auditory Cortex in the Mustached Bat, *Pteronotus parrnellii*

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INTRODUCTION

The auditory pathways of microchiropteran bats, which use echolocation, are enormously hypertrophied. The acoustic signals of echolocation, both sent and received, are well understood, thereby establishing the auditory cortex of the mustached bat, *Pteronotus parrnellii*, as a model system for cortical auditory computation. Behaviorally relevant echolocation signal components are coded in specific cortical regions (reviews: O’Neill 1995; Suga 1984), and it has been shown that these cortical areas exert a strong descending influence on information processing at lower levels of the auditory pathway (review: Suga and Ma 2003). As in other mammals, the auditory cortex of the mustached bat contains a tonotopically organized primary region with neurons that are sensitive to pure tones and secondary regions that do not respond well to pure tones but to other signal combinations important for echolocation or communication. On the basis of physiological properties and anatomical connections, 11 distinct areas were identified by previous studies (Fitzpatrick et al. 1998a,b; Suga 1984), which make the auditory cortex of this species one of the most extensively studied auditory areas. In addition, another auditory area was discovered in the frontal cortex of this species (Kanwal et al. 2000; Kobler et al. 1987). Eight areas, comprising 2/3 of the auditory cortex, contain neurons sensitive to particular components in biosonar signals (Fitzpatrick et al. 1998b). The present study investigates the postnatal maturation of the tonotopically organized primary cortex that comprises the posterior primary auditory cortex (Alp), where low frequencies are represented, the anterior Al (Ala) area with high-frequency representation, and between both, the large Doppler-shifted constant frequency (DSCF) processing area (Fig. 1).

The mustached bat uses multiharmonic echolocation signals that contain a constant frequency (CF) component followed by a downward FM. Most energy of the echolocation call is put into the second harmonic CF component (CF2) at ~61 kHz. To resolve Doppler shifts in returning echoes that are due to the bat’s own flight speed and to wing beat movement of prey insects, a processing priority of the auditory system is high resolution sampling of the CF2 frequency range. This processing is initiated in an auditory fovea in the cochlea (review: Kössl and Vater 1995). Cortical computation of the CF2 echoes takes place in the DSCF area, a disproportionately large area of ~2 mm diameter (Suga and Jen 1976). Here neurons respond to the 61 kHz CF2-frequency echoes with an exceptionally high tuning sharpness and different echo amplitudes are represented topologically (Suga 1977; Suga and Manabe 1982). In addition, there is a huge cortical overrepresentation of a narrow frequency band that covers the range of behaviorally relevant Doppler shifts of CF2 echoes (Suga and Jen 1976). A striking feature of many cortical neurons is their combination sensitivity in terms of facilitatory responses to a combination of specific call and echo components (Kanwal et al. 1999; O’Neill and Suga 1979; Suga et al. 1979, 1983; review: Suga 1984). In the DSCF area, there are facilitatory responses if the CF2-echo component is preceded by a FM1-call component (Fitzpatrick et al. 1993). Adjacent to the tonotopically organized region is the CF/CF region where combination-sensitive neurons preferentially respond to pure tones that correspond to CF2 or CF3 echo components if a CF1 call component is presented simultaneously (Suga et al. 1979). Functionally, in this area, the relative velocity between bat and...
target could be represented because different neurons respond best to slightly different CF2 or CF3 echo frequencies, which are due to different relative velocities between the bat and its target. Target distance is coded in two areas that preferentially respond to a time-delayed presentation between FM1-call components and FM2-4 echo components [FM/FM area and dorsal fringe (DF) area] (O’Neill and Suga 1982; Suga and O’Neill 1979; review: Suga 1984).

To understand how these cortical properties emerge in young bats during postnatal development, it is important to consider the developmental changes, which take place, in the echolocation call components and in the auditory periphery. Some young mustached bats can be induced through imposed whole-body displacements to emit immature CF signals at an age of ~2 days. The low-frequency CF1 harmonic dominates these early CF-calls (Vater et al. 2003). CF signals are first spontaneously emitted at ~9 days of age, and, with increasing age, CF2 becomes the dominant harmonic. Within the first 4–5 wk of postnatal maturation, mustached bats increase their CF2-frequency from ~48 to 61 kHz (Fig. 2) (Vater et al. 2003). This change runs parallel to an increase in the resonance frequency of the cochlea (Kössl et al. 2003). Presumably, during development, the bats adjust the frequency of their dominant echolocation call component so that it equals the region of highest cochlear sensitivity. However, even before the bats spontaneously emit echolocation calls, cochlear adaptations in the region of the adult cochlear fovea already become functional. The present study seeks to establish the temporal sequence during which cortical neurons obtain adult-like characteristics. The aim of this study is to assess the interplay between maturation of the cortex and cochlea in comparison to the developmental time course of the echolocation signal components.

**METHODS**

**Animals**

For the present study, 20 young mustached bats were collected from maternity colonies in Cuban hot caves with permission from local authorities. All experiments were performed at the University of Havana and comply with the Principles of Animal Care, Publication No. 86-23, revised 1985, of the National Institutes of Health and with the Declaration of Helsinki. The young bats had forearm lengths between 21–48 mm. Newborn bats have a forearm length (FAL) of ~20 mm. At an approximate growth rate of 1 mm/day, the adult forearm length of 51–52 mm is reached after ~4–5 wk of development (see Vater et al. 2003). Comparable growth rates are reported for *Rhinolophus rouxi* (Rübsamen 1987: 1 mm per day) and *Myotis lucifugus* (Kunz and Anthony 1982: 0.7–1.4 mm per day). Young mustached bats of forearm lengths <29 mm, i.e., within the first 9 postnatal days, do not spontaneously emit echolocation calls, but some individuals can be induced to emit calls when they are moved rapidly downward (Fig. 2) (Vater et al. 2003). The lowest CF2 frequencies emitted in such situations are at 48 kHz. The CF2 frequency of induced and spontaneous echolocation calls continuously increases within the next postnatal weeks until adult-like values close to 61 kHz are reached (Vater et al. 2003). In the population of the 20 bats that were used for comparative topographic assessment of cortical areas, no induced or spontaneous calls could be recorded for a subpopulation of bats with FALs between 21 and 30 mm (group 1 in Table 1: postnatal day 1–10). Spontaneous or induced calls with CF2-frequencies between 52 and 58.9 kHz could be measured in a second group of bats (group 2 in Table 1: postnatal day 9–19, FALs between 29 and 39 mm). Bats with FALs ≥40 mm (group 3 in Table 1) were most adult-like and showed active flight behavior and high call repetition rates (see Vater et al. 2003) and emitted CF2 frequencies of 58.3–61.8 kHz.

