New Field With Tonotopic Organization in Guinea Pig Auditory Cortex

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INTRODUCTION

Evidence has been accumulating that the auditory cortex is essential in the analyses of complex sounds and in the processing of spatial cues (Pickles 1982; Schnupp et al. 2001). The auditory cortex of many mammals has core areas and surrounding belt regions (Kaas and Hackett 2000; Merzenich and Brugge 1973; Reale and Imig 1980). Although the function of these areas has not been fully elucidated, it is possible that each area plays a specialized role in the processing of acoustic information. In guinea pigs, mapping with microelectrodes (Hellweg et al. 1977; Redies et al. 1989; Wallace et al. 1999) has revealed two core areas, a primary area (AI) and a dorsocaudal field (DC, dorsocaudal to AI), together with a small field (S) rostral and adjacent to AI. A belt area dorsocaudal to DC, a belt area ventrocaudal to DC (VCB) (Redies et al. 1989), and a belt dorsostral to AI, together with a belt ventral to AI (VRB) (Wallace et al. 1999, 2000), surround the DC field and AI with two small gaps rostral and dorsal to AI. Between VRB and VCB, an intermediate zone (T) has also been reported (Wallace et al. 2000).

Optical imaging in vivo using voltage-sensitive dyes has confirmed the existence of AI and DC (Fukunishi et al. 1992; Song et al. 2006; Taniguchi et al. 1992) and has further revealed propagating activity in AI (Song et al. 2006). Recently, Horikawa et al. (2001) have imaged tone-evoked activities at multiple sites of the auditory cortex and have identified seven or eight spot-like areas in the belt regions. However, none of these areas seems to correspond to zone T reported by Wallace et al. (2000). The S field consistently found in single electrode studies has also turned out to be elusive in imaging studies.

To resolve these discrepancies, we have examined the auditory cortex of guinea pigs with a high-resolution in vivo imaging system using the voltage-sensitive dye RH-795 (Grinvald et al. 1994). We largely confirmed previous results (Wallace et al. 2000), but we found that the caudal portion of VRB is actually a novel field with tonotopic organization.

METHODS

Animal preparation

Experiments were carried out according to the guidelines for use of animals in experiments of Osaka University. Experimental procedures followed those reported previously (Nishimura et al. 2006; Song et al. 2006). Briefly, a total of 18 6- or 7-wk-old Hartley guinea pigs were used. The animal was first anesthetized with a mixture of ketamine (46 mg/kg) and xylazine (24 mg/kg), and anesthesia was maintained by injecting half dose of the mixture per hour during surgery and recording. During recording, the animal was paralyzed with pancuronium bromide (0.2 mg) and artificially ventilated. Rectal temperature was maintained at 34–36°C. Heart rate was monitored throughout the experiment (150–170 beats/min). The skull over the left auditory cortex, ~12 mm in rostrocaudal direction and ~10 mm in dorsoventral direction, was removed, and the dura mater was resected. The cortex was then stained for 1 h with the voltage-sensitive dye RH-795 (0.7 mg/ml in saline; Invitrogen, Carlsbad, CA).

Optical recording

The principles of optical imaging using voltage-sensitive dyes have been described elsewhere (Cohen et al. 1978). We used a high-resolution CMOS imaging system (100 × 100 pixels; MiCAM Ultima, BrainVision, Tokyo, Japan) to detect optical signals at a sampling interval of 1.0 ms. The recording system and optics have been described before (Nishimura et al. 2006; Song et al. 2006). With our optics, the sensor array covered a cortical area of 6.25 × 6.25 mm². We aimed to record from an area including the pseudosylvian sulcus and the ventral edge of the cortex.

Stimulation

Pure tones (50 ms; 10-ms onset and offset cosine ramps, 30-ms plateau; 250 Hz to 16 kHz) at sound pressure level (SPL) between 50 to 70 dB (re. 20 μPa) were generated and presented to the right ear of...
the animal in a double-walled soundproof room (Nishimura et al. 2006).