After capture, the bats were kept for 1–3 days in a metal cage (dimensions: 30 × 20 × 17 cm) before the physiological experiments. The metal cage was positioned in a plastic container where humidity was kept high. During this period of captivity, care was taken that the
The electrodes with a skull opening and the blood vessels were made for each animal. Graphs of the electrode tips at the recording sites in relation to the dorsoventral direction. To avoid damage to cortical vessels, successive penetrations were then made at distances of 100–400 μm in the auditory cortex region in rostrocaudal and dorsoventral axes, considering the direction of penetration was perpendicular to the dorsoventral direction. The electrodes were advanced using a Science Products PM-10 Piezo drive. The direction of penetration was perpendicular to the dorsoventral extent of the cortex and due to constraints of the setup tilted by ~15° toward posterior in relation to the anterior-posterior curvature of the cortex. This induces a systematic error of unit position of 130 μm toward posterior in relation to the anterior-posterior curvature of the cortex. This induces a systematic error of unit position of 130 μm in anterior direction at a recording depth of ~500 μm. The neurons were recorded at a cortical depth of 60–840 μm with 86% of the units between 200 and 500 μm thus corresponding to layers 3–5 (Fitzpatrick et al. 1998a). There was no obvious dependence of neuronal tuning properties on penetration depth. After band-pass filtering (0.3–5 kHz), spikes were discriminated using a threshold procedure. The data shown, if not otherwise stated, are from multiunit clusters that contained between 1 and 4 different spike sizes. At certain recording sites where we recorded both large single-unit spikes and smaller multiunit activity, we did not find differences regarding tuning or sensitivity between single and multiunits. If neuronal thresholds deteriorated by >20 dB in comparison to earlier measurements at the same cortical position, the data were excluded from subsequent comparative data analysis. Such control measurements at specific cortical locations were performed regularly, in particular after encountering recording sites that were unresponsive to pure tone stimulation.

**Table 1.** Experimental animals sorted into three groups according to forearm length (FAL) and echolocation behavior

<table>
<thead>
<tr>
<th>Group</th>
<th>Echolocation Calls</th>
<th>FAL mm</th>
<th>CF2 kHz</th>
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<tbody>
<tr>
<td>1</td>
<td>No echolocation</td>
<td>21</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>Induced or spontaneous echolocation</td>
<td>29–39 mm</td>
<td>52.0–52.5</td>
</tr>
<tr>
<td>3</td>
<td>Spontaneous echolocation</td>
<td>&gt;39 mm</td>
<td>58.0–58.9</td>
</tr>
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FAL, forearm length; CF2, second harmonic constant frequency echolocation call component.

Bats’ environment was at a temperature close to 38°C. In the cave chambers where the maternity colonies are found, temperature ranges between 38 and 42°C. The bats were hanging together, and therefore they were able to hear the vocalizations of other bats. This situation is comparable to that in the maternity colony but without exposure to the vocalizations of adult bats. The bats were handled with fat-rich puppy milk 4–5 times a day.

**Surgery and recording procedures**

Before electrophysiological recording sessions, spontaneous or induced echolocation calls were measured with an U30 bat detector (Ultrasound Advice) and a PCMCIA data acquisition card (Computerboards DAS330, 330 kHz sample rate) controlled by Batsound 2.1 Software (Petterson) and the CF2 frequencies were determined from ≥10 echolocation calls. The bats were anesthetized by subcutaneous injection of ketamine-hydrochloride, diluted in saline, at concentrations of 2.5–10 mg ketamine per milliliter with volumes between 0.01 and 0.06 ml. The dose was adapted to the age of the animals, older bats required higher doses, and was between 0.05 and 0.25 mg ketamine per hour per 10 g body wt. The skull of the bats was fixed by dental acrylic to a metal bar and after retraction of muscles on top of the skull. The skull was opened above the auditory cortex with the dura mater remaining intact. The approximate location of the auditory areas was determined from major blood vessels and the Sylvian fissure that marks the anterior limit of the auditory cortex. At the beginning of the recordings, we aimed to place the electrode within the DSCF area the position of which in relation to the Sylvian fissure was derived from published data on adults (Fitzpatrick et al. 1998a,b; Suga 1984; Suga and Jen 1976). Subsequent penetrations were then positioned at distances of 100–400 μm to extend the mapping of the tonotopically organized auditory cortex region in rostrocaudal and dorsoventral direction. To avoid damage to cortical vessels, successive electrode penetrations were not always positioned at regular distances. Up to 26 penetrations were performed in the same animal at different cortical locations. For reconstruction of the recording sites, photographs of the electrode tips at the recording sites in relation to the skull opening and the blood vessels were made for each animal.

To record single- and multiunit activity, custom-made carbon fiber electrodes with a 7 μm carbon filament within a glass pipette were used. The electrodes were advanced using a Science Products PM-10 Piezo drive. The direction of penetration was perpendicular to the dorsoventral plane of 3 ms duration, 0.5 ms Cos² rise/fall, and a bandwidth of ±0.3 octaves. The stimuli were produced at a repetition period of 400 ms by D/A converters of a Microstar DAP 840 data acquisition card at a sampling rate of 250 kHz and then attenuated by Tucker Davies Technologies System 3 attenuators. The voltage response recorded by the electrode was amplified by a custom-made recording amplifier and fed into an A/D converter of the DAP 840 card using a sampling rate of 25 kHz. Frequency-tuning curves were obtained by randomly presented frequency-level combinations. The responses were averaged 4–10 times within a response time window of 30–50 ms depending on the temporal response pattern. The threshold criterion for tuning curves was spontaneous spike rate +2 SD.