Data analyses

Data acquired with the CMOS imaging system were analyzed with custom-made C++ programs. The mean of fractional fluorescence ($\Delta F/F$) signal in 16 consecutive recordings was calculated and filtered with a Gaussian-windowed sinc temporal filter (cutoff frequency: 500 Hz). The latency of tone-evoked responses was defined as the time interval from stimulus onset to when the signal exceeds triple the SD of the baseline (30 ms recording before stimulus application). Response latencies at 60 dB SPL are presented as means ± SD. Wilcoxon rank sum test was used for statistical comparison between groups.

RESULTS

Responses in AI and DC

Tone-evoked optical signal in the auditory cortex exhibited a rapid decrease, followed by a slower increase (Fig. 1, A and B; polarity reversed). The spatiotemporal patterns of responses in the cortex to stimulations of pure tones of 250 Hz, 500 Hz, 1 kHz, 2 kHz, and 4 kHz are exemplified in Fig. 1, C–G, with the response amplitude encoded in color; the responses were superimposed on the cortical surface image.

As reported previously (Fukunishi et al. 1992; Song et al. 2006), the response in the cortex to a pure tone did not appear simultaneously in all places but was rather in most cases first evoked at a dorsal location in AI, immediately caudal to the pseudosylvian sulcus (Fig. 1, C–G, dotted line), and then spread mainly ventrally (Fig. 1, C–G). In other cases, the activity was either initiated at a middle site or at multiple sites of AI and then propagated along the dorsoventral axis (Song et al. 2006). Responses in other areas appeared as activity in AI propagated (Fig. 1, C–G). We first focus on early responses with latencies <33 ms. Responses in AI (Fig. 1, C–G, c1–g1, arrow) occurred with a minimum latency (minimum among pixels) of 25.6 ± 3.8 ms ($n = 17$). Responses in field DC occurred slightly later (Fig. 1, C–G, c2, d1–g1, arrowhead; minimum latency = 26.0 ± 3.5 ms, $n = 11$). To examine the tonotopic organizations, locations, and dimensions of AI and DC, responses to tones of different frequencies at 7 ms after the shortest latency in AI were binarized at the threshold of 25% of the maximum value and were superimposed (Fig. 2A). As the tone frequency increased, there was a clear rostral-to-caudal shift of activation in AI and a shift in DC in the opposite direction (Fig. 2A, far right). Figure 2C shows another example; the existence of AI and DC as well as their tonotopy agree well with the case shown in A, although the exact pattern of activation by tones differed between the animals. The short response latencies and the reversal in frequency axis (the axis along which activation shifted with increasing frequency) of AI and DC agree well with previous single-electrode studies (Redies et al. 1989; Wallace et al. 2000). AI had a maximum dimension of 4–5 mm in dorsoventral direction and ~4 mm in rostrocaudal direction.

The ventral half of AI had larger response amplitude than the dorsal half, as is evident when a representative recording from a dorsal pixel (Fig. 1A) is compared with that from a ventral pixel (Fig. 1B). This is also evident from the reddish color of the ventral half of graphs in Fig. 1, C–G. Another feature of the ventral half of AI was more spread of activity in a rostrocaudal direction (Fig. 1, C–G, c2–g2, white arrows).

Novel field ventral to AI

After ~33 ms from stimulus onset, the activity in AI continued to spread in a dorsal-to-ventral direction out of AI to
a point near the ventral edge of the cortex, forming another activated area ventral to AI (Fig. 1, C–G, c3–g3, c4–g4, white arrowheads). We tentatively identified this area as ventrorostral field (VR). Two features in the response of VR made it different from AI. First, the response peak amplitude exhibited a steep increase on entering VR, as is shown in Fig. 1H where the peak amplitude of each pixel is shown in color (arrow points to VR). Second, the activation in VR was much confined in rostrocaudal direction, compared with that in ventral AI (e.g., Fig. 1, Dd3 and Ee3, broken white line), especially to tones of frequencies <2 kHz. For a quantitative comparison, the activated area by a 1-kHz tone, defined as where a response having an amplitude exceeding 25% of the maximum response, was first identified. The maximum dimension in rostrocaudal direction of activated area was 2.8 ± 0.5 mm in ventral AI, which was significantly larger than that in VR (1.8 ± 0.3 mm; n = 6; P < 0.05). Of the 18 animals studied, 8 had a VR area that satisfied these two criteria, 5 animals had a VR area that satisfied one of the two criteria, 1 animal showed no significant responses in the area ventral to AI, and for the remaining 4 animals, the expected VR area was out of the recorded field. The minimum latency of responses in VR to tones at 60 dB SPL was 33.3 ± 3.6 ms (n = 9), which was usually found at the dorsal end of VR.