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Latency of pure-tone responses was defined as the time at which the onset peak of neuronal activity in poststimulus-time-histograms at the best frequency (BF) and 15–35 dB above threshold reached spontaneous activity ±3 SD. The sharpness of the frequency tuning curves was defined by the Q100 value (BF/bandwidth of tuning curve 10 dB above minimal threshold). The recording sessions lasted ≈26 h during which the body temperature of the bat was kept close to 38°C using an infrared lamp. At the end of the recording session, the animals were killed by an overdose of anesthetic and were decapitated. The head was immersion-fixed in 0.1 M phosphate buffer.

The procedure used to pool maps across different individuals is similar to the methods used in adult mustached bats by Fitzpatrick et al. (1998b). The heads of the bats were placed in a headholder and tilted to a position comparable to that from published data of the adult (Fitzpatrick et al. 1998b) and photographed with a digital camera attached to a stereomicroscope. The pictures were processed with Corel Draw 8 and Corel Photopaint 8. Overlay drawings of major landmarks such as the Sylvian fissure, the midline of the skull roof, and the posterior border of the parietal bone were made for each area of the brain using major blood vessels as landmarks. As noted before (Fitzpatrick et al. 1998b), this procedure neglects the curvature of the brain that is particularly pronounced within anterior cortical regions close to the Sylvian fissure.

RESULTS

General response properties

Tuning curves were measured using pure tone stimuli within a frequency range of 5–100 kHz, and the BF of the recording site was defined as the frequency at the absolute threshold minimum of the tuning curve. Neuronal activity in response to pure tones was classified into four basic types.

Single peaked tuning curves had one distinct region of maximum sensitivity at BF that could be accompanied by a low-frequency tail region or additional high-frequency responses with >20 dB less sensitivity. Multipeaked tuning curves had an additional sensitivity peak at either higher or lower frequency than BF that was within 20 dB of the threshold minimum at BF. In a third population of units, thresholds were >60 dB SPL and a BF could not be defined unambiguously. These units were classified as insensitive and broadly tuned. Recording locations with spike activity but no response to pure tones at levels <90 dB SPL were categorized as unresponsive if in subsequent recordings at other cortical sites sensitive tone responses were still present. In Table 2, the distribution of the different response types is given for the three age groups. There is a clear decrease in the percentage of unresponsive recording sites and of insensitive and broadly tuned units from group 1 (postnatal day 1–10; no echolocation) to group 3 (postnatal day >19).

The latency of pure tone responses decreased with age. In the youngest animals (FAL 21 mm, 1–2 days old), the average latency at 15–35 dB above threshold at the BF was 20.5 ms for low-frequency units and 19.0 ms for units in the DSCF area (minimal latency: 14.7 ms). Animals with a FAL of 29–30 mm (9–10 days old) had mean latencies of 16.7 ms (low frequency) and 14.0 ms (DSCF) with 8.5 ms minimal latency. In the oldest animal group (40–48 mm FAL), the average latency for low-frequency and DSCF units was 13.0 and 8.1 ms, respectively.

Individual maps and frequency tuning properties

GROUP 1. The most immature bats (FAL between 21 and 29 mm) did not emit CF-FM signals. In previous studies on echolocation and peripheral auditory sensitivity on a larger bat population of similar age, some individuals could be induced to echolocate at CF2 frequencies between 48 and 55 kHz (Vater et al. 2003), and their cochlear resonance frequency was similar at 46–53 kHz (Kössl et al. 2003).

Recording sites in newborn bats were restricted to central regions of the auditory cortex targeting the DSCF area plus more caudal locations (Fig. 3, top left). Many of these recording sites were unresponsive to pure tones (Fig. 3, top middle, right). In Pp.903 (top middle; A and B), three units in the posterior part of the recorded region had tuning curves with distinct regions of maximum sensitivity <50 dB SPL, and BFs between 28 and 35 kHz. These tuning curves had shallower high-frequency tails similar to many low-frequency units recorded in older bats (compare Figs. 5–8). For the more rostrally located recording sites of PP203 (Fig. 3, top right; C and D), the threshold was >60 dB SPL comparable to the sensitivity of low-frequency tails in older bats (see following text).

In a slightly older animal (FAL 25 mm), units in the central region of the auditory cortex were tuned to frequencies between 51 and 53 kHz and occupied a large portion of the recorded area (Fig. 4, top). This particular bat did not echolocate but the 51–53 kHz frequency range corresponds to the range of CF2 frequencies for the respective age group (Vater et al. 2003). The tuning curves exhibited distinct tip and tail regions (Fig. 4, A and B), but the tips were elevated with a maximal sensitivity of ~50 dB SPL. At a position ~1,500 µm more posterior, the units were tuned to lower frequencies of 41 and 46 kHz (Fig. 4, C and D). As in younger bats, many recording sites were either insensitive and broadly tuned or unresponsive to pure tones.

Recording sites and tuning curves for a bat with 29 mm FAL are shown in Fig. 5. Units with BFs between 53 and 57 kHz were recorded from a large area of the auditory cortex that was bordered caudally by units with lower BFs between 15 and 41.9 kHz (Fig. 5, top). Although there was a clear progression from low to high frequencies in the anterior direction, the transition between “low-frequency responses” and prospective DSCF-responses was rather abrupt, a phenomenon also ob-

### Table 2. General response properties at the different recording sites (penetrations) in the 3 age groups

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Penetrations</th>
<th>Unresponsive</th>
<th>Broad</th>
<th>Single</th>
<th>Multiple</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>93</td>
<td>35 (37.6)</td>
<td>15 (16.1)</td>
<td>36 (38.7)</td>
<td>7 (7.5)</td>
</tr>
<tr>
<td>Group 2</td>
<td>137</td>
<td>14 (10.2)</td>
<td>10 (7.2)</td>
<td>66 (48.2)</td>
<td>47 (34.3)</td>
</tr>
<tr>
<td>Group 3</td>
<td>85</td>
<td>7 (8.2)</td>
<td>4 (4.7)</td>
<td>53 (62.3)</td>
<td>21 (24.7)</td>
</tr>
</tbody>
</table>

Percentages are in parentheses.
served at later age. The tip to tail ratio in single peaked units with BFs around 53 kHz (Fig. 5C) was increased as compared with younger bats. In close vicinity to units with single peaked tuning curves, units with multipeaked responses were recorded both in the low-frequency range (Fig. 5B) and the prospective DSCF range (Fig. 5D). The example shown in Fig. 5B has two separate fields of almost equal sensitivity at 20 and 40 kHz plus a 20 dB less sensitive response area around 60 kHz. Units in both low-frequency fields had responses that were relatively independent of stimulus level. In this particular bat, latencies ranged from 14.1 to 19.3 ms for low-frequency neurons and from 9.9 to 15.4 ms for units with BFs around a CF2 frequency of 53 kHz. In this age group, the percentage of recording sites unresponsive to acoustic simulation was highest (Table 2) and the occurrence of multipeaked tuning curves was generally scarce (7.2%; Table 2) but increased for DSCF neurons (27%).

GROUP 2. Bats with FALs between 29 and 35 mm emitted echolocation calls, either spontaneously or induced. At a FAL of 32 mm, PP1102 called with CF2 frequencies between 54 and 55 kHz. About half of the units in the recorded area had BFs between 55 and 59 kHz (Fig. 6, top) with either single peaked (C) or multipeaked tuning curves (D). All posterior located units with BFs below CF2 had response areas that reached into the high frequencies above CF2 at sound levels >60 dB SPL as exemplified in Fig. 6, A and B. Two units at the dorsal border of the recorded area were maximally sensitive to frequencies of the third harmonic of the call. One of these units was rather sharply tuned to 84 kHz with responses that were almost independent of level (Fig. 6E); the other had a broad threshold minimum around 79 kHz (F). Low-frequency neurons responded with latencies between 10.7 and 20.9 ms, neurons with BFs of 55–59 kHz had latencies between 8.3 and 17 ms, neurons with higher BFs had latencies around 10 ms.

Recording sites in a bat of 30 mm FAL, with CF2 frequencies in their echolocation calls of 53 to 55 kHz, covered a larger and more dorsally located area than in the previous

FIG. 3. Location and tuning properties of multiunits in 2 newborn bats (21 mm FAL). Top left: outlines of the recorded areas superimposed on the skull. Landmarks include the Sylvian fissure (*) anteriorly, and the posterior limit of the parietal bone. Top middle, right: individual maps. Penetration sites are indicated with different symbols. The term “broad” refers to insensitive, broadly tuned recording sites with thresholds >60 dB SPL. Numbers next to symbols denote the BFs, capital letters refer to tuning curves plotted in A–D. Major blood vessels are indicated. In PP303, neurons located in posterior cortical regions had low-frequency BFs between 28 and 35 kHz with thresholds between 20 and 40 dB SPL (A and B), more rostral recording sites were unresponsive. In the 2nd individual (PP203), the most rostral recording sites exhibited insensitive responses in the low-frequency range (C and D).
examples (Fig. 7). Units with BFs between 54.8 and 56.4 kHz occupied a disproportionally large area that was bordered caudally by low-frequency responses and rostrally and dorsally by high-frequency responses. As in younger bats, most units located in posterior regions of the auditory cortex that were tuned to low frequencies had responses to high frequencies above CF2 (Fig. 7A). In contrast, the multipeaked tuning curve shown in Fig. 7B had two narrow, equally sensitive response areas with upper thresholds at 80 dB SPL. The two examples shown for the DSCF area both had a sharply tuned threshold minimum within the CF2 range and steep high and low-frequency flanks. In these neurons, a pronounced low-frequency tail is present (Fig. 7C) or there is a second high-frequency response area tuned to 88 kHz (D). High-frequency neurons recorded at anterior and dorsal positions either had broadly tuned regions of maximal sensitivity around 88 kHz plus extended low-frequency tails (Fig. 7E) or sharply tuned tips and poorly developed low-frequency tails (F). Units that were located at the most anterodorsal region of the recorded area had robust pure tone responses with BFs of 50 and 22 kHz, respectively, and latencies between 11.3 and 21 ms. These frequencies are in the range of the FM2 and FM1 component of the call. Latencies of neurons tuned to the frequency range around CF2 ranged from 11.3 and 21 ms; low-frequency neurons responded with latencies between 10.5 and 19.2 ms; neurons with BFs >CF2 had latencies between 9.3 and 18.6 ms. In this age group, the percentage of multiple tuned neurons was highest (Table 2), both within and outside the DSCF area.

GROUP 3. Bats with FALs ≥40 mm had CF2 frequencies ≥58 kHz. In a bat with 43 mm FAL, the sampled cortical area extended more dorsally than in most juveniles. In ventral and central aspects of the recorded area, many units were sharply tuned to a frequency range between 59.2 and 60.9 kHz. These were flanked caudally by low-frequency units (BFs 35.9–50 kHz), two of which had single peaked tuning curves (Fig. 8, A and B). One unit at the transition to the prospective DSCF area was tuned to 39.1 kHz and had a secondary less sensitive response field at 61 kHz (tuning curve not shown). At the most anterior recording site, the unit exhibited separate fields of maximum sensitivity to 20, 40, and 94 kHz.

Neurons within the prospective DSCF area had single peaked tuning curves either without or with prominent low-frequency tails (Fig. 8, C and D). One neuron in the central region of the recorded area was tested for tuning in the presence of a probe tone. If a probe tone was presented at the CF1 frequency of 30 kHz, while measuring the tuning curve, the sensitivity at the tip increased by 15 dB relative to single tone stimulation (Fig. 8D).

At locations dorsal to the prospective DSCF area, the tuning properties of neurons were heterogenous. Some sites were unresponsive or insensitive. Others had BFs in the FM2-
frequency range (54 kHz, 58 kHz). The tuning curve of the most dorso-anterior site was multi-peaked with response maxima at 24 and 96 kHz.