FIG. 2. Tonotopic organizations of subfields of the auditory cortex. A: same recordings as those shown in Fig. 1. Cortical responses at 7 ms after the shortest latency in AI were binarized using a threshold of 25% of the maximum response and superimposed on the cortical surface image. Stimulus tone frequency is indicated at the bottom of each graph. In the rightmost graph, binarized response to a lower frequency tone was superimposed on the response to a higher frequency tone, and the superposition was done for all frequencies. Note the gradual shift of responses in AI and DC toward each other with the increase of tone frequency. B: same recordings as those shown in Fig. 1. Cortical responses at 14 ms after the shortest latency in VC were binarized using a threshold of 50% of the maximum response, and superimposed on the cortical surface image. Stimulus tone frequency is indicated at the top of each graph. In the rightmost graph, binarized responses were superimposed as in A. Note the gradual shift in response area with frequency in all activated areas. C: recordings from another animal were processed and shown in the same way as in A. D: same recording as that shown in C processed and shown in the same way as in B. E: rightmost graph in B is enlarged, with the borders of VR, VC, and T depicted with broken lines. Note the reversal of tonotopy in VR and VC, and the vertical frequency gradient in T. F: rightmost graph in D is enlarged, with the borders of VR, VC, and T depicted with broken lines. Note the consistency in response location and in tonotopy in all fields between E and F despite the variability in the exact shape of the fields. G: cortical responses to tone stimulations at 60 dB SPL, at 10 ms after the shortest latency in R, were binarized using a threshold of 25% of the maximum value (g1–g3). Superthreshold responses in other areas were transparently coded in the same color. The superposition of g1–g3 is shown in g4 with the responses in R marked by the arrowhead. The arrow in g1 points to VC. Note that the recorded field in G was more rostral than those shown in other figures; thus field T was not imaged. Field DC had weak responses in this animal. The scale bar in A also applies to B, C, D, and G and that in E also applies to F. All scale bars = 1.0 mm.
An isolated area at a ventrocaudal site close to the edge of the cortex, activated soon after the activation of VR, was newly identified (Fig. 1, C–G, c4–g4, double black arrowheads; minimum latency at 60 dB SPL = 34.9 ± 3.4 ms, n = 11). We named this area as ventrocaudal field (VC). Dorsocaudal to VC, another area was activated with slightly longer latencies (Fig. 1, C–G, c4–g4, double white arrowheads; minimum latencies at 60 dB SPL = 38.3 ± 5.3 ms, n = 11). Because this area was located at the intermediate zone ventral to AI and DC, we call this area the transition zone (T), following the naming of Wallace et al. (2000).

To examine tonotopic organizations, locations, and dimensions of VR, VC, and T areas, cortical responses at 14 ms after the shortest latency of VC were binarized at the threshold of 50% of the maximum response (Fig. 2B) because in these areas response latencies were longer than those in AI and response amplitudes were larger. The superimposed binary images shown in Fig. 2, B (rightmost graph) and E, clearly demonstrate that all of VR, VC, and T had a tonotopic organization. Whereas VR had a frequency axis parallel to that in AI, VC had a frequency axis in opposite direction; T had a frequency axis in a ventral-to-dorsal direction, orthogonal to those in other areas (Fig. 2, B, rightmost graph, and E). Binarization of the response made the tonotopy in T appeared less compelling, but the gradual shift of activated area in T according to tone frequency is evident in the raw data shown in Fig. 1, c4–g4 (double white arrowheads). Figure 2, D and F, shows another example; the existence of VR, VC, and T as well as the tonotopy in these fields agree well with the case shown in Fig. 2, B and E, although the exact pattern of activation by tones differed between the animals. Of the 18 animals studied, VC could be identified in 13 animals; in the rest animals, it was either difficult to identify VC as an isolated area or the VC area was out of the recorded field. Field T was identifiable in all 16 animals in which the field was covered by the recording camera. The dimension in dorsoventral direction was ~1.5 mm for VR and VC, and ~1.4 mm for T. The dimension in rostrocaudal direction was ~3 mm for VR, ~2 mm for VC, and ~1 mm for T.