Latencies of low-frequency neurons ranged from 12.6 to 18.2 ms; neurons with BFs around CF2 had latencies of 5.7 to 19 ms. Of a total of 85 recording sites, 7 (8.2%) were unresponsive, 4 (4.7%) were broadly tuned and insensitive, 53 (62.3%) were tuned to one frequency with a single peak, and 21 (24.7%) had multiple peaks. The decrease in units with multiple peaks relative to group 2 is particularly pronounced within the presumptive DSCF-area: of 40 neurons with BFs in the CF2 range, only 7 (17.5%) had multiple peaks.

Combination sensitive neurons

In adult mustached bats, combinations of different echolocation call components can produce pronounced facilitatory responses (review: O’Neill 1995; Suga 1984). We therefore tested 41 units in bats of all age groups for combination sensitivity by obtaining pure tone response areas in the presence of a probe stimulus. The probe was a 3 ms long FM sweep the frequency range of which was adjusted to equal the FM1 component of the respective age group and was presented just before the onset of the pure tone signal. When presented alone to 9 units with BFs within the bats CF2-frequency range, the probe stimulus did not evoke a neuronal response. FM1-CF2 combination sensitivity was tested for bats with FALs between 25 and 48 mm and a decrease of neuronal threshold to the pure tone by $\geq 10$ dB was seen only in 5 units from bats with FAL $\geq 30$ mm. In two of these units, there was a pronounced pure tone threshold improvement of $\geq 20$ dB (Fig. 9A).

In dorsal regions of the area responsive to the CF2-frequency range, presumably the CF/CF area, combinations of two simultaneous pure tones adjusted to the CF1 frequency and the CF2 or CF3 frequency of the respective age group were used as stimuli. We recorded from animals with FALs between 21 and 48 mm and found an increase in threshold sensitivity of $>10$ dB in 14 of the 32 tested units, but only from animals with FAL $= 39$ mm. A pronounced increase in threshold sensitivity of $\geq 20$ dB was only found in 5 of the 32 units tested, and these strongly facilitated units were from the oldest age group with FAL $\geq 40$ mm (Fig. 9, B and C).
Neuronal tuning sharpness and threshold sensitivity in different age groups

A comparison of neuronal thresholds in the three age groups (Fig. 10, A, C, and E) reveals that sensitive low-frequency neurons with BFs below the CF2 frequency or frequency of cochlear resonance are already present in the youngest age group and reach thresholds close to 0 dB SPL (Fig. 10A). The average threshold for BFs 45 kHz decreased slightly from 31.4 dB SPL in group 1 to 20.1 and 24.2 dB SPL in groups 2 and 3, respectively. This change is not significant (Wilcoxon ranked sum test, P = 0.05). Neurons with BFs within the range of CF2 call frequencies of the youngest age group (Vater et al. 2003) are less sensitive with lowest thresholds close to 20 dB SPL and average thresholds of 40.5 dB SPL. The average thresholds decrease to 37.0 and 35.2 dB SPL in groups 2 and 3, respectively (not significant, P = 0.05). Within group 1, thresholds in the CF2 range are significantly decreased (P < 0.05) from a mean of 49.8 dB SPL in the youngest bats (FAL 21–25 mm) to a mean of 36.7 dB SPL in slightly older ones (FAL 26–30 mm).

Frequency tuning of neurons in the range of the CF2 range is sharp (Fig. 10, B, D, and F), even in the youngest bats (group 1). However, the maximal Q10dB value of 57 is clearly lower than that known for adults (maximal Q10dB ≤ 300) (Suga and Manabe 1982). The maximum Q10dB values rise to 144 in group 2 and to 212 in group 3. The average Q10dB values increase from 31.9 (group 1) to 49.3 (group 2) and 66.1 (group 3). The change from group 1 to group 2 is significant (Wilcoxon ranked sum test P < 0.05); however, the change from group 2 to group 3 is not significant.

High-frequency responses with BFs >65 kHz were found in only one animal (28 mm FAL) of the youngest age group.
These units were tuned to frequencies close to 80 kHz at a threshold of ~50 dB SPL. In the second age group, in contrast to group 1, there are many high-frequency neurons with sensitive thresholds between 22 and 51 dB SPL and with BF ≤90 kHz. In the oldest age group with FAL ≥40 mm, the maximum BFs go ≤95 kHz. A quantitative comparison of threshold or tuning sharpness of the high-frequency neurons across age groups is not possible due to the scarcity of data points and quite different BFs of neurons from the three age groups.

**Topography of primary auditory cortex in different age groups**

The mapped cortices are combined in composite maps for the three age groups (Fig. 11). In the youngest age group (Fig. 11A), the recordings were limited to ventral aspects of the auditory cortex, whereas in the older juveniles (groups 2 and 3; Fig. 11, B and C), we sampled a larger region that also includes more dorsally and ventrally located areas.
GROUP 1. In bats with FAL between 21 and 30 mm, neuronal BFs between 51 and 57 kHz are represented in a large central region of the auditory cortex (Fig. 11A). This frequency range approximately corresponds to the echolocation calls and cochlear resonance frequency of this age group (Kössl et al. 2003; Vater et al. 2003). Within this region, however, many cortical units exhibited broadly tuned insensitive responses or were unresponsive to pure tones. Topographically, this area closely corresponds in location to the presumptive DSCF area of older juveniles who emitted CF-FM calls (Fig. 11B and C). High-frequency responses with BFs close to 80 kHz were only encountered in one individual of 28 mm FAL in an anterior region of the mapped cortex. Units with low BFs between 20 and 45 kHz are found in posterior regions. The overlap between low-frequency units and presumptive DSCF units in composite maps of this age group, and group 2 (see following text) is caused by pooling data and is not present in individual maps.

GROUP 2. In bats with FALs between 29 and 39 mm, neurons with BFs between 52 and 59 kHz, a frequency range which corresponds to the measured CF2 call components (Table 1) were located in a large central area of the auditory cortex (Fig. 11B). Low-frequency responses (<CF2) were found posterior and ventral to the presumptive DSCF area. In the most ventral region, several low-frequency units had long latency responses between 21 and 37 ms, which is above the range of latencies observed at all other recording sites. Anterodorsal to the prospective DSCF area, there is another region containing low-frequency neurons. Neurons with BFs > CF2 were much more abundant than in the youngest age group and their location was widespread. They were found in anterior and dorsoposterior regions. In contrast to younger and older stages, there was a population of high-frequency neurons that was sandwiched between the caudal border of the prospective DSCF area and adjacent low-frequency areas.

GROUP 3. Recording sites in the most mature bats with FALs >39 mm (Fig. 11C) were fewer and covered a smaller area than in group 2 animals. As in group 2, there was a large centrally located area occupied by neurons tuned to the CF2 component. This presumptive DSCF area was bordered ros-
trally by high-frequency responses and caudally and ventrally by neurons tuned to low frequencies. In this age group, the topography of primary auditory cortex is comparable to published data on adults (Fitzpatrick et al. 1998a), and the $Q_{10\text{dB}}$ values of units in the DSCF area approach those of adults.

**DISCUSSION**

**General response properties**

Similar to previous studies of the auditory cortex of the mustached bat (Fitzpatrick et al. 1998b; Suga and Jen 1976), we used well isolated multiunit responses for mapping. The majority of recordings were obtained at a cortical depth of 200–500 μm, which encompasses cortical layers 3–5 in adult bats (Fitzpatrick et al. 1998a). Because thalamic input fibers terminate in layers 3–4, this sample may thus contain thalamic input activity in addition to cortical units. Although the BF of single units within a cortical column are typically similar (e.g., Suga and Jen 1976), the frequency responses of multiunit recordings may differ from the constituent single unit recordings (e.g., South and Weinberger 1995). Multiple tuned responses are a characteristic of many auditory cortical units (cat: Sutter and Schreiner 1991; mustached bat: Suga and Jen 1976; big brown bat: Dear et al. 1993) and are indicative of convergence of different frequency inputs at a given cortical site.

All our recordings were obtained in ketamine anesthetized juveniles, whereas previous mapping studies in adults either used barbiturate anesthesia (Suga and Jen 1976) or recorded from awake animals (e.g., Fitzpatrick et al. 1998b; Suga et al. 1987). Despite differences in anesthesia, cortical topography in adult bats is highly comparable among studies and shows a good correspondence to our results in juveniles (see DISCUSSION in the following text). Other response parameters such as response strength, bandwidth, and variability may well be influenced by anesthesia [e.g., pentobarbital/chloral hydrate (Gaese and Ostwald 2001), ketamine/xylazine (Kisley and Gerstein 1999)]. We interpret the age related shift in BFs of units in the prospective DSCF area of juvenile mustached bats to reflect developmental shifts in cochlear resonance frequency (see DISCUSSION in the following text). It is far larger than the shifts in cochlear resonance frequency induced by cooling body temperature or use of anesthetic (Kössl et al. 2003).

There was a clear decrease in neuronal response latency with age that was most substantial within the first postnatal week. In newborn animals, neurons had average response latencies of 20 ms; in the oldest animal of 48 mm FAL, average latency was 8 ms (range of 6–12.3 ms). These values are slightly higher than multiunit latencies in adults (4.4–5.2 ms) (Suga and Jen 1976). The latter study attributed the shortest latencies to thalamic input and refers to a latency range of 7–10 ms for most single unit cortical neurons in the DSCF area. A decrease in the latency of cortical units within the first postnatal weeks is also found in other mammals (chinchilla: Pienkowski and Harrison 2005a,b, 27–23 ms for AC core P3–P30; rat: De Villers-Sidani et al. 2007, 30–15 ms for high-frequency units within P11–P30; cat: Brugge et al. 1988, 55–20 ms within P10–P30). This decrease could be attributed both to a maturation and increasing myelination of the ascending auditory pathway (e.g., Moore and Guan 2001) as well as to development of intracortical circuitry (review: Sur and Leamey 2001).
A quantitative comparison of the extent of specific functional cortical areas among juveniles and with published data on adult bats is not possible with the present data base. In contrast to mapping experiments in adult bats (Fitzpatrick et al. 1998b), we were not able to map the entire auditory cortex in individual juveniles, and our sample of recording sites differs among age stages with most recordings obtained at intermediate age (group 2). This limitation was caused by fragility of the young bats that often allowed only short recording periods that restricted the extent of the mapped areas. Because we tested for combination sensitivity only in a few recordings, borders between prospective DSCF, CF-CF regions, and FM-FM regions cannot be established precisely. In the following discussion, we therefore focus on the relative locations of specific areas and their frequency response properties.

Development of the DSCF area in mustached bats

Published data on cortical topography in adult mustached bats (Fitzpatrick et al. 1998b; Suga and Jen 1976) allowed us to reliably identify the location of the prospective DSCF area in young animals. This indicates that a basic feature of auditory cortex topography, namely the disproportionately large representation of a behaviorally relevant narrow frequency band that reflects specialized cochlear processing is already present shortly after birth.

Starting with a FAL of 25 mm (postnatal day 5), there was a clear topographic segregation between a centrally located area, which contains a magnified representation of a small frequency band corresponding to the range of the age-specific, cochlear-resonance frequencies, and a more posteriorly located region with tonotopically organized, low-frequency responses. These areas correspond closely in location to the DSCF area and Alp of adult bats as defined by Suga and Jen (1976) and Fitzpatrick et al. (1998b).

The high-threshold (>65 dB SPL) low-frequency responses (20–35 kHz) recorded in the prospective DSCF of newborn bats could be due to the relatively more sensitive tuning curve tails rather than to the insensitive tips, of immature DSCF neurons because in slightly older bats (25 mm FAL), comparable low-frequency response areas together with a more sensitive tip are found at similar cortical positions. The emergence of CF2-tuned tips in such tuning curves is likely to be a consequence of cochlear maturation. While cochlear microphonic (CM) potentials in newborn bats already have offset responses that indicate the presence of a mechanical resonator (Kössl et al. 2003), CM threshold sensitivity is poor and clearly defined threshold minima in the CF2 range are seen only in slightly older bats of ~24 mm FAL (Kössl et al. 2003). A comparable neuronal maturation has also been reported for CF2 neurons in the cochlear nucleus and inferior colliculus of hipposiderid and rhinolophid bats: in early stages of postnatal...
development only low-frequency tails are observed and in later stages sharply tuned tips appear (Rübsamen 1992; Rübsamen and Schafer 1990; Rübsamen et al. 1989). This interpretation favoring immature CF2 neurons as source of the low-frequency tails in anterior cortical locations, however, has to be treated with caution. From the cochlear nucleus of adult mustached bats, it is known that comparable low-frequency tails are not only found for CF2 neurons but also for neurons with higher BFs (Marsh et al. 2006). Therefore it is also possible that the cortical low-frequency tails are from more anterior high-frequency regions (A1a).