**Rostral small field**

Responses in a small spot-like area (<1 mm in dorsoventral direction) rostral to AI (Fig. 1Gg4, double arrow) were observed in 9 animals (among the 18 studied animals, the spot area was out of the recorded field in 6, and no response was observed in the expected area in 3 animals), with a minimum latency of 33.0 ± 1.8 ms at 60 dB SPL (n = 6). We tentatively named this area as field R. Field R appeared as an isolated activation only when the stimulus tone frequency was >1–4 kHz. The R area responding to 4 kHz in Fig. 1 was well within the area of AI responding to 250- or 500-Hz tones (compare Fig. 1, Gg4 to Cc4, and Dd4). Thus the R area responding to low-frequency tones was engulfed in AI.

Figure 2G illustrates the tonotopy in field R. Unlike AI, field R appeared to have a frequency gradient in a caudal-to-rostral direction (Fig. 2Gg4). In Fig. 2G, the recorded area was more rostral compared with other figures; zone T was thus out of the recorded area and VC was partially visible in the response to a 1 kHz tone stimulation (Fig. 2Gg1, arrow); To tones of 8 and 16 kHz, responses in VR and VC merged together (Fig. 2G, g2 and g3), as in other animals. The response was weak in field DC in this animal.

To illustrate the variability among animals in response patterns in all auditory fields identified here, we superimposed response contours that outline tone-activated areas (Fig. 3A). Response contour was defined using the same threshold as those used in Fig. 2. It can be seen from Fig. 3A that the shape of response contours varied among animals, but the relative location of fields AI, DC, VR, and VC, as well as the tonotopy in these fields were consistent among animals. Notably, responses in field VC showed little variability in location and in tonotopy among animals. There appeared to be more variability in the responses in field T (Fig. 3A).

**DISCUSSION**

All auditory cortical fields identified in this study are summarized in Fig. 3B. The major finding of this study is the discovery of the VC field. Thus the previously reported VRB (Wallace et al. 1999, 2000) can be subdivided into two areas, VR and VC, with opposite frequency axis. Further, other than successful imaging of field R, we have shown that the previously identified zone T does exist and has a tonotopic organization.

The isolated response in VC from that in VR, and the opposite frequency axis of VC to that of VR, make the identification of VC unequivocal. Previously reported areas ventral to AI, VRB (Wallace et al. 1999, 2000), and VA/V (Horikawa et al. 2001) do not correspond to VC demonstrated here because none of these fields has a frequency axis equal to that in VC. VR and VC are different from the two areas (VA, V) identified ventral to AI in Horikawa et al. (2001) because of the facts that VR and VC are aligned in rostrocaudal direction, whereas VA and V are aligned in dorsoventral direction, and that VR and VC are close to the ventral edge of the cortex, whereas VA and V are quite dorsal to the edge. From the dimension and location of VC, a caudal portion of VRB should be identified as VC. It is not clear what fields VA/V reported in Horikawa et al. (2001) correspond to.
Field S has been consistently reported in single electrode studies (Redies et al. 1989; Wallace et al. 1999, 2000), but it has not been observed in imaging studies. Here we were able to record the activity in a small rostral field, field R. The size and tonotopic organization of field R found here both agree with those found for field S in previous studies (Redies et al. 1989; Wallace et al. 1999, 2000), although the tonotopy in field R requires further investigation at a better resolution. Our results also suggest that the low-frequency region of R is engulfed in AI, again in agreement with field S (Redies et al. 1989). Field R, however, appears not equal to field S because field S has response latencies comparable to those in AI (13.9 vs. 14.1 ms on average) (Wallace et al. 1999), whereas field R has response latencies much longer than those in AI (33.0 vs. 25.1 ms on average).

Zone T has been reported to separate the belt ventral to AI and that ventral to DC in single electrode studies (Wallace et al. 2000). No such an area, however, was reported in imaging studies. Here we have confirmed the existence of zone T, and have further demonstrated for the first time the tonotopic organization in the zone, with a unique ventral-to-dorsal frequency axis. Considering the small size of VR, VC, R, and T field, successful identification of the fields can be attributed to the effective suppression of heartbeat and respiration interferences and the high spatial resolution of our imaging system (62.5 × 62.5 vs. 250 × 250 μm² in Horikawa et al. 2001). The expansion of stimulus tone frequency to lower frequencies (0.25–16 vs. 1–16 kHz in previous imaging studies) was also important in identifying field VC and in demonstrating the tonotopy in the field. Field VR, VC, R, and T were difficult to recognize during recording; they were identified only after off-line analyses. Because these fields are on the edge of the recorded area and are of small size, a small shift of the camera will make some of the fields out of the recorded area. This is why some of the fields were not recorded in some animals.

The maximum dimension in dorsoventral direction of AI identified here (4–5 mm) disagrees with Horikawa et al. (2001) where AI has a dorsoventral dimension <3 mm but agrees well with Wallace et al. (1999, 2000). Clear differences in response amplitude, response latency, and rostrocaudal activity spread between the dorsal and ventral part of AI, however, were observed here. All these agree with our previous imaging study using a photodiode array (Song et al. 2006). The more widespread activity in rostrocaudal direction in ventral AI may suggest neurons in the ventral half being tuned to a broader frequency range. This speculation needs to be examined by recording unit activities along the isofrequency axis. Such studies may meanwhile reveal the functional significance of the difference in response amplitude between the dorsal and ventral part of AI as well as the functional significance of activity propagation along the dorsoventral axis (see Fig. 1) (Song et al. 2006).

With the identification of the VR, VC, and T areas, the belt region ventral to AI in guinea pigs becomes most elaborate in rodents. Ventral to AI, no auditory area has been reported in the rat (Rutkowski et al. 2003), and in the well-studied gerbil, only one area is found (Thomas et al. 1993). In other species, the squirrel monkey for example, two areas ventral to AI have been identified (Luethke et al. 1988), but they are aligned dorsoventrally unlike the rostrocaudal alignment of VR, VC, and T in guinea pigs. Because VR has a frequency axis parallel to that in AI and activation of VR is a result of sequential activation in a dorsal-to-ventral direction from AI, identification of VR can be problematic. Actually an early single-electrode study did not discriminate AI and its ventral belts (Redies et al. 1989). Wallace et al. (1999, 2000a), however, have clearly shown that VRB neurons have long response latencies and biphasic, sustained responses to pure tone stimulations. It is true that regions close to the ventral edge of the cortex have long response latencies, but it is difficult to define a border between AI and its ventral belt on a latency basis alone because of the propagating nature of activity in the area. We have tentatively identified VR based on spatial response amplitude profiles and on rostrocaudal activity spread. Because VRB is now subdivided into VR and VC, two areas having tonotopic organizations mirror-symmetric to each other, it would be interesting to study whether the sustained activities of “VRB neurons” are a common feature of VR and VC neurons. A sustained response would make these neurons more likely be involved in spectral rather than temporal analysis of sounds.

Qualitatively, all fields identified in this study had tonotopy (see Fig. 3B for a summary). It would be interesting in future studies to examine how cell response characteristics differ quantitatively in different fields. Another fundamental question is on the structural relationships among the fields identified here and the functional roles of each field in acoustic information processing. Although previous studies have revealed the anatomical relationship between some fields (Wallace et al. 2002), it is necessary to further examine the connections among the fields, in light of the current discovery of the VC field. To this end, experiments combining electrophysiological, anatomical, and imaging techniques would be a useful approach.

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