With increasing age, the BFs of sharply tuned neurons in the DSCF area increase from ~51 to 61 kHz. A corresponding shift is seen for CM threshold minima in the CF2 range (Kössl et al. 2003) and is due to cochlear maturation, e.g., in the form of an increase in stiffness of components of the cochlear resonator. Concomitantly, the sharpness of frequency tuning of cortical DSCF neurons increases from $Q_{10dB}$ values with a maximum of 57 in bats <10 days in age to 212 in the oldest age group, which still is slightly below the maximum $Q_{10dB}$ values found in adults (Suga and Manabe 1982). This developmental gradient of neuronal $Q_{10dB}$ compares well with an increase in $Q$ values of the cochlear resonance in the same age groups from ~60 to 300 (Kössl et al. 2003). Thus maturation of the neuronal tuning properties in the DSCF area appears to be determined through the maturation of the specialized peripheral tuning of the cochlea. The development of sensitive sharply tuned responses of the DSCF area is clearly delayed relative to the development of sensitive low-frequency responses in posterior cortical regions. A high initial sensitivity to the low-frequency range prior to emergence of sharply tuned neurons at the CF2 frequency has also been reported in a developmental study of the auditory midbrain of horseshoe bats (Rübsamen and Schäfer 1990). In newborn mustached bats with 21 mm FAL, caudally located cortical units are tuned to low frequencies between 20 and 40 kHz with minimal thresholds of 22–45 dB SPL. This compares to a cochlear microphonic and compound action potential threshold of ~40–50 dB SPL measured in the same frequency range in an individual of similar age (Kössl et al. 2003). Cochlear evoked potentials require simultaneous action potentials from a population of auditory nerve fibers or the summation of extracellular receptor currents from a larger number of outer hair cells and are therefore generally more insensitive than single- or multi-unit recordings in the brain. Nonetheless, the high sensitivity at the cortical level is surprising. This implies that shortly after birth at least some cortical units would be able to process the low frequencies that are contained in communication signals of adult mustached bats (Kanwal et al. 1994) and in the dominant first harmonic of CF-FM calls of infants (Vater et al. 2003).

Development of basic cortical topography in mustached bats

The prospective DSCF-area of juveniles was bordered anteriorly by units with high-frequency responses. This area probably corresponds to A1a of the adult (Suga and Jen 1976), but its extent was not systematically mapped in our study due to its close vicinity to the Sylvian fissure, which would have required a different stereotactic approach (Asanuma et al. 1983). Furthermore, units tuned to high frequencies (above CF2) were generally sparse in the youngest age group possibly as a conse-
quence of the poor high-frequency sensitivity of the cochlea at this developmental stage (Kössl et al. 2003).

Similar to adult bats (Fitzpatrick et al. 1998b; Suga and Jen 1976), additional high-frequency responses were found dorsal to the prospective DSCF area in the intermediate and oldest age group. According to Suga and Jen (1976), this area is separate from the primary cortex. It probably encompasses the rostral part of the dorsomedial cortex (DM) as defined by Fitzpatrick et al. (1998b).

In the intermediate age group, many high-frequency responses (BFs > CF2) were sandwiched between the caudal border of the prospective DSCF area and Alp. This representation is not observed in our oldest age group and is also not a robust feature of the adult mustached bat cortex. Suga and Jen (1976) reported a lack of units with BFs between 64 and 79 kHz on the main tonotopic axis through Alp and DSCF, however, Fitzpatrick et al. (1998b) reported that four locations in two of nine mapped hemispheres at the transition of Alp and DSCF responded to frequencies between 65 and 69 kHz. Thus although the foundations of tonotopy in the auditory cortex of the mustached bat are laid out early, these results suggest that the representation may undergo a subtle process of focusing within the first 20 postnatal days. This suggestion is also corroborated by our finding that many multiple tuned units occur within the prospective DSCF area in the intermediate age group. Such responses are comparatively rare in the older age group and very rarely encountered in adult mustached bats (Suga and Manabe 1982). This may indicate a developmental reduction of overlap across frequency channels with age, similar to findings in the pallid bat (Razak and Fuzessary 2007a) where multipeaked neurons had a more widespread occurrence across the juvenile cortex than in the adult.

It is noteworthy that in anterodorsal parts of the area tuned to the individual’s CF2 some neurons were clearly facilitated by combinations of CF1 and CF2 stimuli. Because the young bats used in the present study were not yet capable of full flight and hence of leaving the maternity colony and catching insects (juvenile bats leave the cave at 5–6 wk of age), this indicates that basic properties of this functional area (Suga et al. 1979) are already present in juvenile bats prior to active foraging guided by Doppler-sensitive sonar. It is also noteworthy that in the intermediate and oldest group of juveniles, we found neurons tuned to FM2 frequencies in an area anterodorsal to regions tuned to CF2 frequencies. A comparable representation of neurons tuned to frequencies between 50 and 60 kHz (FM2) was also noted by Suga and Jen (1976). The corresponding area was later identified as the specialized FM-FM-region of the adult mustached bat cortex (O’Neill and Suga 1979; Suga and O’Neill 1979).

It is not possible to clearly establish borders between posterior primary cortex (Alp) and the adjacent dorsomedial (DM), and ventroposterior areas (VP) in the juveniles based on available criteria for the adult bats. With only one exception, all neurons with BFs below CF2 in juvenile bats responded to frequencies >55 kHz at levels >60 dB SPL. In the adult, Alp neurons typically do not respond to frequencies above CF2, whereas at moderate to high intensities, DM and VP often respond across the entire audiogram, and the best frequencies can lie anywhere in this range (Fitzpatrick et al. 1998b). Furthermore, all low-frequency neurons in juveniles irrespective of location possessed highly reliable pure tone responses, whereas in adults, putative secondary cortical areas exhibited less consistent responses (Suga and Jen 1976). In juveniles, a significantly longer response latency as typically found in nonprimary areas of the adult cortex (Suga and Jen 1976) was only observed in most ventrally located low-frequency neurons, which may indicate that these recordings were obtained from VP.

Taken together, the data from young mustached bats corroborate the finding that most functional areas of the auditory cortex of mustached bats are in a constant predictable spatial relationship to each other (review Fitzpatrick et al. 1998b; Suga 1984). They also show that the basic architecture is laid out early in development prior to the bats use of sonar. Up to about postnatal day 20 there are still substantial changes in peripheral hearing properties, both cochlear microphonic and auditory nerve evoked potentials become more sensitive (Kössl et al. 2003). Cochlear resonance that is responsible for sharp tuning in the CF2 range is present at birth but up to an age of ~20 days is not as stable and sharply tuned as in adults (Kössl et al. 2003). This could contribute to some of the variability of cortical CF2-frequency representation but cannot explain a high percentage of multiply tuned neurons within the CF2-range. At about the 20th postnatal day, when the cortical frequency representation has matured, the bats’ echolocation changes profoundly. At younger age stages, the bats rarely echolocate and the CF2 frequency of the call is quite variable (Vater et al. 2003). After day 20, the CF2 call component is stabilized in frequency and increases in duration and adult-like calls are emitted (Vater et al. 2003). The bats now really seem to make use of their echolocation system because the call repetition rates increase and echo Doppler-effects are compensated (Vater et al. 2003).

Combination-sensitive neurons in immature bats

A striking feature of cortical physiology in adult mustached bats are combination sensitive neurons that respond to echolocation call components, which are, e.g., important for target distance calculation and calculation of echo Doppler-shifts (reviews: O’Neill 1995; Suga 1984). The discovery of such neurons in the 1970s was a strong impetus for cortical auditory physiology. Some of the underlying convergent neuronal circuitry is, however, as in case of delay-sensitive FM call combination responses, already created in the inferior colliculus (Mittmann and Wenstrup 1995; Portfors and Wenstrup 1999; Yan and Suga 1996). In our study, we were only able to test certain forms of combination sensitivity in a subset of neurons. FM1-CFn combination sensitivity, i.e., the enhancement of a neuronal response to a CF2 (echo) stimulus by a preceding FM1 (call) stimulus, was first found in bats of intermediate age at postnatal day 10 (FAL 30 mm). This implies that facilitatory interactions, which are specific for echolocation components, are implemented before the bat’s echolocation call characteristics and echolocation behavior is adult-like. In the developmental phase (starting at postnatal day 19, FAL 39 mm) when Doppler-effect compensation behavior first appears (Vater et al. 2003) and echolocation calls became more adult-like, we found CF/CF facilitation in dorsal locations of the recording area, presumably the CF/CF- region. Other forms of combination sensitivity, such as delay-tuned FM-FM facilita-
tion, are also present in young bats and are the subject of another study (in preparation).

Comparison with other mammals

In the pallid bat, the only other bat species whose cortical auditory development has been investigated so far, cortical tonotopy is present at the 14th postnatal day (the earliest age that was studied) when the bat has acquired its full auditory range (Razak and Fuzessery 2007a). However, a frequency dependent sharpening of tuning curves and an increase in binaural facilitation as well as the formation of adult-like inhibitory sidebands that are responsible for a FM-direction sensitivity takes place at later age stages (Razak and Fuzessery 2007b; Razak et al. 2008). Both side-band inhibition and FM-direction sensitivity can be reduced by interfering with the bats’ vocalizations during ongoing maturation (Razak et al. 2008), which shows that even if adult-like tonotopicity is already present at the earliest age investigated, other features of auditory processing emerge during critical periods of development.

In rats, initially diffuse tonotopic maps are progressively refined after hearing onset and the establishment of tonotopy critically depends on the acoustic environment (De Villers-Sidani et al. 2007; Zhang et al. 2001). In the rat model, it is argued that progressive changes in tonotopy of A1 may not reflect developmental shifts in cochlear frequency representation (Zhang et al. 2001). Rather the developmental refinement of topography seems to occur through neural activity-dependent mechanisms that can be interfered with during a critical period of development (Chang and Merzenich 2003; De Villers-Sidani et al. 2007; Zhang et al. 2002). In the mustached bat, however, ongoing postnatal cochlear maturation exerts a pronounced effect on cortical tonotopy (see preceding txt). Chinchillas that are born with a mature hearing capability possess well ordered and sharply tuned topographic representations of sound frequency already at postnatal day 3 (Pienkowski and Harrison 2005a). Here the establishment of tonotopic maps must occur in utero, where environmental sounds are considerably attenuated and unlikely to contribute to mechanisms of refinement, especially in the high-frequency range. Thus intrinsic genetically determined mechanisms may suffice to establish a topographic map (Pienkowski and Harrison 2005a). In cats, where auditory cortical responses to high-intensity clicks can be recorded at postnatal day 5 (Rose et al. 1957), a general tonotopic organization is present at the 14th postnatal day. With increasing postnatal age, the tonotopic frequency gradient decreases, the tonotopic area increases and the bandwidth of neurons increases at certain locations suggesting that cortical pruning and selection mechanisms of thalamic input underlie the changes in tonotopy (Bonham et al. 2005a). In addition, there is ample evidence for profound developmental plasticity during critical periods (Kral et al. 2001; Rauschecker 1995, 1999).

Conclusions

In mustached bats, the maturation of threshold sensitivity, tuning sharpness as well as development of cortical overrepresentation of CF frequencies is largely determined by the bandwidth and sensitivity of the cochlea. This dependency is particularly obvious in the first postnatal week when the bats do not yet actively echolocate. Cochlear properties also seem to govern the frequency range of the echolocation calls during further postnatal development. Cortical properties in young bats that are not related to cochlear maturation are a high incidence of multiple tuned neurons at an intermediate age and the emergence of neurons that are facilitated by combination of components of the echolocation signal.

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